Vitamin D, an Immunomodulator for Eosinophils in Asthma and Allergy

by

Caroline Denyse Ethier

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

EXPERIMENTAL MEDICINE

Department of Medicine

University of Alberta

ABSTRACT

Epidemiological studies correlate vitamin D deficiency with asthma severity. Calcitriol modulates receptor and cytokine expression from various leukocytes in an anti-inflammatory manner. Despite eosinophil activity during mucosal inflammation, knowledge on the direct effect of calcitriol on this granulocyte is very limited. We propose that calcitriol exerts direct effects on eosinophil biology by modulating its programmed cell death pathways and other functions. Purified peripheral blood eosinophils from atopic donors were incubated with calcitriol (10nM) for up to 14 days with and without IL-5. We observed that calcitriol significantly reduced eosinophil necrosis and cytolytic release of EPX in media when co-incubated with IL-5. The addition of calcitriol also significantly decreased CRTh2 and CCR10 expression on eosinophils.

Eosinophil cytotoxic mediator release into mucosal tissues, resulting from necrotic cytolysis, as well as eosinophil recruitment to lungs are major contributors to airway inflammation in allergic asthma. Hence, the reduction of mucosal inflammation achieved by decreasing eosinophil necrosis and recruitment unveil new anti-inflammatory mechanisms for calcitriol in the context of airway inflammation.

PREFACE

This thesis is an original work by Caroline D. Ethier. This research project received ethics approval from the University of Alberta Research Ethics Board, Immunoregulating activity of vitamin D on eosinophil, Pro00005581, April 2012.

I was primarily responsible for the recruitment of blood donors, collection of primary cells, acquisition of data, analysis as well as data interpretation. Dr. F. Davoine and I collaborated on overall experimental design. Dr. P. Lacy and Dr. L. Cameron provided important intellectual input regarding the scientific design of the study as my co-supervisors. Y. Wu contributed specifically to the design of the EPX colorimetric assay (See Chapter 3) and RSV experiments (See Chapter 4). Dr. P. Lacy was involved in statistical data analysis of cell debris and EPX colorimetric data (See Chapter 3). Dr. F. Davoine and I wrote the manuscript presented in Chapter 3. Dr. F. Davoine was my primary supervisor and principal investigator for this study.

I would like to dedicate this body of work to my parents…

Je voudrais dédier cette thèse à mes parents ...

May my life achievements be a reflection of their dedicated efforts

which have made me the woman I am today.

Que mes succès soient le reflet de leurs efforts et dévouements

qui font la femme que je suis.

ACKNOWLEDGEMENTS

Throughout my graduate experience, I was privileged to have a genuine support system along the way. First, I must thank Dr. Francis Davoine, my supervisor. From awakening my curiosity about immunology in my 2nd year of undergraduate studies, to guiding me through graduate studies while giving me the liberty to become an autonomous researcher; I deeply thank you for nursing my potential and ambitions. I will also thank my committee members. My co-supervisor, Dr. Lisa Cameron, who consistently inspired me by offering new perspectives and genuine advice towards my academic and career goals. As well, my mentor, Dr. Paige Lacy, who not only kept her office door open to me, but also her heart. Thank you for talking science and talking life! I would also like to express my appreciation towards the entire Pulmonary Research Group, particularly the Airway Inflammation Team. For contributing to my training and knowledge as well as offering me sincere friendships and role models, I thank each PRG member for providing me with a work environment most students wish for. All of you have made me into a better scientist!

I am also thankful for the Agricultual, Life and Environmental Sciences (ALES) Food and Health Innovation Initiative (Vitamin Fund), which financially sustained this study. Furthermore, many thanks to my blood donors for trusting me with a needle while expressing genuine interest in my project. Research projects like these could not move forward without this support.

Finally, I recognize how cherished I am for the family and friends that I have. I thank you all for keeping me sane as well as motivated. You all believed in me, when often that is all I needed!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMPs: Antimicrobial peptides

BAL: Bronchoalveolar lavage

Bcl-2: B-cell lymphoma 2

BSA: Bovine serum albumin

CCL: Chemokine (C-C motif) ligand

CCR: Chemokine (C-C motif) receptor

CD: Cluster of differentiation

CLA: Cutaneous lymphocyte associated antigen

CRTh2: Chemoattractant receptor-homologous molecule expressed on Th2

DBD: DNA binding domain

DBP: Vitamin D binding protein

DCs: Dendritic cells

DPBS: Dulbecco's phosphate buffered solution

ECP: Eosinophil cationic protein

EDTA: Ethylenediaminetetraacetic acid

EPX: Eosinophil peroxidase

FasL: Fas ligand; CD95 ligand

FasR: Fas receptor; CD95

FBS: Fetal Bovine Serum

FEV1/FCV: Forced Expiratory Volume in One Second/Forced Vital Capacity; Tiffeneau-Pinelli index

FS/SS: Forward Scatter / Side Scatter; flow cytometry light scattering property

GM-CSF: Granulocyte macrophage colony-stimulating factor

GR: Glucocorticoid receptor

GREs: Glucocorticoid responsive elements

HBSS: Hank's balanced salt solution

IFN-γ: Interferon gamma

IFNGR: Interferon gamma receptor

IL-: Interleukin-

IL-5R : Interleukin-5 receptor

IL-5 : IL-5 cytokine

JAK: Janus kinase

JNK : c-Jun N-terminal kinase

LAMP-3: Lysosome-associated membrane protein -3

LBD: Lipid binding domain

LC3: Microtubule-associated protein 1 light chain 3

MAPK: Mitogen-activated protein kinase pathway

MBP: Major basic protein

MFI: Mean fluorescence intensity

MKP-1: MAPK phosphatase 1

mTOR: Mammalian target of rapamycin (serine/threonine kinase)

OPD: O-phenylenediamine HCl

PAF: Platelet activating factor

PAFR: PAF receptor

PCD: Programmed cell death

PFA: Paraformaldehyde

PGD_2: Prostaglandin D_2

PI: Propidium iodide

PMD: Piecemeal degranulation

PRG: Pulmonary Research Group

PRRs: Pathogen-recognition receptors

PS: Phosphatidylserine

RIP: Receptor-interacting serine/threonine-protein kinase

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute; culture medium

RSV: Respiratory syncytial virus

RXR: Retinoic X receptor

SDF-1: Stromal cell-derived factor 1

SNARE: soluble NSF attachment receptor

TARC: Thymus and activation regulated chemokine; CCL-17

TCR: T cell receptor

TGF-β: Transforming growth factor beta

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TNFR: TNF receptor

Ulk-1: Serine/threonine-protein kinase ULK1

UVB: Ultraviolet B

VAMP : Vesicle associated membrane protein

VDR: Vitamin D receptor

VDRE: Vitamin D responsive elements

CHAPTER 1: Introduction

1.1 Vitamin D

1.1.1 Vitamin D Physiology and Synthesis

Vitamin D functions have generally been discovered due to effects related to its deficiency. Thus, it was originally categorized as a vitamin. Although still controversial, vitamin D deficiency is determined by 25(OH) D_3 peripheral blood levels, where < 50 nM is considered deficient. Vitamin D was first linked to bone metabolism, specifically calcium absorption, due to the development of rickets and osteoporosis in patients suffering from hypovitaminosis D. In the 1990's, associations were made between vitamin D deficiency and cancer prevalence [1]. Later, it was demonstrated that vitamin D induces cancer cell death through cell cycle arrest [2-4]. Recently, functional links have been emerging in the literature between vitamin D and the human immune system. Overall, vitamin D is a multifactorial molecule with diverse roles depending on tissue type [1, 2, 4-11].

In contrast to its name, vitamin D is actually a secosteroid hormone [12]. Secosteroids are structurally very similar to steroids, like corticosteroids, where the only difference is the lack of a bond between C9-C10 to form a fourth carbon ring [10] (**Figure 1**). Both groups share some precursors, and are both intra-nuclear signaling factors [8]. Vitamin D can be obtained through diet, yet humans mainly rely on ultraviolet B (UVB)-based vitamin D_3 photosynthesis. UVB light exposure enables conversion of 7-dihydrocholesterol into pre-vitamin D_3, which later becomes inactive vitamin D_3 (cholecalciferol) through an isomerisation reaction. Next, inactive vitamin D_3 is transformed by 25-hydroxylase, from hepatocytes, to produce 25(OH) D_3, commonly known as calcifediol [13]. The latter molecule, which is bound to the Vitamin D-binding protein (DBP) while in circulation, is known as our reservoir which is available for reactions with vitamin D metabolic enzymes. DBP acts as a transport protein, primarily using blood circulation to deliver calcifediol to targeted tissues for its activation [6]. Calcifediol is modified by 1α-hydroxylase which adds a hydroxyl group to the latter precursor to form 1,25 dihydroxyvitamin D_3, referred to as calcitriol, which is the most physiologically active form of vitamin D [14]. The latter pathway is summarized in **Figure 2**.

The enzyme, 1α-hydroxylase, is traditionally known to reside in proximal convoluted tubule cells of the kidneys [14], yet has recently been found constitutively expressed in airway

epithelial cells [15]. Alveolar macrophages, dendritic cells and lymphocytes have been shown to execute this conversion as well [16]. Consequently, extra-renal 1α-hydroxylase suggests significant concentrations of calcitriol in the airway mucosa microenvironment [16-18].

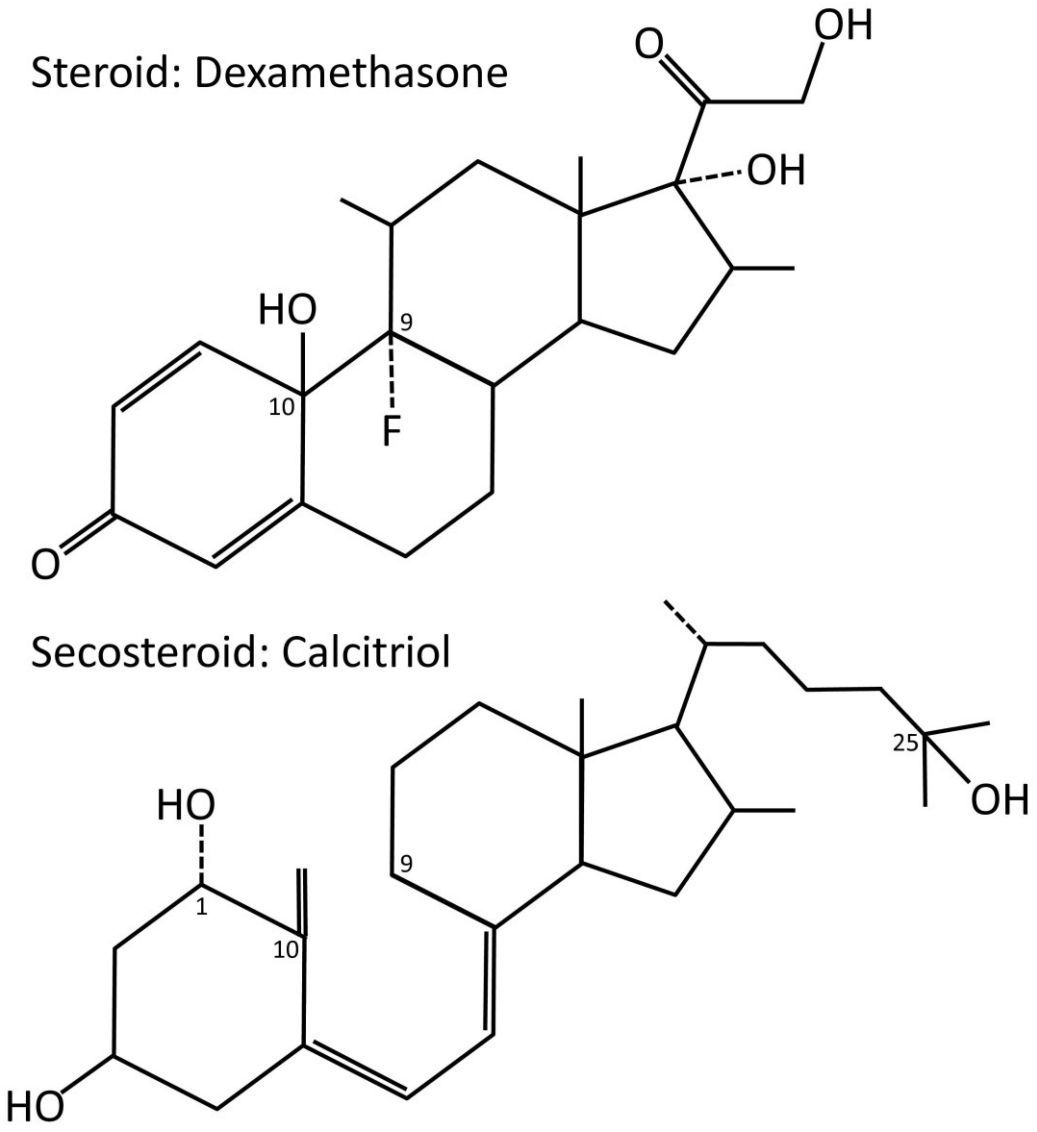


Figure 1: Structural differences between a steroid (e.g. dexamethasone) and a secosteroid (e.g., calcitriol). Adapted from Norman *et al.* [10].

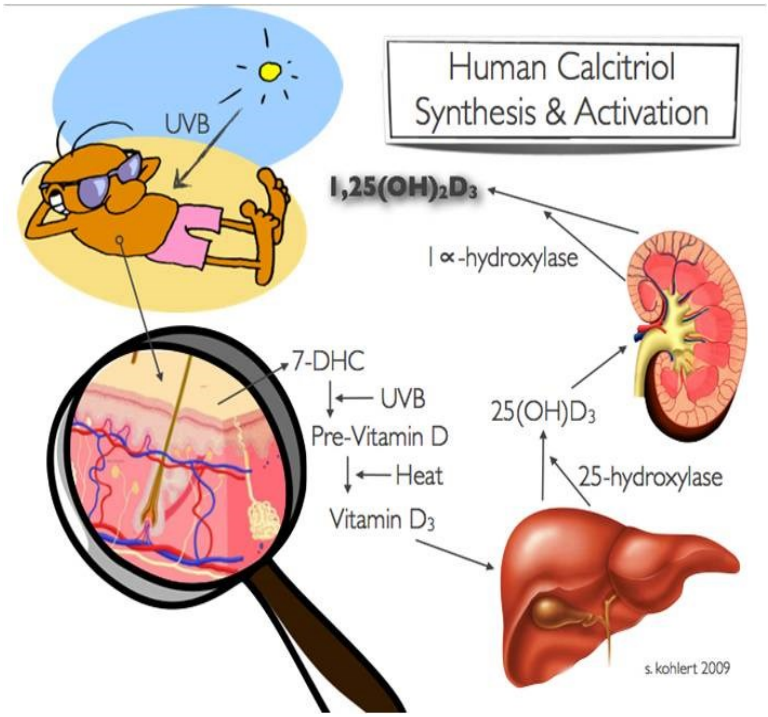


Figure 2: Schematic representation of UVB-dependent vitamin D_3 synthesis and activation.

1.1.2 Vitamin D Signaling Pathway

Calcitriol mediates its activity by binding to vitamin D receptor (VDR) which resides at the nuclear membrane. Although ligands to VDR include calcifediol, calcitriol exerts the highest affinity for VDR out of all vitamin D metabolites. Thus calcitriol is considered the active metabolite of vitamin D, even though it is found in low concentrations in blood [19]. VDR monomer contains a lipid binding domain (LBD) and a DNA binding domain (DBD). When VDR exists as a monomer it is in its inactive form. VDR heterodimerizes with the retinoic X receptor (RXR), upon ligand-binding activation, through recognition by alpha-helices of both monomers [9]. The DBD of VDR allows it to act as a transcription factor for vitamin D responsive elements (VDREs) [9, 11, 20]. VDR signaling is said to influence the transcription of roughly 3% of the human genome (directly and indirectly), including approximately 900 genes, where some target genes regulate cell differentiation, cell growth, immunomodulation and hormonal systems, which are beyond calcium regulation [12, 21].

1.2 Vitamin D and Inflammatory Diseases

1.2.1 Implications of VDR

Studies on VDR in animal models support a role for vitamin D in immune functions. VDR knockout mice are unable to generate airway hyperreactivity in response to standard experimental allergen challenge, and are defective in Th2-type cytokine release [22-24]. Only one other study demonstrated elevated Th2 cytokines and IgE levels in these mice, yet still failed to induce experimental asthma [25]. These studies imply that eliminating vitamin D function in mice is beneficial against allergic airway inflammation. This finding from allergic asthma mouse models contradicts allergy- and autoimmune-related outcomes associated with vitamin D deficiency observed in humans. Removing VDR function from mice has other consequences on mouse airway physiology. VDR-deficient mice have overall reduced lung function, decreased lung size and lung structure compared to wild type [26, 27]. Therefore, current evidence suggests that a mouse animal model remains satisfactory to study physiological roles of vitamin D on lung development, perhaps not for investigating vitamin D immunomodulation properties. In other words, mice do not seem to utilize vitamin D metabolites for immunological functions in the same manner as humans do. Hence, it is important to work with primary human immune cells when investigating vitamin D effects.

In human populations, a large number of VDR polymorphisms identified exist between exons 8 and 9 of the VDR gene, which have unknown function while other polymorphisms are found in regulatory sections of the gene [4]. Overall, researchers seem to agree that identified VDR polymorphisms are less related to changes in the protein structure, but more to differences in stability and/or translation efficiency of the RNA [4]. Still, VDR polymorphisms significantly correlate with asthma prevalence [28-32]. Hence, the current consensus in human epidemiology is that normally functioning and expressed VDR is protective in action against allergic asthma, and probably other atopic diseases [14].

1.2.2 Human Clinical Evidence for Vitamin D Immune Functions

Vitamin D deficiency has been linked with an increase (and/or adverse outcome) in autoimmune diseases, infections, cardiovascular diseases, cancer and apparently linked with a higher risk of mortality in all conditions [1]. There is also evidence supportive of sunshine (in relation to vitamin D production) as a protective factor against human atopic disease, known as the 'sunshine hypothesis'. For example, the USA National Hospital Ambulatory Medical Care Survey reported significantly higher incidence of acute allergic reactions in north-eastern regions compared to southern regions [33]. Specific to lung health, current literature associates vitamin D deficiency with airway hyperresponsiveness, lower pulmonary functions, worse asthma control and possibly, corticosteroid resistance [17]. Also, many studies report a relationship between low serum levels of calcifediol (*i.e.,* described as serum vitamin D) and FEV_1/FVC ratio, a diagnostic measurement for obstructive and airway disease [14]. Although still controversial due to contradictory reports, some researchers have demonstrated that low calcifediol, in sera of pregnant women, predicts higher prevalence of asthma, wheezing and other atopic diseases in childhood [14]. In contrast, a similar study contradicted these findings but revealed that higher maternal vitamin D lowered the risk of respiratory tract infections in early life [34]. In general, the prevalence of vitamin D deficiency observed in Western society associates with its increases in asthma prevalence. A meta-analysis study revealed that 61% of American youth suffering from asthma are clinically vitamin D deficient [35].

1.2.3 Calcitriol Exerts Direct Effects on Leukocytes

Although human studies, thus far, remain ambiguous towards directly linking vitamin D to allergic diseases, *in vitro* research has been able to enlighten the matter. As reviewed by Hart and

colleagues [36], calcitriol has direct effects on various immune cells, including: monocytes, macrophages, dendritic cells, T cells and B cells (**Table 1**). In general, calcitriol is said to activate innate immune responses while suppressing adaptive immunity mechanisms [36].

On the one hand, calcitriol has the capacity to upregulate innate immunity components, such as antimicrobial peptides (AMPs), and anti-inflammatory strategies. Calcitriol directly promotes the synthesis of AMPs, cathelicidin and β-defensin 2, and encourages the production of tolerogenic dendritic cells (DCs) and T cells [2, 37]. Hewison *et al.* [38] showed that monocytes, macrophages, neutrophils and epithelial cells produce AMPs upon calcitriol exposure *in vitro*. Additionally, calcitriol seems to alter immune cell differentiation in an anti-inflammatory manner. Baeke *et al.* [5] demonstrated that calcitriol enhances the differentiation of monocytes into macrophages with increased phagocytic capacity as well as an altered cytokine profile that encourages resolution of inflammatory immune responses. Baeke and colleagues also showed direct effects of calcitriol on T cells. They report that T cells must be properly stimulated through their T cell receptor (TCR) in order to express VDR. Nonetheless, calcitriol directly and selectively induces the development of Treg cells, while it failed to enhance Th1 and Th17 cells [5, 39]. The same team of researchers also observed increases in chemokine (C-C motif) receptor (CCR) 10 and CCR5 expression in T cells as a result of calcitriol treatment, which the researchers suggest may improve skin and mucosal immunity [5, 39]. Furthermore, van der Aar *et al.* [40] observed enhanced FOXP3-expressing and interleukin (IL) -10 secreting Treg cells when monocytes, Langerhans and dermal DCs were incubated with calcitriol.

On the other hand, it predominantly downregulates pro-inflammatory elements, such as cytokine, co-stimulatory cell surface receptors and antibody production. Baeke *et al.* [5] report that, even though calcitriol induces monocytes to differentiate into DCs, calcitriol downregulates cytokine release and co-stimulatory molecule expression on cultured monocytes. In relation to DCs, van der Aar *et al.* [40] showed that calcitriol modulates co-stimulatory molecules expressed by mature DCs while reducing the production of pro-inflammatory cytokines as well as induction of Th1 cells *in vitro*. Calcitriol seems to affect pathogen recognition as well. Khoo *et al.* [41] demonstrated a reduction in pathogen-recognition receptors (PRRs) toll like receptor (TLR) 2 and TLR4 in mononuclear cells during *M. tuberculosis* infection. Finally, calcitriol directly dampens antibody production. Baeke *et al.* [5] showed reduced B cell differentiation into memory and

plasma B cells (thus discouraging antibody production) upon calcitriol exposure. Also, incubated B cells showed reduced capacity to produce antibodies [5]. In respect to eosinophil biology, Hiraguchi *et al.* have confirmed that eosinophils do express VDR [42]. At present, little is known about the direct effects of calcitriol on granulocytes, such as eosinophils.

Table 1: **Effects of calcitriol on myeloid and lymphoid leukocytes in vitro.** (Adapted from Hart *et al.* [36]).

Effects on myeloid cells		
	Upregulates	**Downregulates**
Monocytes	- Antimicrobial peptides: Cathelicidin and β-defensin 2	- Co-stimulatory molecules: CD40, CD80, CD86, HLA-DR
Macrophages	- Antimicrobial peptides: Cathelicidin and β-defensin 2 - Phagocytosis - Activin A	- Cytokines: IL-1, IL-6, IL-8, IL-12 and TNF
Dendritic cells	- ILT3 - Cytokines: TGF-β and IL-10 - Treg cell differentiation	- Co-stimulatory molecules: CD1a, CD14, CD40, CD83, CD86 - Cytokines: IL-12 and IL-23 - Th1 and Th17 cell differentiation
Effects on lymphoid cells		
$CD4^+$ T cells	- Treg cell differentiation: FOXP3, IL-10, CTLA4, TLR9, OX40L - Homing to inflamed tissues: CCR5, CXCR3, CXCR6 - Homing to skin: CCR4, CCR10	-Th1 and Th17 cell differentiation - Cytokines: IL-17, IL-21, IL-2, IFN-γ -Homing to lymph nodes: CD62L, CCR7
B cells	- Homing to skin: CCR10	- Plasma cell development - Antibody secretion - Memory B cell differentiation

1.3 Asthma - A Complex Inflammatory Disease

Asthma is a chronic pulmonary inflammatory disease with many phenotypes. Clinically, asthma is recognized as increased airway hyperresponsiveness leading to events of wheezing and/or shortness of breath. Allergic asthma is currently agreed to result from inappropriate Th2 immune responses, triggered by an allergen. As our understanding of the complexity of asthma etiology and pathogenesis increases, so does its prevalence. Today's growing asthma epidemic has prompted research to focus on various target immune cells involved in airway allergic inflammation, with needed emphasis on Th2 cells and eosinophils [16, 43]. In addition, current therapies rely heavily on glucocorticosteroids to manage asthma symptoms, yet there remains variability in the response to these treatments [44].

Allergic asthma is described as a Th2 type of inflammatory disease which is characterized by the involvement of specific IgE to an allergen and a major involvement of mast cells. The classic asthmatic immune reaction is said to occur in two phases. The first is an immediate bronchoconstriction caused by degranulation of mast cell histamines, leukotrienes, prostaglandins and PAF. Phase 1 is dependent on the activation of mastocytes, which is essential for the subsequent phase [45]. The second phase of bronchoconstriction is probably more dependent on cytokines and chemokines (*e.g.,* IL-5, TNF, GM-CSF and CCL11). Factors that specifically trigger the delayed phase, or those which elicit degranulation of eosinophils once it is found in the bronchial lumen, are not yet fully defined. Such factors cause the recruitment and activation of pro-inflammatory cells to be trafficked toward bronchi where eosinophils are activated to release inflammatory factors [46]. As reviewed by Erle *et al.* [47], the cell biology of asthma relies on many complex relationships between immune cells and non-immune cells where most mechanisms are initiated by airway epithelium upon stimulation from environmental triggers, such as allergens, pollutants and viruses. Major cells involved are notably epithelial cells, nerve cells, smooth muscle cells, mast cells, DCs, eosinophils/basophils and Th2 cells [47].

1.4 Eosinophils in Allergic Airway Inflammation

Allergic airway inflammation, as described in allergic asthma pathogenesis, is a multifaceted condition involving diverse acute and chronic mechanisms. Although many immune

and non-immune cells participate in allergic airway inflammatory responses, the eosinophils are known to be a major player [48, 49]. In animal models, eosinophil functions have been shown to be implicated in most aspects of asthma pathogenesis, in particular: airway hyperreactivity and excess mucus production, which result in reversible bronchoconstriction [50, 51]. In clinical studies, eosinophil sputum counts associate with asthma exacerbations [52]. Furthermore, eosinophils are associated with lung tissue remodeling, a permanent consequence of chronic airway inflammation [53, 54]. Accordingly, eosinophils have been and remain a target in asthma pathogenesis and therapeutic research [43, 50, 55, 56]. Many studies, which pertain to asthma, focus on eosinophil biology according to three major categories: eosinophil survival, phenotyping eosinophil populations and modulation of eosinophil activities [43].

1.4.1 Eosinophilia and Eosinophil Survival

Eosinophils originate from bone marrow and temporarily circulate in blood, where their short life is suggested to range from 8 to 18 hours. Once eosinophils reach tissues, their lifespan increases, dependent on the tissue, from 2 to 5 days. Various *in vitro* studies have shown that eosinophil survival can be sustained for up to 14 days by supplementing with cytokines. In general, eosinophils are known to be sensitive to death without sufficient anti-apoptotic stimulus [57].

Lung eosinophilia is a key characteristic of allergic asthma, and consequently its mechanisms have been a main focus in eosinophil research [58, 59]. IL-5 is distinct and imperative to the differentiation as well as survival of eosinophils, therefore is a central factor in airway eosinophilia [60]. Furthermore, IL-5 has been shown to induce eosinophil activation, which is an additional relevant eosinophil function in the context of airway inflammation [57]. IL-5 receptor (IL-5R) is composed of two subunits termed alpha and beta. IL-5Rα is the subunit which renders specificity to IL-5 [61]. Hence, changes in α subunit expression provides insight on IL-5 sensitivity, specifically. IL-5, as well as its family members; IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF), signal through the common β chain leading to anti-apoptotic effects. The IL-5Rβ subunit is the signaling component of the IL-5R [61]. As mentioned, this β signaling chain is common for IL-5, IL-3 and GM-CSF receptors [43, 49, 57]. Therefore, expression modulation of the β subunit is indicative of the responsiveness to all three respective cytokines. IL-5 signaling relies on β receptor activation to phosphorylate Janus kinase (JAK) 2 to mediate its downstream effects [62]. In general, common β chain activation causes prolonged

eosinophil survival by inhibiting the apoptotic pathway, primarily by increasing B-cell lymphoma 2 (Bcl-2) expression [43, 63]. An increased expression of IL-5R at the cell surface renders eosinophils more responsive to IL-5 cytokine. Thus, an increase in IL-5R would intensify the anti-apoptotic signal, when exposed to IL-5 cytokine. Indeed, IL-5 is commonly present in the airway mucosa of asthmatics and allergic rhinitis patients [64]. Hence, eosinophils in asthmatic individuals are continually stimulated to inhibit apoptotic death resulting in increased eosinophil survival.

Other cytokines with anti-apoptotic properties, including certain inflammatory factors, have been shown to increase eosinophil survival as well [65]. Particularly, Interferon (IFN)-γ has been studied in eosinophils due to its inhibition of apoptosis. IFN-γ mediates its anti-apoptotic effects through the Interferon gamma receptor (IFNGR) 1 and 2. Its intracellular signaling initiation relies on JAK1 and 2 activation [62]. In contrast to IL-5, IFN-γ sustains eosinophil survival by a different mechanism than increasing Bcl-2 expression [66]. Both IL-5 and IFN-γ have been shown to successfully inhibit spontaneous apoptosis and Fas-induced apoptosis, in eosinophils, by blocking caspase-3 and -8 proteolysis, yet fail to alter mitochondrion membrane potential [67]. Overall, IFN-γ utilizes a pathway which is independent of the IL-5 pathway to inhibit apoptosis in eosinophils.

Regardless of the manner in which apoptosis is inhibited, delayed-apoptosis or insufficient apoptosis of eosinophils is now recognized as an important factor in asthma pathology [55, 56, 59, 68]. Although prolonged eosinophil survival may provoke general eosinophilia in asthma, anti-IL-5 therapy seems successful in reducing blood eosinophilia but not sufficient to resolve lung tissue eosinophilia and related symptoms in asthmatics [54, 69, 70]. Therefore, therapeutic alternatives against eosinophilic airway inflammation, beyond the systemic elimination of eosinophils, have been explored in regards to eosinophil recruitment into pulmonary tissues.

1.4.2 Death Signaling Pathways and Inflammation

1.4.2.1 Apoptosis is Anti-Inflammatory

Apoptosis is a caspase-dependent pathway, since it is suppressed by wide-spectrum caspase inhibitors such as zVAD-fmk [71]. Although various caspases have been said to be

involved, caspase-8 becomes predominantly activated to become an effector molecule [72]. Its downstream targets include pro-apoptotic proteins from the Bcl-2 family. At large, these molecules aim to increase mitochondrial membrane permeabilization to release cytochrome c into the cytosol. The latter molecule dictates intracellular changes such as fragmentation of nuclear DNA as well as plasma membrane alterations, such as phosphatidylserine (PS) exposure [71].

Apoptosis is the classical programmed cell death (PCD) pathway, which has been thoroughly studied in eosinophils. Most of our current knowledge of eosinophil apoptosis stems from studies on the role of IL-5 in eosinophilopoiesis and eosinophil survival [57, 73, 74]. Apoptosis is morphologically characterized by a reduction in cell volume, chromatin condensation, nuclear fragmentation, plasma membrane blebbing and expression of cell surface components which facilitate engulfment by phagocytes [71]. Therefore, apoptotic eosinophils and their apoptotic bodies are actively phagocytosed by other immune cells, such as macrophages, to avoid granule release, which allows resolution [75]. Apoptosis is an important component of resolution of inflammatory responses. Consequently, the most popular therapies against asthma, which target eosinophils, aim to induce apoptosis in this cell type (*e.g.,* anti-IL-5 therapies and corticosteroids) [72, 75-77]. In contrast to this anti-inflammatory pathway, necrosis is commonly known as the opposite of apoptosis, since it promotes inflammation [71].

1.4.2.2 Necrosis is Pro-Inflammatory

Necrosis is mainly characterized by an increase in cell volume, swelling of organelles and loss of plasma membrane integrity. The term ‘necrosis’ was previously described as an unregulated form of cell death which induces inflammation due to the release of intracellular contents. More recently, the concept of necrosis is understood to be more complex and regulated than traditionally thought. Therefore, it is now termed ‘necroptosis’ [71, 78, 79]. Current scientific literature on the matter tends to utilize three different words: cytolysis, secondary necrosis and necroptosis. Cytolysis is a general term used to describe cell death through lysis, as a result from damaged plasma membrane, thus expelling their intracellular contents. Secondary necrosis is said to occur within a cell that has first been in the apoptotic state for an extended period of time. In contrast, necroptosis is a newer concept which describes a highly regulated death signaling pathway [71]. It is now known that necroptosis can be initiated, similarly to apoptosis, such as by Tumor Necrosis

Factor (TNF), Fas Ligand (FasL) and DNA damage. [80-83]. It has also been demonstrated that necrosis can be specifically triggered through PRRs.

Since it induces inflammation, necroptosis is beginning to be studied in some immune cells, such as T cells [78, 84]. It has been proposed that immune cells are more prone to die by necrosis than other cell types [79]. As reviewed by Han *et al.* [78], there are many reports suggesting that necrosis is a backup death pathway, which directly competes with apoptosis, in order to prompt immune responses [78]. It is distinct from apoptosis by the formation of receptor-interacting serine/threonine-protein kinase (RIP) 1 and 3 protein complex, known as necrosome, which inhibits caspase-8 in order to initiate downstream cytolytic effects [78, 80]. In general, our familiarity with apoptosis-necroptosis cross-talk remains basic, yet there is increasing evidence that both PCD pathways inhibit each other [78, 85, 86]. (This information was adapted from a review by Ethier *et al.* [87])

1.4.2.3 Autophagy Induces Survival

Besides its complicated relationship with apoptosis, necrosis has also been shown to influence cell survival through autophagy. Necrosis has been associated to changes in autophagy through intracellular reactive oxygen species (ROS) production [75]. Autophagy is an intracellular programmed pathway which specifically promotes cell survival [3, 84, 88]. Even though cell death can occur during autophagy induction, the suppression of autophagy by genetic knockout and knockdown of autophagy essential genes, termed *atg*, results in accelerated cell death. Therefore, autophagy was categorized as a pro-survival pathway [71]. Briefly, autophagy is characterized by the formation of autophagosome fusion to lysosome for cytoplasmic material degradation where redistribution of microtubule-associated protein 1 light chain 3 (LC3) fusion proteins in vesicular structures, including autophagosomes and autolysosomes, is observed [71]. Autophagosomes are distinctly two-membraned vesicles which degrade degenerating cytoplasmic organelles and cytosol components by acidic lysosomal hydrolases. Although autophagy is characterized by massive autophagic vacuolization of the cytoplasm, accumulation of autophagosomes and autolysosomes is deemed insufficient in identifying autophagic cells [71]. Researchers rely heavily on intracellular signaling molecules which have been identified as distinct to autophagy to induce cell survival. Its intracellular signaling pathway is distinctive due to phosphorylation of serine/threonine-protein kinase ULK1 (Ulk-1) which permits interaction with mammalian target

of rapamycin (mTOR) to directly induce survival [89-91]. Although the molecular mechanism is not fully understood, it has been recommended to identify Beclin-1 dissociation from Bcl-2, by co-immunoprecipitation, and $p62^{Lck}$ degradation, by immunoblotting, as they are events which occur when autophagy is induced.

It is important to note that research pertaining to death and survival signaling pathways is still a fairly new field in cell biology. PCD pathways have not yet been fully described mechanistically. Furthermore, apart from apoptosis, PCD pathways, resulting in death or survival, have not yet been studied specifically within the context of eosinophil biology. Most of our current knowledge of PCD pathways relies on studies using epithelial cell lines and transgenic animal models [71]. Therefore, above mentioned signaling may slightly differ in human peripheral blood eosinophils. Investigation of eosinophil PCD pathways warrants more attention due to lack of knowledge and therapeutic potential.

1.4.3 Corticosteroid-Induced Eosinophil Death

Current therapies in asthma management largely rely on corticosteroids, due to their potent anti-inflammatory effects. One of the known mechanisms by which corticosteroids improve asthma symptoms is through drastic reduction of active eosinophils. This reduction is achieved by inducing eosinophil-apoptosis (among other cells, such as: mast cells and T cells) resulting in increased eosinophil clearance [92]. Kawabori *et al.* [93] were the first to demonstrate that intraperitoneal injections of dexamethasone in rats induced eosinophil apoptotic death and disappearance from the intestinal mucosa. In humans, clinical improvement of asthma by corticosteroid therapy was associated with a higher proportion of apoptotic eosinophils in bronchial biopsies [94], in sputum samples [95, 96], and with eosinophil apoptotic bodies within macrophages [96]. *In vitro*, the detection of apoptotic characteristics on peripheral blood eosinophils, such as PS exposure, is usually detectable after 24-72 hours of exposure to clinically effective concentrations of corticosteroids [97]. Specifically, Dexamethasone at 1 μM has been shown to induce eosinophil apoptosis within 24 hours *in vitro* [98]. Although corticosteroids are commonly used to treat asthma, knowledge on the intracellular signaling of such molecules leading to eosinophil apoptosis is limited. At present, evidence suggests that dexamethasone-induced apoptosis, although associated with the activation of c-Jun N-terminal kinase (JNK) and p38

MAPK, is regulated by unknown caspases (*i.e.,* not through apoptosis-related caspase-3 and -8), in human eosinophils [98].

Regrettably, current corticosteroid treatments in asthma are not perfect, in particular for corticosteroid-resistant asthmatics [44, 92], which has prompted research toward optimizing corticosteroid response. Interestingly, vitamin D has been linked to differences in the response to corticosteroid treatment. Vitamin D deficiency is currently discussed as a factor in steroid-resistance [99]. Since corticosteroid and calcitriol are structurally similar as well as share signaling properties [8], direct interactions between these two molecules is plausible. Corticosteroids, also known as glucocorticoids, mediate their effects through the Glucocorticoid receptor (GR). GR translocates from the cytosol to the nucleus once activated by ligand binding leading to dimerization and direct interactions with glucocorticoid-responsive elements (GREs) to regulate expression of specific genes, such as mitogen-activated kinase phosphatase 1 (MKP-1). MKP-1 is known to inhibit pro-inflammatory mediator production via the MAPK pathway. Recently, Leung *et al.* [100] showed that vitamin D enhances GR activity in monocytes by stimulating glucocorticoid induction of MKP-1. Even though pro-apoptotic effects of corticosteroids have been studied in eosinophils, the relationship between VDR and GR is yet to be examined.

1.4.4 Recruitment of Eosinophils to the Lung

Eosinophils are produced in the bone marrow, yet are primarily tissue-dwelling cells, which rely on various mechanisms in order to enter tissues [50, 57]. Eosinophils are primarily trafficked toward inflamed lung tissue by chemoattractants produced by the airway epithelium and lung-residing immune cells during inflammatory responses.

Although a variety of eosinophil chemokine receptors may be involved in eosinophil recruitment, a few have been prioritized in asthma research, such as CCR3 and chemoattractant receptor-homologous molecule expressed on Th2 (CRTh2) [43, 50]. CCR3, which binds a total of 11 ligands, is selectively and abundantly expressed on eosinophils [43]. Accordingly, evidence demonstrates that CCR3, when bound to chemokine (C-C motif) ligand (CCL) 11, is crucial in the recruitment and accumulation of eosinophils in experimental models of asthma, and other eosinophilic disorders [43, 101, 102]. Furthermore, CCR3 antagonists reduce airway eosinophilia in chronic experimental asthma [103, 104]. In humans, increased expression of CCR3 associates with greater asthma severity [105].

Similarly, CRTh2 has been shown to mediate eosinophil recruitment to lung in experimental modes of asthma [106, 107]. This receptor binds specifically to prostaglandin D_2 (PGD_2), a product released by activated inflammatory cells during an allergic response [43]. Consequently, CRTh2 antagonist therapies do partially attenuate pulmonary eosinophilia in various models [108, 109]. Although several CRTh2 antagonists are currently available, their efficacy against asthma in humans continues to be studied. Thus far, the use of a CRTh2 antagonist OC000459 in patients with chronic asthma, who were not on other asthma management treatments, improved: FEV_1, quality of life, night-time symptom scores and eosinophil counts [110].

There is extensive literature surrounding the roles of CCR3 and CRTh2 on eosinophils in allergic inflammation. It has been shown that both chemokine receptors mediate eosinophil recruitment to lungs in experimental asthma. Hence, CCR3 and CRTh2 are classic markers in asthma research.

Interestingly, there are a variety of under-studied eosinophil receptors which have recruitment functions and, thus, therapeutic potential. One chemokine receptor of interest, which is expressed by eosinophils, is CCR4. It is known that CCR4 particularly binds to inflammatory mediators, such as: Stromal cell-derived factor 1 (SDF-1) and Thymus and activation regulated chemokine (TARC). Furthermore, eosinophils collected from bronchoalveolar lavage (BAL) in allergic patients are known to highly express CCR4 [111]. Unfortunately, CCR4 remains to be functionally described in eosinophils. Otherwise, CCR4 has been shown to propagate airway inflammation when expressed on Th2 cells. Recently, the elimination of CCR4 in T cells was considered sufficient to control allergic airway inflammation as well as airway hyperresponsiveness in mice [111]. Consequently, an extensive review on chemokines in airway inflammation has named CCR4 as a new target for asthma therapy [112].

Another receptor which is gaining attention in allergy research is CCR10. CCR10 is a chemokine receptor which is primarily known to serve as a mucosal homing receptor. Thus far, it has mainly been studied within the context of skin immunity on lymphocytes [113]. Two major cell populations seem to express CCR10: T cells and B cells. Although various sub-types of T cells may express CCR10, Homey *et al.* demonstrated a significant expression of CCR10 in cutaneous lymphocyte-associated antigen (CLA) positive T cells from human blood, dermal microvascular endothelium and fibroblasts [114]. In respect to B cells, Kunkel *et al.* [115] have shown that IgA

antibody-producing B cells (*i.e.,* plasma cells) express CCR10 for their migration and maintenance at mucosal sites. Although not functionally defined, CCR10 is also known to be expressed by Langerhans, melanocytes and dermal endothelial cells [113]. Recent studies have revealed that CCR10 (and its known ligands: CCL27 and CCL28) are factors in several atopic diseases [113]. CCL27 is predominantly expressed by keratinocytes while CCL28 is expressed by epithelial cells from various mucosal tissues [113]. Thus, CCR10 expression allows cells to enter specific mucosal tissues.

Some chemokine receptors also activate eosinophils to release pro-inflammatory mediators. The subject of eosinophil activation and release of mediators has incited research into another area of eosinophilic asthma, termed eosinophil degranulation [43].

1.4.5 Eosinophilic Degranulation

1.4.5.1 Eosinophil Granules and Modes of Degranulation

Eosinophils are generally known to be potent inducers of inflammation due to the release of granule content. Eosinophilic granules contain over 35 different types of chemokines, cytokines and growth factors in addition to cytotoxic granule proteins [75]. Major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPX), in particular, are considered to be involved in asthma pathogenesis [116]. From all of the studied eosinophil granule proteins, EPX is recognized to be the most specific to the eosinophil granulocyte lineage. Lee *et al.* [51] developed an eosinophil-deficient transgenic mouse, which they named PHIL, by silencing the EPX gene. While other eosinophil granule protein genes caused decreases in other lineages, EPX silencing selectively reduced eosinophil counts. EPX, and other granule proteins, are released through a process termed 'degranulation' which refers to the release of preformed, granule-stored components. In general, there are four degranulation modes described for eosinophils: compound exocytosis, classical exocytosis, cytolysis and piecemeal degranulation (PMD). Current literature seems to focus on the latter mode, since granule exocytosis is rarely documented during inflammatory responses while PMD and cytolysis are more frequently documented during human diseases [117]. According to Erjefalt *et al.*, the release of mediators from eosinophils in airway lumen occurs by PMD, in approximately 67% of tissue eosinophils, and by cytolysis, characterized by a break in the integrity of the cell membrane, in 33% of eosinophils [118, 119].

PMD is defined as selective release of eosinophil granules through secretory vesicles, resulting in intact eosinophils harboring empty granules [120]. During PMD, activated human eosinophils execute vesicular trafficking which directs transport of granule-stored proteins from secretory granules to the cell surface [117]. In short, stimulus-induced release of preformed eosinophil granule proteins occurs from granule-derived vesicles, not granules themselves. Loaded vesicles dock at specific cell membrane areas, and release their contents through membrane fusion. Although mechanisms involved in the docking and fusion of eosinophil secretory vesicles at the cellular membrane remain to be fully elucidated, a few key molecules have been identified. Lacy *et al*. [121] demonstrated that eosinophil secretory vesicles express vesicle-soluble NSF attachment receptor (v-SNARE) vesicle associated membrane protein (VAMP)-2, which co-localizes with CCL5 during IFN-γ-induced PMD of CCL5. Furthermore, Logan *et al.* [122, 123] showed that v-SNARE mediates specific membrane docking through interactions with plasma membrane t-SNARES, soluble NSF attachment protein (SNAP)-23 and syntaxin-4. In contrast, cytolysis causes intact granules to be released due to plasma membrane disintegration. Unlike PMD, where eosinophils remain viable, cytolysis results in cell demise [120]. Although eosinophil cytolysis has been thoroughly observed, mechanisms regulating this mode of degranulation in eosinophils have yet to be eluded.

1.4.5.2 PAF-induced Eosinophil Degranulation

Platelet activating factor (PAF) is a phospholipid mediator released by activated macrophages, mast cells and basophils, which motivates pathophysiological inflammation. PAF-mediated responses in human eosinophils include: chemotaxis, superoxide production, adhesion, and release of cationic granule proteins [124-131]. This mediator initiates the latter actions through PAF receptor (PAFR) signaling. PAFR is a G-coupled receptor present on human eosinophils, which has been extensively studied in relation to anaphylaxis [132-136].

As previously elaborated, eosinophil degranulation is of high importance in allergic inflammation [63, 75, 137]. Usually, asthma therapies mainly aim to eliminate eosinophils in order to decrease lung eosinophilia as well as asthma symptoms [43]. Hence, maintaining eosinophil survival in asthma and allergy is beyond traditional therapeutic approaches. It may be suggested that vitamin D metabolites could cause lower responsiveness to common stimuli, such as PAF (*i.e.,* eosinophils may enter an anergic-like state). As mentioned, human studies seem to suggest that

vitamin D drives anti-inflammatory processes [14]. Anergy is defined as a generalized functional inactive state, which occurs intrinsically, where immune cells remain viable yet desensitized. Consequently, anergy is broadly known as a mechanism of tolerance in immunology. Although it has primarily been studied in T cells, anergy is believed to be possible in most immune cells [138]. Studying PAF sensitivity of eosinophils, in the context of vitamin D, is pertinent for clarifying unresponsiveness to degranulation stimuli, in the form of anergy, as an anti-inflammatory mechanism.

1.4.5.3 Eosinophil Cytolysis as a Cell Demise Process

Even though most reports do not boldly make this link, cytolytic death can also be termed 'necrosis'. Against traditional outlook of PCD, various studies have shown that the necrotic process is also regulated by signaling pathways (newly termed 'necroptosis' to imply its regulated nature), which heavily cross-talk with apoptosis while having specific immune functions [78, 85]. Unfortunately, the topic of eosinophil cytolysis is often not taken into account in leading reviews whereas studies observing cytolysis lack proper discussion of the matter [75]. While necrosis has yet to be studied in eosinophils, our team had the privilege to present possible functional mechanisms related to observations of eosinophil necrosis and cytolysis. This review is included in **Appendix 1**. While the subject is debated, mounting evidence of eosinophil cytolysis and cytolytic release of granules in inflammatory conditions compels us to carry out more research on these mechanisms.

1.4.5.4 Eosinophil Cytolysis in Airway Inflammation

As previously reviewed, the presence of cytolytic eosinophils is a common observation in tissues exhibiting eosinophilic inflammation [139]. Specifically, there is evidence that cytolytic eosinophils as well as clusters of free granules are present in inflamed airway mucosa [75]. Furthermore, allergen exposure induces mucosal eosinophilia, PMD and significant amounts of eosinophil cytolysis and deposition of granules in patients [137]. In fact, microscopic observations of human nasal polyps have shown that one-third of the mucosal tissue eosinophil population appears to be undergoing necrosis/cytolysis. The presence of clusters of eosinophil free granules in the bronchial mucosa also correlates with the severity of asthma [75, 140, 141]. Eosinophil cytolysis is also observed in other disorders, such as eosinophilic esophagitis, where eosinophilic inflammation is prominent [142]. Since cytolysis is a distinct mode of degranulation while causing

cell demise, eosinophil cytolysis may be a key mechanism in airway inflammation due to significant release of cytotoxic granule proteins [75, 139].

1.5 The Relationship between Respiratory Syncytial Virus and Eosinophils

In respect to eosinophil pathogen-related functions, various reports suggest that eosinophils exercise anti-viral capacities. The idea that eosinophils can exert anti-viral properties is not new. This perspective of eosinophil biology originates from a collection of studies since the 1990's demonstrating that eosinophil functions are beneficial for viral clearance [143]. The majority of these studies focus on eosinophil granule proteins having RNase activity, such as ECP and EDN, allowing RNA virus break-down [144]. In particular, respiratory syncytial virus (RSV) has received a lot of attention in research due its high prevalence in infants and children. Additionally, RSV had become controversial following vaccination studies from the 1960's which demonstrated the development of hypersentivity towards RSV antigens [143].

The direct response of eosinophils to RSV is recently gaining attention in the field of allergic airway inflammation [145].One study actually demonstrated that eosinophils are recruited and induced to degranulate in lung tissue in response to human RSV infection [146]. There is accumulating evidence that RSV respiratory tract infections in infants are probably linked to the development of recurrent wheezing and childhood asthma. RSV is also interconnected with asthma exacerbations. Newer studies suggest that RSV infection may play a role in the inception of asthma. Furthermore, latent persistent respiratory virus infections may sustain airway inflammation and hyperresponsiveness in a chronic manner [147]. Overall, a recent review on the matter concluded that, although associations have been made between primary RSV infection and pulmonary eosinophilia as well as progression of asthma, there is still no clear link, such as a pathophysiological mechanism [145].

Interestingly, human studies imply an association between vitamin D and respiratory infection clearance. For example, vitamin D deficiency and insufficient UVB light, due to time of year and season, are factors which have been correlated with peaks in respiratory tract infections [148, 149]. More recently, numerous epidemiological studies have found an association between low serum vitamin D and increased susceptibility to respiratory infections [16]. Furthermore, studies have shown that vitamin D seems to dampen inflammatory chemokine expression while potentiating innate immunity, through upregulation of cluster of differentiation (CD) 14 and

cathelicidin, for viral pathogen clearance [15, 150]. The common opinion remains that vitamin D seems to be beneficial against respiratory viruses. One may question whether there is a direct relationship between vitamin D, eosinophil function and RSV infection. At present, all three variables have not been studied together.

1.6 Hypothesis and Objectives

Given the established associations between eosinophil functions and airway inflammatory pathologies, and the complex role of VDR in allergic asthma, we ask the question: "does vitamin D play a modulatory role in eosinophil biology?" Explicitly, we questioned whether the commonality of vitamin D deficiency in allergic inflammatory diseases could be linked to characteristic eosinophil functions involved in immune responses, at the cellular level.

1.6.1 Main Hypothesis

The vitamin D active metabolite, calcitriol, exerts direct effects on human eosinophil functions.

1.6.2 Specific Objectives

We chose to investigate direct effects of calcitriol on human peripheral blood eosinophils from mildly eosinophilic donors, *ex vivo*. We were specifically interested in the capacity of calcitriol to modulate three functional aspects of eosinophil biology:

1) **Define the effect of calcitriol on eosinophil survival**

   We wanted to investigate whether calcitriol could influence eosinophil survival in a short-term (*i.e.,* 24 hours) or long-term (*i.e.,* 14 days) manner. We also tested if calcitriol would have a combined effect with anti-apoptotic cytokines, including IL-5 and IFN-γ. We hypothesized that calcitriol may decrease eosinophil survival, as supported by the ability of calcitriol to induce apoptosis in cancer cells [3].

2) **Investigate if calcitriol can modulate eosinophil cytotoxic mediator release**

Our aim was to examine the role of calcitriol in cytolytic release of pro-inflammatory factors. Thus, we decided to evaluate the effect of calcitriol on spontaneous release of granules and granule protein EPX. Moreover, we also tested the effect of calcitriol on eosinophils' response to PAF, a degranulation stimulus. According to current suggestive literature that vitamin D has anti-inflammatory properties, we hypothesized that calcitriol would reduce the release of eosinophilic pro-inflammatory mediators.

3) **Determine calcitriol-induced eosinophil phenotype alterations in cell surface receptor expression.**

We were interested in calcitriol possibly modulating eosinophil cell surface receptor expression. We focussed on receptors relevant to asthma pathology: by survival, such as IL-5R, and recruitment, such as CRTh2, CCR10, CCR3 and CCR4. We hypothesized that calcitriol would decrease the expression of these receptors, due to their established or potential role in inflammatory responses.

4) **Determine if calcitriol alters dexamethasone killing of eosinophils.**

Our goal was to investigate whether calcitriol could directly influence the efficacy of dexamethasone in killing of eosinophils. Population studies associate vitamin D deficiency with poor responses to corticosteroids, which suggests a relationship between corticosteroids and calcitriol. Therefore, we hypothesized that calcitriol would improve dexamethasone killing of eosinophils.

5) **Determine if calcitriol effects cytolysis of eosinophils in response to respiratory syncytial virus infection.**

We wished to explore the possibility that eosinophil cytolysis may be a programmed anti-viral response. We wanted to determine if eosinophil viability would decrease in response to RSV infection and reveal if calcitriol could protect against eosinophil death in the presence of RSV. We hypothesized that calcitriol would inhibit RSV cytolytic killing of eosinophils.

CHAPTER 2: MATERIALS AND METHODS

2.1 Blood Donors

Adult blood donors were recruited through advertisement in the Edmonton, Alberta area (**Figure 3**). Recruitment efforts were primarily utilized on the University of Alberta North Campus. Volunteers would contact our research group by email (*i.e.,* pulmres1@ualberta.ca). All donors belonged to the 20-40 years age group. A database was constructed with collected personal information, such as: telephone number, email address, history of allergies/asthma, and current medications. A total of 33 blood donors (17 women and 15 men) were enlisted for this study. All participants were self-reported allergic and/or asthmatic (40% allergic, 15% asthmatic and 45% both), where allergies included were seasonal allergy, food allergy, drug allergy, Hay fever and Rhinitis. Every donor was confirmed to be mildly eosinophilic by white blood cell count (*i.e.,* > 4% total white blood cells). Approval for the study was obtained from the local Ethics Research Board at the Faculty of Medicine and Dentistry (University of Alberta) and all adult subjects gave their informed consent according to the Helsinki protocol.

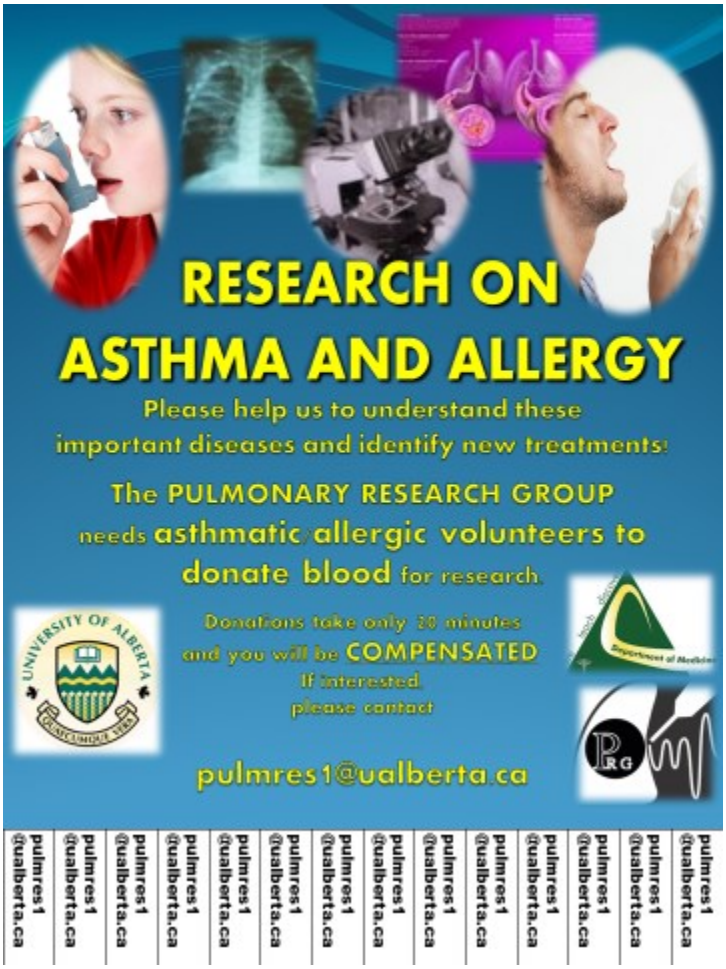


Figure 3: Public advertisement for asthmatic/allergic blood donor recruitment.

2.2 Venous Blood Collection

Donors were monetarily compensated for every visit to the laboratory. A maximum of 100 mL of peripheral venous blood was collected using a winged infusion set (Gauge 25) (BD Vacutainer®, Franklin Lakes, New Jersey, USA) from either the Cephalic or Median Cubital vein in 10 mL EDTA-coated plastic vacutainers (BD Vacutainer®, BD Hemogard™, Franklin Lakes, New Jersey, USA). Blood collection was executed on non-fasting individuals only. Donors were called back following a standard 4-6 week recovery period.

2.3 Peripheral Blood Eosinophil Isolation and Purification

Erythrocytes are sedimented using a dextran gradient. Dextran from Leuconostoc mesenteroides, in solution (6% dextran in RPMI 1640 medium) (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada) was added to fresh blood (*i.e.,* 10 mL of dextran solution per 50 mL of blood). Following a 30 min room temperature incubation, plasma is removed. Granulocytes are separated from mononuclear cells by centrifugation (at 250g for 20 min) using Ficoll-Paque™Plus (GE-HealthCare Ltd. Uppsala, Sweden). The resultant cell pellet is resuspended in molecular biology grade sterile water (HyClone®, Thermo Scientific Inc., Logan, Utah, USA) for a few minutes to lyse remaining contaminating erythrocytes. Cells are then rested on ice in a 1% EDTA and RPMI 1640 medium solution (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada). Mixed granulocyte population is then subdued to a negative selection process in 2% Fetal Bovine Serum (FBS) and 0.2% EDTA in Dulbecco's phosphate buffered solution (DPBS) 1X (GIBCO® Invitrogen, Grand Island, New York, USA; Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada; Hyclone™ Thermo Scientific, Logan, Utah, USA).

This cell-rich fraction was purified using STEMCELL Technologies' EasySep Human Eosinophil Enrichment Kit (Vancouver, British Colombia, Canada) in order to obtain eosinophils only. Briefly, the negative selection is done by adding a cocktail of tetrameric antibodies which recognize: CD2, CD3, CD14, CD16, CD19, CD20, CD36, CD56, CD123 (IL-3Rα) and glycophorin A. As per manufacturer's product information, this formulation of antibodies is specifically targeted for the removal of T cells, NK cells, monocytes, macrophages, neutrophils, dendritic cells, B cells, platelets, erythrocytes, hematopoietic progenitors, myeloid progenitors and basophils (**Table 2**). It must be noted that there are limitations with the particular use of anti-CD16 and anti-CD123 in an eosinophil enrichment process. Firstly, human peripheral blood eosinophils

express CD16. Davoine *et al.* [151] have demonstrated that eosinophils from allergic rhinitis and asthma patients express higher CD16 than non-allergic controls. Furthermore, the expression of CD16 increases on eosinophils during allergen exposure and has been shown to correlate with IFN-γ levels in mild allergic asthmatics. The subpopulation of $CD16^+$ eosinophils were also found to express more IL-5R [151]. Secondly, CD123 (IL-3Rα) is also expressed on circulating eosinophils in normal as well as atopic donors. In fact, eosinophils from allergic dermatitis patients have been shown to have an elevated IL-3R gene expression profile [152]. Therefore the presence of CD16 and CD123 antibodies in the negative selection method contributes to a loss of eosinophils. These select eosinophil subpopulations exhibit distinct phenotypes and probably different activity levels and functions. The use of this antibody cocktail therefore negatively selects for $CD16^-/CD123^-$ eosinophils which alters the final composition of the purified eosinophil population.

Table 2: **Markers recognized by tetrameric antibody cocktail for the removal of selected cells.**

Positive selection markers recognized by tetrameric antibody cocktail	Selected cells for removal
CD2	T cells, NK cells
CD3	T cells
CD14	Macrophages, neutrophils, dendritic cells
CD16	NK cells, neutrophils, monocytes, macrophages, eosinophil subpopulation
CD19	B cells
CD20	B cells
CD36	Platelets, erythrocytes, monocytes
CD56	NK cells
CD123	Hematopoietic progenitor cells, myeloid progenitor cells, eosinophil subpopulation, basophils
glycophorin A	Erythrocytes

The granulocyte-rich cell population is incubated with the latter antibody cocktail for 10 min on ice in 5 mL polystyrene tubes (BD Falcon™, BD Bioscience, Franklin Lakes, New Jersey, USA). Magnetic beads are added in order to allow conjugation with the diverse antibodies to form immunomagnetic beads. Subsequently, polystyrene tubes containing eosinophils, antibodies and magnetic beads are rested in magnetic chambers (EasySep™, STEMCELL Technologies, Vancouver, British Colombia, Canada) for 5 min. During this incubation, immunomagnetic beads bound to unwanted cells remain within the polystyrene tube while unbound eosinophils linger in the solvent, which is poured out. Purified eosinophils are resuspended in RPMI 1640 medium (Sigma-Aldrich Canada Ltd. Oakville, Ontario) and 10% FBS (GIBCO® Invitrogen, Grand Island, New York, USA) for subsequent experiments. Only eosinophil preparations with 98% purity or higher were utilized (contaminating cells were neutrophils and/or lymphocytes).

2.4 White Blood Cell Counts

White blood cell counts were done at three distinct time points (*i.e.,* on freshly collected blood, following the Ficoll step, and subsequent to the negative selection process) during the isolation procedure using Kimura stain (0.05% Toluidine Blue, 0.03% Light Green and saturated saponin 50% ethanol solution in a phosphate buffer; pH 6.4). The specific formulation of Kimura stain components and functions are indicated in **Table 3**.

Cells were visualized by light microscopy using a total magnification of 50X. Counting was done using a total volume of 10 µL in a Hemocytometer, where the total cell count is estimated based on the number of cells present in 4 quadrants of the counting chamber.

Table 3: Kimura stain formulation and specific functions.

Solutions within Kimura stain	Ingredients	Function
Toluidine Blue (0.05%)	• Toluidine Blue (0.05 g) • 1.8% NaCl (50 mL) • 95% Ethanol (10 mL) • Distilled water (40 mL)	Metachromatic stain (*i.e.,* stains nucleus blue)
Light Green (0.03%)	• Light Green (0.03g) • Distilled water (100 mL)	Acidic dye which binds to basic proteins (*i.e.,* stains eosinophil granules green)
Saturated Saponin solution	• Saponin • 50% Ethanol	Lyses red blood cells
Phosphate buffer (pH 6.4)	• Na_2HPO_4 (2.13 g) • K_2HPO_4 (2.04 g) • Distilled water (100 mL)	Solvent

2.5 Eosinophil Incubation Conditions

In all experiments, eosinophils were incubated at 37°C in 5% $CO_{2\ (gaseous)}$ in 24-well flat bottom plates (BD Falcon™, BD Bioscience, Franklin Lakes, New Jersey, USA). Calcitriol (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada) was reconstituted in 100% non-denaturated ethanol, as per manufacturer's instructions. Calcitriol dose (10 nM) used for all experiments within this study was selected as per a dose response. As elaborated in Chapter 3, calcitriol at 10 nM was the smallest concentration which yielded significantly higher eosinophil survival than media alone while having no significant difference compared to IL-5 (1 ng/mL) after a 24 hour dose response (**Figure 4**). Since IL-5 is a crucial factor for eosinophil survival, we used IL-5 (1 ng/mL) as a positive control in each experiment. Its dose was selected as per a previous study which investigated the influence of IL-5 on eosinophil survival in response to glucocorticoids [153]. IFN-γ (100 ng/mL) dose was selected as per a comparative study on the anti-apoptotic ability of IL-5 and IFN-γ [66]. IL-5 and IFN-γ were reconstituted in DPBS 1X with 0.2% Bovine serum albumin (BSA) (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada), as per manufacturer details. All treatments were executed in 24-well flat bottom plates, where ~300,000 eosinophils were incubated per well in 1 mL of RPMI 1640 medium (Sigma-Aldrich Canada Ltd. Oakville, Ontario) with 10% FBS (GIBCO® Invitrogen, Grand Island, New York, USA). Calcitriol (10 nM) (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada), IL-5 (1 ng/mL) (R&D Systems, Minneapolis, Minnesota, USA), IFN-γ (100 ng/mL) (R&D Systems, Minneapolis, Minnesota, USA), Dexamethasone (1 μM) (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada) and RSV (4.73×10^7 PFU/mL) (ATCC®, Manassas, Virginia, USA) were added directly to media using a micropipette at day 0. Media was not changed or supplemented during incubations.

2.6 Viability Assay

Viability levels were measured using Invitrogen's Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen™, Life Technologies, Eugene, Oregon, USA) according to manufacturer's instructions. Briefly, ~300,000 eosinophils were pelleted into a 5 mL polystyrene tube (BD Falcon™, BD Bioscience, Franklin Lakes, New Jersey, USA) by centrifugation (at 200 g for 6 min). Cells were resuspended in Binding Buffer 1X, provided in kit. Annexin-V and Propidium Iodide (PI) were added to eosinophils.

Annexin-V is a cellular protein which binds to PS. Its physiological function is mainly to bind PS expressed by apoptotic cells *in vivo* to inhibit coagulation and encourage inflammation [154]. In the laboratory, annexin-V is commonly used as an early apoptosis marker. During apoptotic death, PS becomes exposed from the inner leaflet onto the outter leaflet of the plasma membrane prior to the formation of apoptotic bodies [154, 155]. There are limitations to this method as apoptosis is a transient cell state, hence its cellular characteristics can be unstable. For example, PS exposure has been shown to be reversible in neutrophils [156]. Nevertheless, PS exposure detection by annexin-V remains a valid method for identifying apoptotic cells as per the Nomenclature Committee on Cell Death's latest recommendations [71]. The annexin-V used in this study was conjugated to the fluorochrome Alexa488 for detection by flow cytometry. Therefore, cells which are positive for annexin-V-Alexa488 are counted as apoptotic.

PI is a fluorescent chemical DNA intercalating agent. It is regularly used to stain DNA, yet is also utilized to identify dead cells [157]. Hence, PI is often referred to as a vital dye [71]. PI does not gain access to cellular DNA unless the cellular membranes have become permeable. During cytolysis, as a result from necrosis, PI gains access to intracellular compartments where it intercalates nuclear and mitochondrial DNA [157]. During membrane breakdown, annexin-V is also able to bind to PS within the inner leaflet of dead cells. Therefore, cells that stain for both annexin-V and PI are necrotic. In contrast, cells which are annexin-V-Alexa488$^-$/PI$^-$ are viable.

Staining reaction was stopped by adding DPBS 1X (HycloneTM Thermo Scientific, Logan, Utah, USA). Acquisition was performed using BD FACS-CANTO flow cytometer. Gating of events was limited to high FS/SS events corresponding to eosinophil cell size and density. Apoptotic cells were single positive for annexin-V. Necrotic cells were double positive for annexin-V and PI, while viable cells were double negative.

2.7 Eosinophil CD63$^+$ Granule and EPX Staining

Eosinophils were incubated in the presence of anti-CD63 or matched isotype control antibodies (AbD Serotec, Kensington, Oxford, England), in order to detect eosinophil granules. These eosinophil preparations were co-stained with anti-EPX-biotin (gift from Lee Lab Mayo Clinic, Arizona, USA) and streptavidin (Affymetrix eBioscience, Inc., San Diego, California, USA). Eosinophils were recovered from the incubation plate into 5 mL polystyrene tubes (BD Falcon^{TM}, BD Bioscience, Franklin Lakes, New Jersey, USA) and washed with Flow buffer (2%

FBS in DPBS 1X) (GIBCO® Invitrogen, Grand Island, New York, USA; HycloneTM Thermo Scientific, Logan, Utah, USA). Cells were centrifuged to form pellets as to remove the supernatant. Eosinophils were incubated with 4% Paraformaldehyde (PFA) solution (PFA in DPBS 1X) (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada; Hyclone^{TM} Thermo Scientific, Logan, Utah, USA) for 20 min to allow fixation. Cells were washed again with Flow buffer before permeabilization on ice using 1% Saponin and 1% FBS in Hank's balanced salt solution (HBSS) 1X (GIBCO® Invitrogen, Grand Island, New York, USA; HycloneTM Thermo Scientific, Logan, Utah, USA). Antibodies were added directly to the latter permeabilization solution. Subsequently, eosinophils were incubated for 20 min at 4^{o}C. Cells were then washed with the permeabilization solution before adding Streptavidin-APC (Affymetrix eBioscience, Inc., San Diego, California, USA). Following another 20 min incubation at 4^{o}C, stained eosinophils were resuspended in Flow buffer with 0.5% PFA (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada). Stained and fixed cells were kept at 4^{o}C and sheltered from direct light before acquisition using BD FACS-CANTO flow cytometer. Gating of events was limited to high FS/SS events corresponding to eosinophil cell size and density. Marker expression was calculated by subtracting isotype fluorescence from specific antibody fluorescence using FACS-CANTO analysis program.

CD63, also called lysosome-associated membrane protein-3 (LAMP-3), is a glycoprotein belonging to the tetraspanin family. It is a membrane bound receptor abundantly found on intracellular vesicles [158]. In research, CD63 is used as a granule membrane marker. Mahmudi-Azer *et al.* [159] have demonstrated that CD63 is expressed on resting human peripheral blood eosinophils, predominantly on crystalloid granule membranes. The latter group also demonstrated an association between CD63 and piecemeal degranulation as CD63 mobilization coincided with selective mobilization of CCL5, before its release [159]. Evidence involving CD63 with eosinophil mediator release is increasing. Recently, CD63 has been shown to colocalize with Ras-related protein Rab27a, a GTPase involved in protein transport, on eosinophil crystalloid granule membranes [160].

EPX was selected as an eosinophil granule protein release marker due to its specificity to eosinophil granulocytes. When Lee *et al.* produced an eosinophil-deficient transgenic line (PHIL) mouse, EPX was the only eosinophil granule protein gene which, when silenced, produced mice lacking eosinophils without effecting the production of other hematopoietic cell types [51].

Although EPX can be a difficult protein to work with due to its negative charge (*i.e.,* EPX is a basic protein), its release can only originate from eosinophils.

The amounts of $CD63^+$ granules and EPX^+ cell debris were obtained by BD FACS-CANTO flow cytometer analysis. Gating of events was limited to low FS/SS events corresponding to cellular debris properties.

2.8 EPX Colorimetric Assay

Eosinophil suspensions in phenol red-free RPMI 1640 medium (Sigma-Aldrich Canada Ltd. Oakville, Ontario) were distributed in a 96-well flat bottom plate (BD Falcon™, BD Bioscience, Franklin Lakes, New Jersey, USA) to contain ~30,000 cells per well. Lysed control consisted of eosinophils subject to -80°C freezing and mechanical stress. The latter processing caused eosinophil cytolysis where maximal granule protein release was attained. PAF_{C16} (Sigma-Aldrich, St. Louis, Missouri, USA) was added to respective wells for 5 min prior to the addition of the peroxidase substrate solution. The peroxidase substrate solution consisted of O-phenylenediamine HCl (OPD) 0.9mg/mL, $CaCl_2$ 1.6mM, 0.004% H_2O_2 in HBSS 1X (HycloneTM Thermo Scientific, Logan, Utah, USA). As per Thermo Fisher Scientific Inc. product information, OPD is a water-soluble substrate for peroxidase which produces a yellow-orange breakdown product. Plate was incubated for 2 min before the reaction was stopped using 4M H_2SO_4. All reactions were executed in isotonic solutions as to avoid washing and removing incubated cells.

The latter described method consists of an improvement developed by Adamko *et al.* to the classic EPO/EPX assay [161]. Since EPX is negatively charged it has a tendency to stick to positively charged surfaces, such as plastics (*i.e.,* the bottom and sides of wells in an incubation plate). Therefore, measuring EPX activity from removed supernatant does not reveal all EPX released in one well. By using isotonic solutions, we were able to directly add OPD to eosinophils, yet avoid eosinophil cytolysis, which permits to exclusively measure extracellular EPX. Also, since OPD has access to both EPX in solution and EPX bound to the plate, results are more accurate.

Each experiment was done in triplicates. Absorbance at 450 nm wavelength was read using BIO-TEK®PowerWaveXS plate reader. All values were normalized to blank readings. Percent

EPX released from non-stimulated eosinophils was obtained using Formula #1. Percent EPX released from PAF_{C16}-stimulated eosinophils was calculated using Formula #2.

Formula #1:

$$\%\ EPX\ released\ into\ media = \frac{EPX\ activity\ in\ media\ (with\ intact\ cells)}{EPX\ activity\ in\ media\ of\ cell\ lysate} \times 100\%$$

Formula #2:

$$\%EPX\ released\ into\ media = \frac{EPX\ activity\ in\ media\ with\ PAF\ (with\ intact\ cells) - EPX\ activity\ in\ media\ (with\ intact\ cells)}{EPX\ activity\ in\ media\ of\ cell\ lysate} \times 100\%$$

2.9 Cell Surface Marker Staining

Surface marker expression was measured using the above listed antibodies following respective manufacturers' instructions. Briefly, treated eosinophils were pelleted as per treatment by centrifugation (at 200g for 6 min). Cell pellets were resuspended in Flow Buffer, which consisted of 2% FBS (GIBCO® Invitrogen, Grand Island, New York, USA) in DPBS 1X (HycloneTM Thermo Scientific, Logan, Utah, USA). Separate antibodies were added to each treatment sample (**Table 4**). Following incubation on ice, Flow buffer was used to wash cells while 4% PFA solution, containing PFA (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada) diluted in DPBS 1X (HycloneTM Thermo Scientific, Logan, Utah, USA), allowed cells to be fixed. Samples were stored at 4°C prior and sheltered from direct light acquiring results. Acquisition was performed on stained eosinophils using BD FACS-CANTO flow cytometer. Gating of events was limited to high FS/SS events corresponding to eosinophil cell size and density. Marker expression was calculated by subtracting isotype fluorescence from specific antibody fluorescence using FACS-CANTO analysis program. Data for percent eosinophils positive as well as Mean fluorescence intensity (MFI) were included in our investigation. On one hand, the percentage of eosinophils positive for a marker provides information about the amount of eosinophils which express the receptor among the total population. On the other hand, MFI reveals the average intensity for this receptor which indicates the average amount of receptor expression per eosinophil. Both measurements are needed to understand how many eosinophils express a certain marker, and with what relative intensity.

Table 4: Cell surface receptors were detected using monoclonal antibodies and isotype controls.

Receptor / Isotype control	**Antibody and Source**
CRTh2	Anti-Human CD294 (CRTh2) Alexa Fluor® 647 (Clone BM16), Rat IgG2a, κ (BD^{TM}Pharmingen, San Diego, California, USA)
CRTh2 Isotype control	Rat IgG2a, κ Alexa Fluor® 647 Isotype Control (Clone R35-95) (BDTMPharmingen, San Diego, California, USA)
CCR10	Anti-Human CCR10 Allophycocyanin MAb (Clone 314305), Rat IgG2A (R&DSystems, Minneapolis, Minnesota, USA)
CCR10 Isotype control	Rat IgG2A Allophycocyanin Isotype Control (Clone 54447) (R&DSystems, Minneapolis, Minnesota, USA)
CCR3	Anti-Human CD193 (CCR3) Alexa Fluor® 647 (Clone 5E8), Mouse IgG2b, κ (BDTMPharmingen, San Diego, California, USA)
CCR3 Isotype control	Mouse IgG2b, κ Alexa Fluor® 647 Isotype Control (Clone 27-35) (BDTMPharmingen, San Diego, California, USA)
CCR4	Anti-Human/Rat CCR4 Phycoerythrin MAb (Clone 205410), Mouse IgG2B (R&DSystems, Minneapolis, Minnesota, USA)
CCR4 Isotype control	Mouse IgG2B Phycoerythrin Isotype Control (Clone 133303) (R&DSystems, Minneapolis, Minnesota, USA)
IL-5Rα	Human IL-5 R alpha Fluorescein MAb (Clone 26815), Mouse IgG1 (R&DSystems, Minneapolis, Minnesota, USA)
IL-5Rα Isotype control	Mouse IgG1 Fluorescein Isotype Control (Clone 11711) (R&DSystems, Minneapolis, Minnesota, USA)
IL-5Rβ	Anti-Human CD131 Phycoerythrin (Clone 1C1), Mouse IgG1κ (affymetrix eBioscience, Inc., San Diego, California, USA)
IL-5Rβ Isotype control	Mouse IgG1κ Isotype Control Phycoerythrin (Clone P3.6.2.8.1) (affymetrix eBioscience, Inc., San Diego, California, USA)

2.10 Dexamethasone Assay

Purified eosinophils were treated with water soluble Dexamethasone (1 μM) (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada). Dexamethasone stock solution was diluted in RPMI 1640 medium and added directly to media. Besides its commonality in current literature, dexamethasone dose (1 μM) was selected as per a study focussed on eosinophil survival in response to dexamethasone and IL-5 by Bloom *et al.* [153]. Viability levels were measured using Invitrogen's Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen^{TM}, Life Technologies, Eugene, Oregon, USA) according to manufacturer's instructions. Acquisition was performed using BD FACS-CANTO flow cytometer. Viable cells were double negative while dead cells were double positive for annexin-V and PI.

2.11 Respiratory Syncytial Virus Assay

Eosinophils were incubated with RSV at a 157:1 virus to cell ratio (*i.e.,* 4.73 x 10^7 PFU/mL) (ATCC®, Manassas, Virginia, USA). The stock of RSV used was a gift from the Adamko lab (Saskatoon, Saskatchewan, Canada) and dosage was based on their previous experience with RSV [162]. RSV was diluted in DPBS 1X (HycloneTM Thermo Scientific, Logan, Utah, USA). RSV control group consisted of UV-inactived RSV (UV). Briefly, 10 μL of active RSV in DPBS 1X (HycloneTM Thermo Scientific, Logan, Utah, USA) was exposed to UV-light for 30 min. Viability levels were measured using Invitrogen's Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen^{TM}, Life Technologies, Eugene, Oregon, USA) according to manufacturer's instructions. Acquisition was performed using BD FACS-CANTO flow cytometer. Viable cells were double negative while dead cells were double positive for annexin-V and PI.

2.12 Statistical Analysis

All results are expressed as mean ± standard error of the mean. Comparison between groups was done using analysis of variance with one-way ANOVA and paired *t*-test as statistical tests (Graph Pad Prism Software®; Origin 9®) where p-value <0.05 was considered significant.

CHAPTER 3: Manuscript in preparation

Calcitriol (1,25 Dihydroxyvitamin D_3) reduces eosinophil necrosis leading to diminished release of cytotoxic granules and eosinophil peroxidase (EPX).

3.1 Introduction

The eosinophilic granulocyte is one of the most prominent inflammatory cells that characterize allergic airway inflammation. Classically, lower numbers of eosinophils in lungs and blood correlate with an improvement in allergic symptoms, and has been generally considered as a sign of improved control of bronchial inflammation [50]. Eosinophils are especially characterized for their capacity to release (*i.e.,* degranulate) highly cytotoxic granules and a variety of mediators, which can cause direct tissue injury as well as locally incite inflammation [43, 163-165]. One major mode for eosinophil degranulation is cytolysis [75, 139, 166]. During cytolysis, mediator release appears to happen spontaneously (often referred to as cytolytic degranulation) or is linked to necrosis, which results in a cytolytic death. Fairly recent work has reported an association between the regulation of eosinophil death signalling pathways and the pathological role of eosinophils in the progression of allergic inflammation [167, 168]. More specifically, it is now accepted that delayed-apoptosis or insufficient apoptosis of eosinophils is an important factor in the pathology of asthma [55, 56, 59]. In fact, airway inflammation caused by asthma-associated insults, such as epithelial shedding and allergic immune responses, induces rapid death of eosinophils causing granule deposition in airway tissues [139]. Cytolysis, causing release of intracellular components, is the characteristic result of necrotic death. Necrosis seems to be a significant process since eosinophil cytolysis has often been observed in human [75, 137, 169, 170] and animal [58] tissues undergoing eosinophilic inflammation. Therefore, we developed an interest in modulating eosinophil cell death to avoid eosinophilic cytotoxic mediator release with the goal of minimizing the presence of pro-inflammatory components originating from these granulocytes within their surrounding environments. One candidate of interest is the vitamin D_3 active metabolite, due to extensive literature of successful cell cycle arrest as well as an increase in apoptotic cancer cells [1, 3] as well as a single recent report that 1,25-dihydroxyvitamin D_3 was able to maintain the survival of peripheral blood eosinophils from healthy donors [42].

Vitamin D is a fat-soluble lipid within the secosteroid group, primarily known to play an essential role in the regulation of calcium absorption from the gastrointestinal tract. There is considerable evidence supporting alternative functions of vitamin D, beyond calcium regulation. In fact, more than 1000 genes are under the control of the VDR, which is essential for directly mediating the various effects of vitamin D [171]. Accordingly, vitamin D deficiency in humans has been associated with an increase (and/or adverse outcomes) in autoimmune diseases,

infections, cardiovascular diseases, cancer, and is apparently linked to a higher risk of mortality by all causes [1]. Furthermore, direct evidence supporting a role for vitamin D in asthma has been revealed from genetic studies of VDR polymorphisms as a risk factor in different human populations [32, 172-174]. Vitamin D deficiency has also been studied in relation to the emergence of airway diseases, in accordance with the hypothesis that a deficiency in vitamin D is associated with immune system impairment leading to inflammatory and autoimmune manifestations. In multiple studies, low serum vitamin D (25-(OH)D_3) has been associated with poor lung function, as well as increased airway hyperactivity and asthma, in adults as well as adolescents [17]. Also, lung epithelial cells express the enzyme 1α-hydroxylase, which catalyzes the conversion of vitamin D precursor calcidiol (25-(OH)D_3) to the active form, calcitriol (1α,25-$(OH)_2D_3$) [15]. This function of lung epithelia suggests that local concentrations of calcitriol are able to rise independently from circulating levels, and may reach higher concentrations than systemic vitamin D. It also implies an immunologic role for this particular microenvironment, beyond calcium regulation. Despite the well-known role of calcitriol in immune responses, the mechanism(s) by which calcitriol may mitigate allergic inflammation in asthma is largely unknown.

In this study, we report that calcitriol can directly regulate eosinophil inflammatory effects by reducing necrotic release of intact granules as well as active EPX. We propose that reducing eosinophil cytolytic degranulation of proinflammatory mediators could minimize mucosal tissue injury in airway allergic inflammatory pathologies.

3.2 Materials and Methods

3.2.1 Blood Donors

Adult blood donors were recruited through general publicity in the Edmonton, Alberta area. Blood collection was restricted to adult donors (*i.e.,* minimum 18 years old) who had mild eosinophilia (*i.e.,* >4% of total white blood cell count) as well as self-reported allergies and/or allergic asthma, while not currently using corticosteroids. Approval for the study was obtained from the local Ethics Research Board at the Faculty of Medicine and Dentistry (University of Alberta) and all adult subjects gave their informed consent according to the Helsinki protocol.

3.2.2 Peripheral Blood Eosinophil Isolation

Peripheral blood eosinophils from atopic donors were purified as previously described [162]. Briefly, venous blood (100 ml) was collected from the Cephalic or Median Cubital veins in EDTA-coated vacutanors (BD Vacutainer®, BD Hemogard™, Franklin Lakes, New Jersey, United States of America). Erythrocytes were sedimented using a Dextran gradient (6% in RPMI) (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada). Granulocytes were separated from mononuclear cells by centrifugation using Ficoll-Paque™Plus (GE-HealthCare Ltd. Uppsala, Sweden). Eosinophils were further purified using EasySep Human Eosinophil Enrichment Kit (Stemcell Technologies™, Vancouver, British Columbia, Canada) as per manufacturer's instructions. Purity of eosinophil preparations were greater than 98%: (contaminating cells were neutrophils and/or lymphocytes).

3.2.3 Viability Assay

Viability levels were measured using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen™, Life Technologies, Eugene, Oregon, USA) according to manufacturer's instructions. Acquisition was performed using BD FACS-CANTO flow cytometer. Viable cells were double negative, while necrotic cells were double positive for annexin-V and PI.

3.2.4 EPX and Eosinophil Granule Staining

Eosinophils were incubated in the presence of anti-CD63 (AbD Serotec, Kensington, Oxford, England) or matched isotype control (AbD Serotec, Kensington, Oxford, England) antibodies, in order to detect granules. These eosinophils were co-stained with anti-EPX-biotin (gift from Lee Lab Mayo Clinic, Arizona, USA) and streptavidin (Affymetrix eBioscience, Inc., San Diego, California, USA). Both detections were done using the manufacturers' protocols. The levels of free EPX as well as intact granules were obtained by BD FACS-CANTO flow cytometer analysis.

3.2.5 EPX Colorimetric Assay

Measurement of EPX release has been previously described [162]. Briefly, eosinophil suspensions in phenol red-free RPMI (Sigma-Aldrich Canada Ltd. Oakville, Ontario) were distributed in a 96-well plate to contain ~30,000 cells per well. For lysed control, eosinophils were subject to -80°C freezing and mechanical stress. PAF_{C16} (Sigma-Aldrich, St. Louis, Missouri, USA) was added to respective wells for 5 min prior to the addition of the peroxidase substrate solution OPD 0.9mg/mL, $CaCl_2$ 1.6mM, 0.004% H_2O_2 in HBSS) 1X as positive control. Plate was incubated for 2 minutes before the reaction was stopped using 4M H_2SO_4 (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada). Results were calculated as:

$$\%\ EPX\ released\ into\ media = \frac{EPX\ activity\ in\ media\ (with\ intact\ cells)}{EPX\ activity\ in\ media\ of\ cell\ lysate} \times 100\%$$

Each experiment was done in triplicate and absorbance at 450 nm wavelength was read using BIO-TEK®PowerWaveXS plate reader. All values were normalized to blank readings.

3.2.6 Statistical Analysis

All results are expressed as mean ± standard error of the mean. Comparison between the groups was done using analysis of variance with one-way ANOVA and paired *t*-test statistical tests (Graph Pad Prism Software®) where p-value <0.05 was considered significant.

3.3 Results

3.3.1 Calcitriol Modulates Eosinophil Survival

To determine if vitamin D influences eosinophil survival, we investigated whether the most active metabolite of vitamin D_3, calcitriol (1α,25-$(OH)_2D_3$), may influence apoptotic and necrotic cellular states of eosinophils. Blood eosinophils from atopic donors were incubated with physiologically relevant concentrations of calcitriol (*i.e.,* 0.05 to 100 nM). Although vitamin D sufficiency is clinically defined by calcifediol blood concentrations, which are always higher than calcitriol [1], calcitriol is suggested to be at even higher levels at the mucosal level [15]. We observed a dose-dependent reduction of necrotic eosinophils which resulted in an increase of viable eosinophils in response to increasing doses of calcitriol after 24 hours of incubation. Interestingly, calcitriol did not cause a significant shift in levels of apoptosis within these

eosinophil populations (**Figure 4A**). Although the effect of calcitriol on eosinophil survival was perceptible at 0.05 nM, it was significantly increased from media, yet not significantly different than IL-5, at 10 nM. Therefore, the latter concentration was selected for future experiments (n = 5, IL-5 *vs.* Calcitriol, $p < 0.05$) (**Figure 4B**). Thus, we demonstrate that calcitriol is able to independently sustain the viability of human blood-derived eosinophils for 24 hours *ex vivo*, in the absence of an anti-apoptotic factor.

In order to evaluate the duration of this effect, we performed eosinophil survival assays using calcitriol (10 nM) over a 14 day time period. Using this design, we observed that calcitriol (10 nM), on its own, is insufficient in sustaining eosinophil viability above 75% in a long term manner (*i.e.,* > 3 days). We consistently observed that results for Media and VD treatments grouped together while IL-5 and IL-5+VD treatments congregated, over time. After 3 days of incubation, there was no significant difference between non-treated and calcitriol (10 nM)-treated eosinophils (day 3, VD 33.5% ± 9.3% *vs.* Media 15,6% ± 8.5%, $p > 0.05$, n = 5). However, when calcitriol is added to IL-5, we observe a marked increase of viable eosinophils. We observed a significant potentiating effect of calcitriol when co-incubated with IL-5 (1 ng/ml) after 4 days of culture. This effect not only persists, but upsurges over the following 10-day period. This difference between IL-5 (1 ng/ml) and calcitriol (10 nM) *versus* IL-5 (1 ng/ml) alone respectively occurred at: 7 days; 20%, 10 days; 24% and 14 days; 32%. Hence, the latter finding depicts a synergistic effect of calcitriol with IL-5 beginning at day 7 (IL-5+VD 86.4% ± 1.5% *vs.* IL-5 66.0% ± 2.4% and VD 4.5% ± 1.5%, $p < 0.05$, n = 12) (**Figure 5A**). To determine if this coupled effect is specific to IL-5R, which signals using the IL-5, IL-3 and GM-CSF common β chain [62], we tested if calcitriol could potentiate the pro-survival of another type of cytokine. Thus, IFN-γ was chosen to repeat the viability assay. We monitored the effect of both IFN-γ (100 ng/ml) in combination with calcitriol (10 nM) on eosinophil survival on a 7-day time course. Although IFN-γ (100 ng/ml) is overall less potent than IL-5 (1 ng/ml) in maintaining the viability of eosinophil populations, we were able to reproduce an analogous trend of effects in comparison to IL-5 and calcitriol experiments through the addition of both IFN-γ (100 ng/ml) and calcitriol (10 nM) treatment over 7-day long experiments. Again, the difference in percentage of viable eosinophils between IFN-γ (100 ng/ml) and calcitriol (10 nM) *versus* IFN-γ (100 ng/ml) alone indicates a synergistic effect on viability at day 7 (IFN+VD 43.6% ± 8.6% *vs.* IFN 21.9% ± 7.5% and VD 6.6% ± 2.0%, $p < 0.05$, n = 5). Accordingly, we confirm that the effect of calcitriol on eosinophil

survival is not IL-5 dependent (**Figure 5B**). We further observed, despite the increase in eosinophil survival and complimentary decrease in necrosis, no significant increase in apoptosis (*i.e.,* eosinophil annexin-V single positives) throughout the long-term time course for both combination treatments (*i.e.,* IL-5+VD and IFN+VD) (**data not shown**).

3.3.2 Calcitriol Influences Necrotic Release of Pro-Inflammatory Components

We then explored the idea whether the observed effects of calcitriol on increasing eosinophil viability are caused by inhibition of eosinophil necrosis which, consequently, would reduce the release of granules and pro-inflammatory mediators into media caused by this cytolytic process. This avenue was investigated due to observations made in cellular debris, which was assumed to result from eosinophil necrotic death, which can be discriminately visualized due to their low FS/SS properties compared to intact cells using flow cytometry. As demonstrated in **Figure 6A,** treatment with IL-5 and calcitriol for a duration of 7 days results in increased amounts of high FS/SS events (left panel, upper right quadrant), corresponding to intact eosinophils, compared to low FS/SS events (left panel, lower left quadrant), corresponding to eosinophilic cell debris. Furthermore, the amounts of cell debris appear to be larger with an increased incidence of necrosis (right panel, upper right quadrant). The most visually significant and relative decrease in cell debris occurs within the IL-5 (1 ng/ml) and calcitriol (10 nM) treatment population (**Figure 6A**). The percentage of cell debris in media resulting from co-incubation of IL-5 (1 ng/ml) and calcitriol (10 nM) is significantly lower than IL-5 alone treatment (IL-5+VD 10.8% ± 1.8% *vs.* IL-5 17.7 % ± 2.6%, $p = < 0.05$, n = 10) (**Figure 6B**). We observed that IL-5 (1 ng/ml) and calcitriol (10 nM) combination limits the amount of eosinophilic cell debris in a pattern which is reflective of eosinophil necrosis levels (**Figure 6A&B)**.

Since the nature of cellular debris is complex, we wished to confirm the presence of granules as well as cytotoxic mediators originating from eosinophil granules. Therefore, we stained permeabilized and non-permeabilized eosinophil preparations following a 7-day long treatment for granules in media only, using the CD63 granule surface marker, and EPX, a cytotoxic protein unique to eosinophil granules. We confirmed the presence of intact granules as well as free-EPX+ particles in the cell debris from these non-treated eosinophil preparations (**Figure 7**).

To investigate the spontaneous release of active EPX from eosinophils, we measured levels of active EPX in the media following treatments. These results revealed a lower abundance of

active EPX in the groups containing calcitriol (10 nM) in comparison to their respective controls (**Figure 8A**). Moreover, all treatment groups had significantly more EPX release compared to IL-5+VD group (IL-5+VD 7.9% ± 1.2% *vs.* IL-5 24.4% ± 3.1%, *vs.* VD 64.3% ± 8.0%, *vs.* Media 72.3% ± 7.1%, $p < 0.01$, n = 9). We observe a unanimously consistent decrease in EPX release by adding calcitriol (10 nM) directly compared to IL-5 (1 ng/mL) alone (**Figure 8B**). These results depict a tendency for EPX release which is analogous to the trends in cell debris (and necrosis) levels, yet complementary to viability patterns (**Figures 5A,6&7**). We also determined if calcitriol would impact eosinophil sensitivity to a stimulus, specifically in relation to eosinophil degranulation, such as PAF. We report that calcitriol treatment did not affect viable eosinophil EPX release in response to PAF at day 7. In fact, treatment groups containing IL-5 (1 ng/mL) were still capable of releasing EPX upon PAF stimulus while other groups were not (**Figure 9**). When we explored eosinophil degranulation, we found differences in EPX release in response to PAF among most treatment groups. Media only and calcitriol (10 nM)-treated eosinophils released significantly less EPX than groups containing IL-5 (1 ng/mL). Media and VD did not differ in percentage EPX released. Likewise, IL-5 and IL-5+VD groups were not significantly different from one another. Hence, Media and VD data grouped together while IL-5 and IL-5+VD results were very similar. Overall, these results show that calcitriol's effect on EPX release obtained through PAF stimulation had an inverse trend to results from spontaneous EPX discharge. Again, sensitivity to PAF seems to reflect the levels of viable eosinophils in all treatment groups at day 7 (**Figures 5A&8A**). By matching data from the various EPX assays to their respective viability data, we observed an inverse correlation between the amount of EPX observed in media and eosinophil viability ($r^2 = 0.9854$, $p < 0.05$) (**Figure 10**).

3.4 Discussion

Eosinophils are usually known to release their granules by exocytosis, which can occur through the regulated process of PMD [117, 175]. The discharge of eosinophil granules can also be the result of cytolysis (related to necrotic death). Necrosis is often defined as a pro-inflammatory event since the liberation of intracellular contents into tissues acts as natural adjuvants which can initiate various immunological activation mechanisms [78]. This is in contrast to apoptosis, which is a prominent anti-inflammatory mechanism for controlling immune cell populations. In asthmatics, apoptotic eosinophils can be detected in the sputum during recovery of acute asthma

exacerbations, requiring corticosteroid treatment. This suggests that eosinophil apoptosis is important in the clearance of airway eosinophils for resolution of inflammatory responses [96]. Hence, promoting the apoptotic pathway, in preference to necrosis, is one of the current therapeutic approaches for asthma management [50, 176]. Interestingly, observations of bronchial tissue seem to lack apoptotic eosinophils. Research by Kodama *et al.* failed to show apoptotic eosinophils in mouse tissues following allergic inflammation [177]. Moreover, evidence demonstrates that cytolytic eosinophils and eosinophilic granules are commonly found in allergic airway mucosa [75]. In humans, allergen exposure causes mucosal eosinophilia, accompanied by eosinophil PMD, eosinophil cytolysis, and deposition of granules [137]. Microscopic observations of bronchial mucosa suggest that 33% of mucosal tissue eosinophils are undergoing cytolysis, while the remaining 67% were undergoing PMD. The presence of cell-free granules in the bronchial mucosa also correlates with asthma severity [139-141]. In fact, epithelial damage and loss as a result of specific eosinophil granule proteins: MBP, ECP and EPX, are collectively believed to play a significant role in asthma pathology. It is proposed that MBP and EPX are directly involved in epithelial damage, bronchospasm as well as increasing vascular permeability in the lung [116].

Calcitriol was able to significantly reduce eosinophil spontaneous release of EPX but not PAF-induced EPX release, when combined with an anti-apoptotic cytokine. PAF induces many eosinophil functions in inflammatory responses, thus it may be an important factor for eosinophil degranulation *in vivo*. Examining eosinophil response to PAF offers insight into eosinophil pro-inflammatory capacity through cytotoxic mediator release upon a stimulus. Furthermore, investigating the effect of calcitriol in relation to PAF-sensitivity may elucidate vitamin D anti-inflammatory mechanisms. In our study, we showed that calcitriol did not affect eosinophil PAF-sensitivity. This finding contradicted our hypothesis, since PAF stimulates eosinophil degranulation. Interestingly, eosinophil PAF-induced degranulation of EPX followed a similar trend to viability patterns, at day 7 (**Figure 5A**). Groups marked by more abundant viable eosinophils (*i.e.,* IL-5 and IL-5+VD) maintained PAF sensitivity, indicating the PAF responsiveness was due to more viable cells. This paralleled finding confirms that viable eosinophils, whether exposed to calcitriol or not, are able to release cytotoxic mediators. This interpretation is strengthened by the fact that IL-5 and IL-5+VD groups were not significantly different in EPX release upon PAF stimulus. Our original thought was related to calcitriol possibly affecting eosinophil responsiveness to degranulation stimuli. In contrast, our results demonstrate

that such anergy is an unlikely causative mechanism of calcitriol exposure. It is more likely that calcitriol selectively modulates eosinophil responsiveness to extra-cellular factors. We thus conclude that calcitriol does not selectively modulate eosinophil degranulation response to PAF. Other aspects of PAF signaling in eosinophils in the presence of calcitriol need further investigation. It is of interest to decipher whether calcitriol does affect the PAF signaling pathway as a whole or effects certain downstream outcomes of PAFR activation. Overall, our results support the idea that calcitriol does not modulate PAFR expression to alter responsiveness, yet this remains to be directly investigated.

Interestingly, calcitriol on its own was not able to execute the latter effects on peripheral blood eosinophils. The fact that calcitriol did not have these effects independently is of minor concern, since anti-apoptotic cytokines, such as IL-5, are typically present in high concentrations in asthmatics [43]. Hence, eosinophils present during chronic asthmatic inflammation are continuously stimulated by IL-5. More importantly, the pattern in which EPX was released into media was strongly suggestive that eosinophil cytolytic death was predominant (*i.e.,* based on viability and necrosis results). This modulation in eosinophil function by calcitriol supports current evidence of vitamin D as an anti-inflammatory immune regulator [36]. Also, calcitriol may directly decrease EPX release, independently of necrosis, such as through PMD, yet our data does not infer this due to the association made between mediator release and eosinophil necrosis. Future studies would render clarification on this matter.

We also determined the presence of eosinophil granules in cell debris, which gives insight into pro-inflammatory functions of eosinophilic cell demise through necrosis. Besides confirming the presence of free EPX, the dispersion of granules within cell debris is suggestive of other granule protein release. The association between cell debris levels and necrotic cell counts in our study is in line with the documented phenomenon of cell-free granules in eosinophilic disease [139]. Cytolysis, as cell demise, causes discharge of diverse cellular components into the surrounding environment. Granules may readily deposit themselves in tissues and be more resilient to clearance, in comparison to other debris elements, while continually releasing cytotoxic granule proteins, causing chronic inflammation in the lung.

Relating to eosinophil survival, we first confirmed a previous report showing that calcitriol (100 nM) sustained eosinophil viability up to 72 h [42]. However, we have extended on this finding

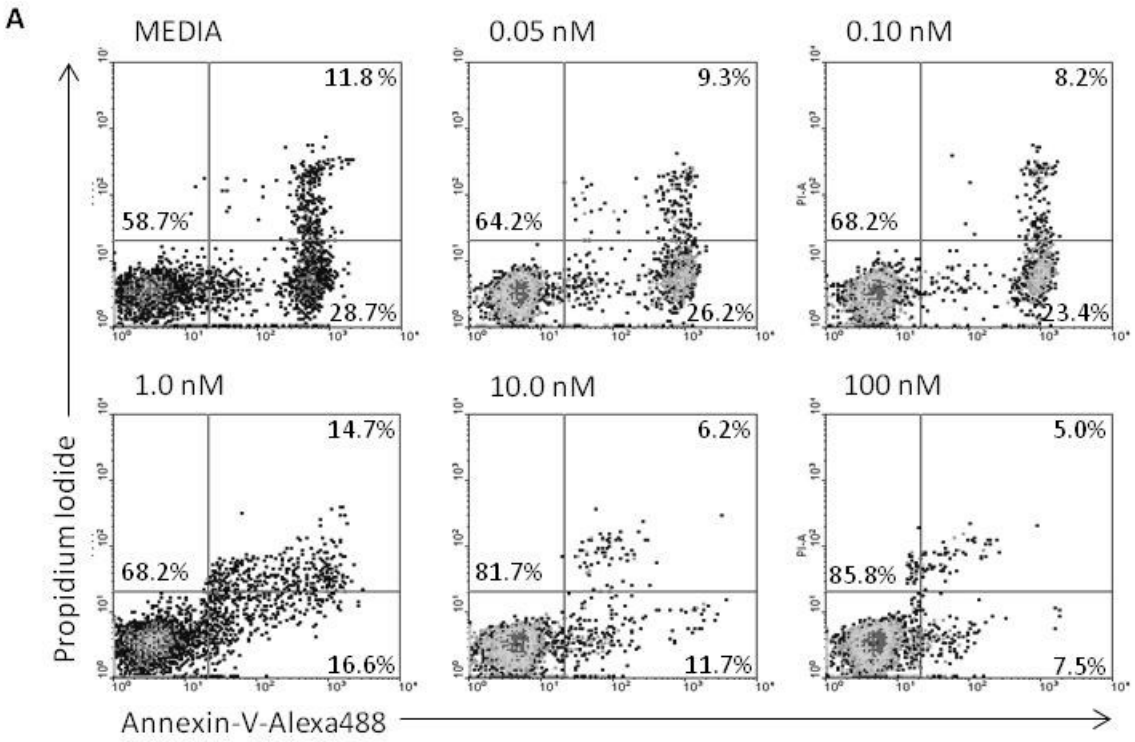
by showing a similar effect with a 10-fold lower concentration of calcitriol (10 nM). Both concentrations of calcitriol are plausible for the lung mucosal microenvironment since lung epithelium, containing 1α-hydroxylase, has been reported to convert calcifediol into calcitriol at much greater levels, *in vitro*. Hansdottir *et al.* observed that approximately 1000 nM of calcifediol was transformed into 0.6 nM of calcitriol after 24h in a constitutive manner allows calcitriol accumulation) [15]. As expected, local generation of active vitamin D has been linked to the regulation of various pulmonary immune responses. This conversion can also be done by alveolar macrophages, dendritic cells and lymphocytes, which implies that extra-renal expression of 1α-hydroxylase is specific to the immune system [16]. In addition, we show a synergistic effect by combining calcitriol with anti-apoptotic cytokines (*i.e.,* IL-5 and IFN-γ). We tested IFN-γ in order to demonstrate that the potentiating effect of calcitriol on eosinophil survival was not exclusive to IL-5R signaling. IFN-γ was chosen due to its signaling initiation, which relies on IFNGR1 and 2 (which interact with JAK1 and 2, respectively), being independent from the IL-5 β chain signaling (which relies on β receptor activation to phosphorylate JAK2 exclusively) [62]. Both cytokines resulted in anti-apoptotic outcomes. The reproducibility in survival trend observed with IL-5, when using IFN-γ confirms that calcitriol modulates signaling components downstream the initiation pathways of both cytokines. This explanation is rational since VDR is localized at the nuclear membrane and acts as a transcription factor once activated by ligand binding [178]. Furthermore, the synergism observed, when calcitriol and tested cytokines were combined, is indicative that calcitriol triggers a cell survival/death pathway distinct from apoptosis. In combination with the lack of changes in apoptotic eosinophil levels and production of cell debris at this same time point, we propose a necrotic pathway as being a target for calcitriol intracellular modulation on eosinophils. In general, our results contradicted our original hypothesis that calcitriol may decrease eosinophil survival similar to its effects on cancer cells. Due to the lack of literature in this field specific to eosinophil biology, more studies on eosinophil programmed death signaling are needed.

Although the modulation of eosinophil apoptosis is a fairly well-described area of eosinophil-targeting therapy, eosinophil necrosis and cell survival pathways are poorly understood. These under-studied mechanisms may provide insight in new therapeutic approaches, specifically for severe, acute and/or steroid-resistant asthmatics, as well as facilitate current diagnosis practices in relation to vitamin D deficiencies. More research is needed in the field of eosinophil cytolysis in order to clarify prominent pathways and identify major signaling molecules

which manage pro-inflammatory cell demise. Although reports on the effects of calcitriol on other leukocytes have offered insight on receptor expression, chemokine and cytokine production as well as cell differentiation, leukocyte survival and death have not been studied [36].

As a whole, our data demonstrate analogous trends across all assay types (*i.e.,* groups with higher necrosis have higher cell debris, containing granules as well as free mediators, and, specifically, increased spontaneous EPX release). Moreover, EPX release inversely correlates with eosinophil survival. The relationship between all latter elements reinforce the notion that eosinophil necrosis/cytolysis is an important pro-inflammatory mechanism in eosinophilic inflammatory disorders. We also propose a new anti-inflammatory function for calcitriol, and possibly other vitamin D metabolites. Our study provides insight into the link between vitamin D deficiency and eosinophilic inflammatory diseases observed in human populations.

3.5 Figures


A
MEDIA
11.8 %
58.7%
28.7%
0.05 nM
9.3%
64.2%
26.2%
0.10 nM
8.2%
68.2%
23.4%
1.0 nM
14.7%
68.2%
16.6%
10.0 nM
6.2%
81.7%
11.7%
100 nM
5.0%
85.8%
7.5%
Propidium Iodide
Annexin-V-Alexa488

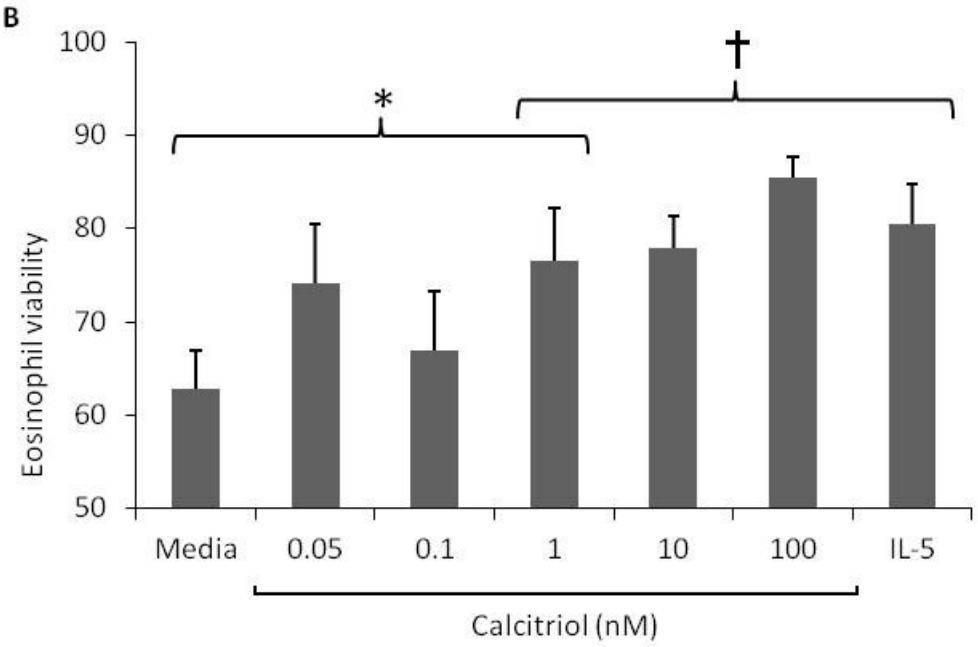

B
100
90
80
70
60
50
Eosinophil viability
*
†
Media
0.05
0.1
1
10
100
IL-5
Calcitriol (nM)

Figure 4: Increasing concentration of calcitriol (1,25-(OH)2VitD3) improves survival of human blood-derived eosinophils *ex vivo* over a 24h period. (A) The number of double positive eosinophils for AnnexinV and PI (*i.e.,* necrotic cell population in right upper quadrant) decreases while the amount of double negative eosinophils increases through augmentation in concentration of calcitriol. (B) The percentage of viable eosinophils is improved after 24h treatment with calcitriol in a dose dependant manner. Eosinophil survival after 24h calcitriol treatment (0.05 – 1 nM) was significantly lower than IL-5 treatment (1 ng/ml) (n = 5, * $p < 0.05$). Viability with calcitriol (1 -100 nM) was significantly higher than Media (n = 5, † $p < 0.05$). Viability was determined by Annexin-V binding and PI exclusion by flow cytometry.

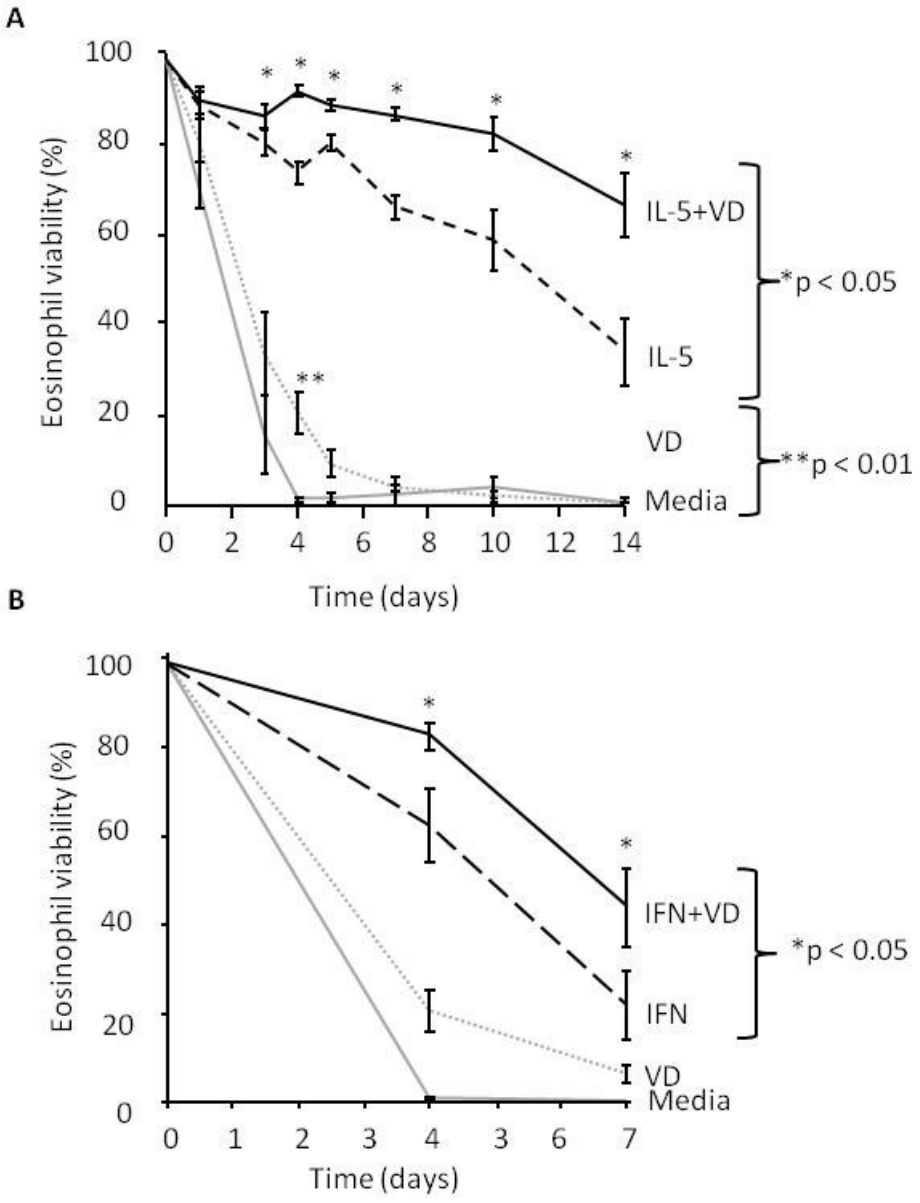
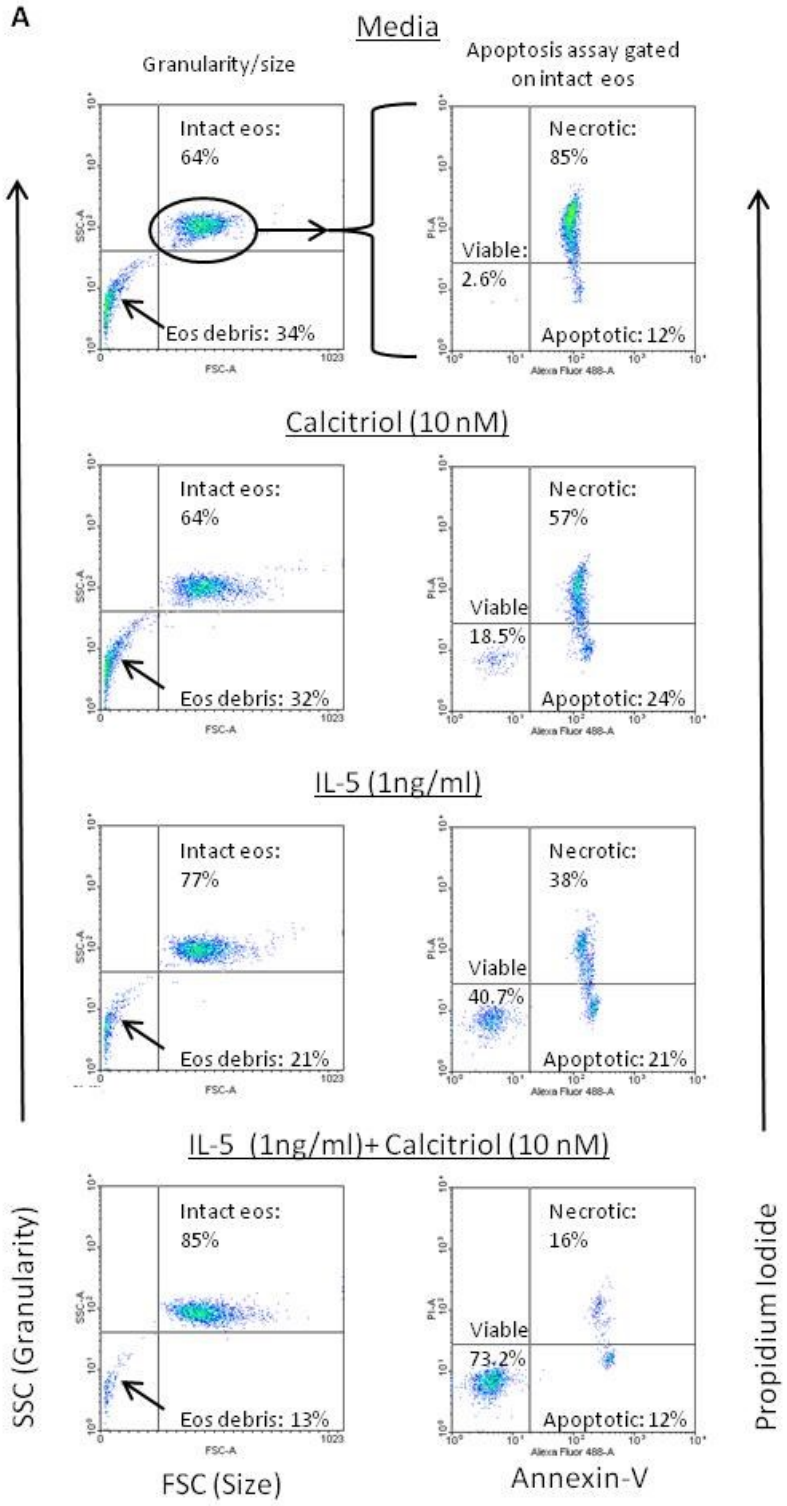


Figure 5: Calcitriol potentiates the survival effect of anti-apoptotic cytokines, IL-5 and IFNγ, on human peripheral blood eosinophils *ex vivo*. (A) IL-5 (1 ng/ml) with calcitriol (10 nM) had an additive effect from day 4, and synergistically prolonged eosinophil viability as of day 7 of incubation (IL-5 + VD *vs* IL-5; * $p < 0.05$, n = 12). (B) Calcitriol (10 nM), in combination with IFN-γ (100 ng/ml), also had a synergistic effect on eosinophil survival at day 7 of incubation (IFN + VD *vs* IFN; *p < 0.05, n = 5).


A
Media
Granularity/size
Apoptosis assay gated on intact eos
Intact eos: 64%
Eos debris: 34%
Necrotic: 85%
Viable: 2.6%
Apoptotic: 12%
Calcitriol (10 nM)
Intact eos: 64%
Eos debris: 32%
Necrotic: 57%
Viable 18.5%
Apoptotic: 24%
IL-5 (1ng/ml)
Intact eos: 77%
Eos debris: 21%
Necrotic: 38%
Viable 40.7%
Apoptotic: 21%
IL-5 (1ng/ml)+ Calcitriol (10 nM)
Intact eos: 85%
Eos debris: 13%
Necrotic: 16%
Viable 73.2%
Apoptotic: 12%
SSC (Granularity)
FSC (Size)
Annexin-V
Propidium Iodide

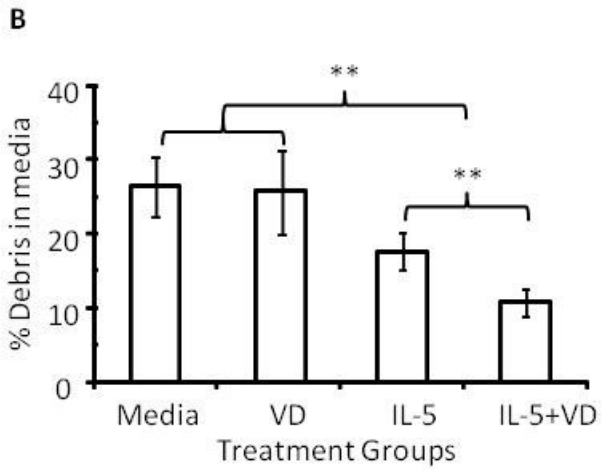


Figure 6: The quantity of eosinophilic cell debris is greatly reduced in IL-5 (1ng/ml) and calcitriol (10 nM) treatment group. (A) Eosinophilic cell debris (left panel, left lower quadrant), are defined as lowSS/lowFS events using flow cytometry light scattering properties. The amount of cell debris corresponds to the percentage of necrotic eosinophils (right panel, upper right quadrant). (B) IL-5 + VD treatment reduced the relative percentage of cell debris in comparison to all other treatments (** $p < 0.01$, n = 10).

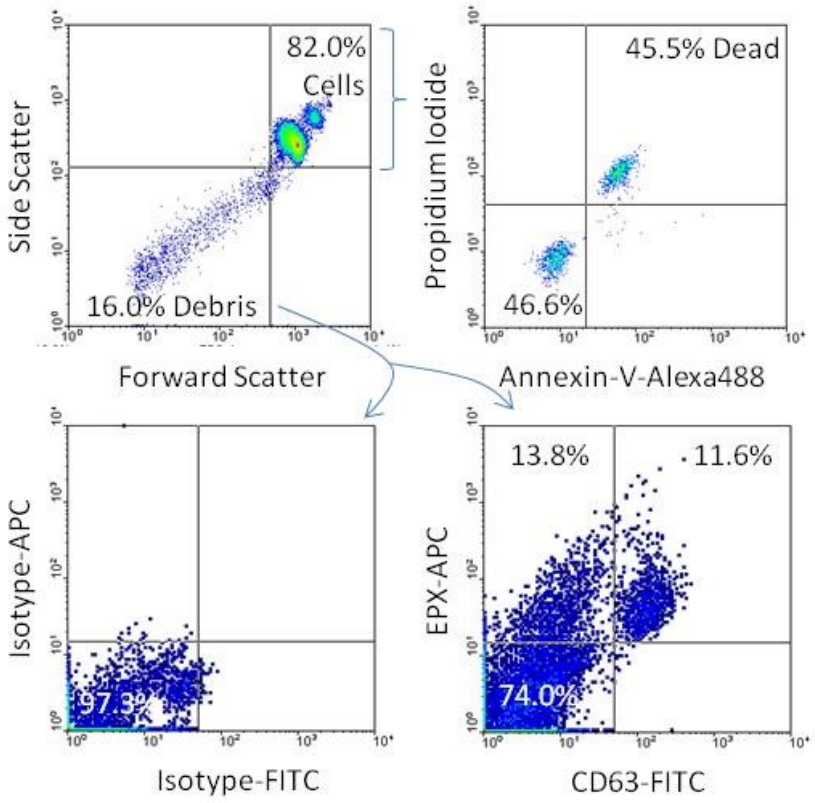


Figure 7: Eosinophilic cell debris contains CD63+ granules as well as EPX-positive particles. Eosinophilic cell debris from non-treated group at day 7 of incubation contained considerable amounts of granules (Upper left panel, lower left quadrant), defined as double positive events for CD63 and EPX (Lower right panel, upper right quadrant) as well as free EPX, defined as single positive events for EPX (Lower right panel, upper left quadrant). Representative figure of 5 independent experiments. FACS plots were obtained by gating on low SS / low FS event population using light scattering properties by flow cytometry.

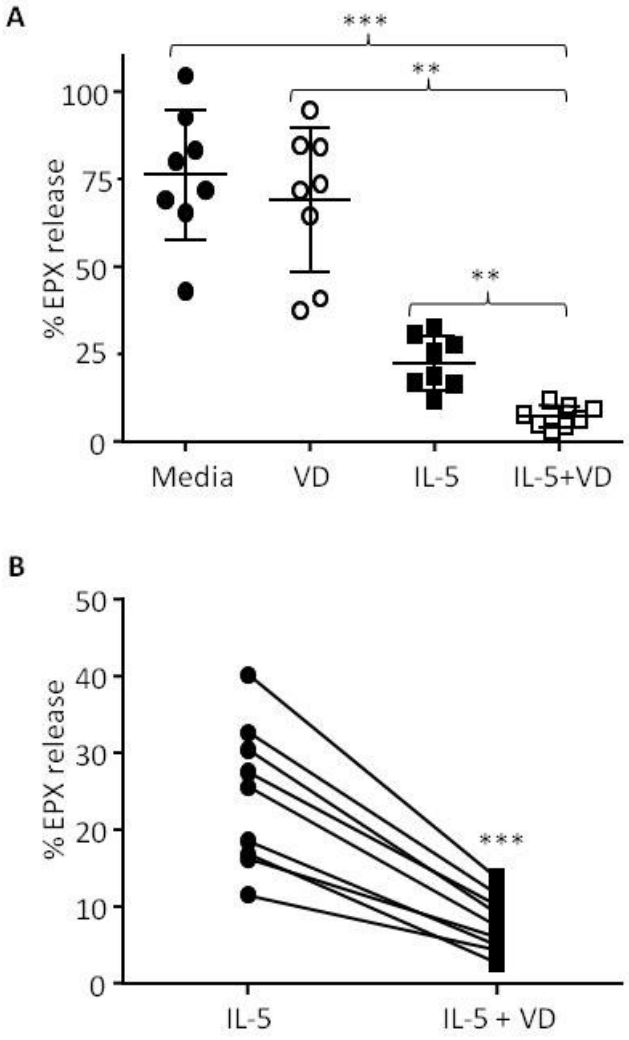


Figure 8: Calcitriol, when combined with IL-5, minimizes the spontaneous release of active EPX in culture media. (A) All treatment groups had significantly less active EPX released in comparison to IL5+VD group (ANOVA; $^{**}p < 0.01$, $^{***}p < 0.001$, n = 9). (B) The relationship between IL-5 and IL-5+VD treatments consistently depicts a respective decrease in EPX percentage within each experiment in a significant manner (Paired *t*-test; $^{***}p < 0.001$, n = 9). EPX activity in media was measured using an OPD-based colorimetric assay.

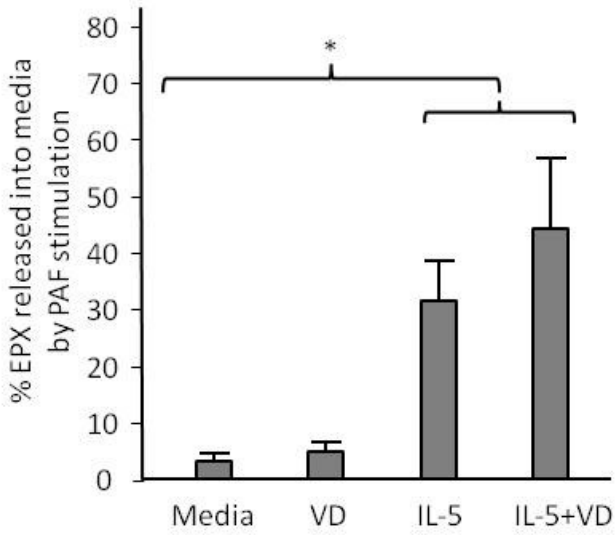


Figure 9: The addition of calcitriol to IL-5 treated eosinophils maintains eosinophils' sensitivity to PAF *ex vivo*. Treatment with IL-5 and IL-5 + VD caused significantly higher PAF induced EPX release than Media and VD (day 7; *$p < 0.05$; n = 9). IL-5 and IL-5 + VD were not significantly different. EPX activity in media was measured using an OPD-based colorimetric assay.

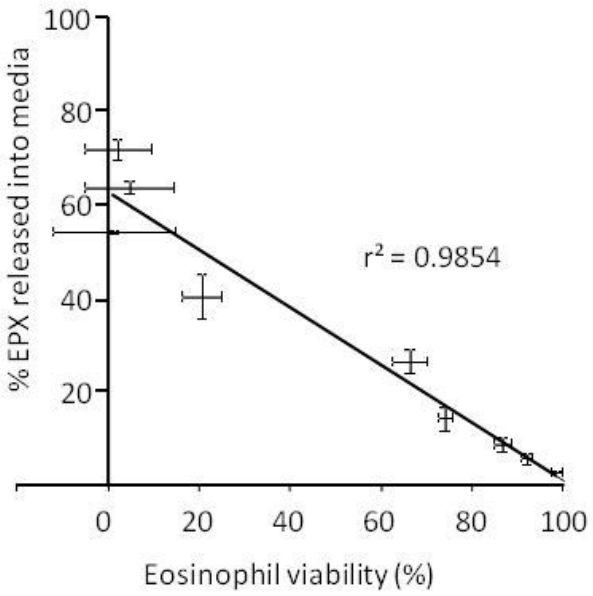


Figure 10: EPX activity in media is inversely correlated to eosinophil survival. The percentage of EPX release into media and levels of eosinophil survival are inversely correlated to eachother with high significance. ($r^2 = 0.9854$, $p < 0.05$, n = 4-11). Results from EPX colorimetric assays were paired with corresponding eosinophil viability data from AnnexinV-PI assays. EPX activity in media was measured using by OPD-based colorimetric assay. Viability was determined by annexin-V binding and PI exclusion by flow cytometry.

CHAPTER 4: Results

Calcitriol selectively reduces eosinophil receptor expression, yet does not influence dexamethasone or RSV killing of eosinophils

4.1 Introduction

Since the effect of calcitriol on eosinophil biology remains novel in current literature, we wanted to explore other aspects of eosinophil function (*i.e.,* beyond cell survival). It should be noted that some of these additional findings may provide indirect support or clarification to evidence presented in the previous chapter, while other experiments serve as pilots. At large, this added information was beyond the scope of the scientific manuscript in preparation "Calcitriol (1,25 Dihydroxyvitamin D_3) reduces eosinophil necrosis leading to diminished release of cytotoxic granules and (EPX)" from Chapter 3. While Chapter 3 presented findings respective to the research objectives 1 and 2, this chapter addresses objectives 3, 4 and 5. Therefore, we present this supplemental data as a separate entity. In general, these results are preliminary as they mostly represent trends, yet offer indications as well as stepping stones for future experiments concerning the role of calcitriol on eosinophil biology.

4.2 Results

4.2.1 Eosinophil Phenotype - Assessed by Cell Surface Receptor Expression

Our goal was to specifically assess eosinophil phenotype in the context of calcitriol modulation. Analysis of cell surface expression of receptors offers a broad indication of cell activation state as well as cell function. In this way, phenotyping through receptor expression paints an inclusive picture of cell properties. We selected eosinophil markers that have been shown to be involved in allergic inflammation, as well as under-studied receptors with inflammatory potential.

4.2.1.1 IL-5 Receptor Alpha Subunit and Common Beta Chain

When analyzing IL-5Rα expression on eosinophils, it was apparent that both completed experiments (*i.e.,* Exp1 *vs.* Exp2) contradicted each other in outcomes (**Figure 11A&B**). Experiment 1 demonstrates a higher expression of IL-5Rα in the IL-5 group, with an inverse lower expression when calcitriol is added, at day 4 (**Figure 11A&B**). This difference is lost at day 7. In contrast, Experiment 2 demonstrates no difference between treatments at both time points at population and cellular levels (**Figure 11A&B**). It should also be mentioned that IL-5Rα detection was extremely low compared to expected levels.

Likewise, IL-5Rβ expression levels were generally lower than anticipated. The percentage of IL-5Rβ positive eosinophils increased overtime (*i.e.,* from 4 to 7 days incubation) independent from calcitriol exposure (**Figure 11C**). MFI of IL-5Rβ expressing cells did not follow the latter trend. In contradiction, IL-5β MFI of eosinophils was lowest in groups supplemented with calcitriol at day 4, yet increased MFI by day 7 (**Figure 11D**). Treatment groups lacking calcitriol showed an opposite effect. Precisely, IL-5 groups had the highest MFI at day 4 followed by decreased expression by day 7 (**Figure 11D**). Accordingly, all treatment groups from both experiments cluster together at day 7 (**Figure 11C&D**).

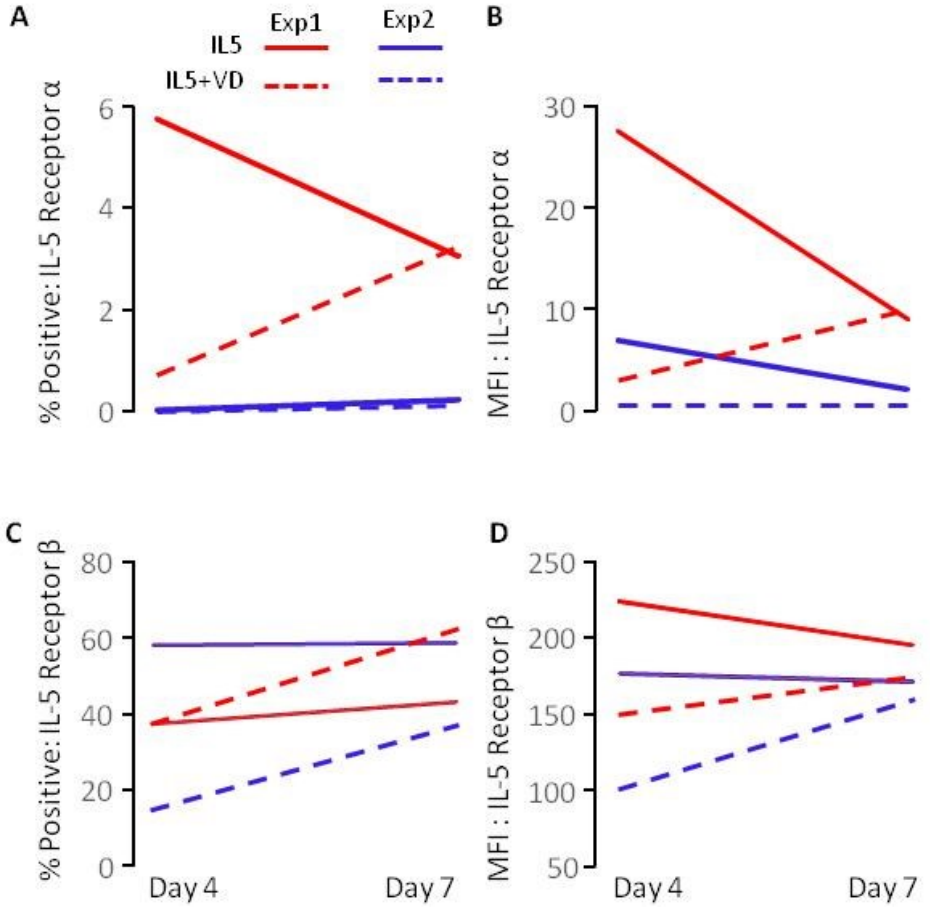


Figure 11: Calcitriol does not influence IL-5R surface expression on eosinophils *ex vivo* (days 4 & 7; n = 2). (A) Percent and (B) MFI of IL-5Rα positive eosinophils in response to the addition of calcitriol (10 nM). (C) Percent and (D) MFI of IL-5Rβ positive eosinophils following calcitriol (10 nM) treatment.

4.2.1.2 CRTh2 Expression

Using our experimental design, approximately 50% of total eosinophils expressed CRTh2 on day 4. This amount declined to roughly 25% at day 7 (**Figure 12A**). A nearly significant difference in gated percentage was observed at day 4 between treatment groups, where the addition of calcitriol induced a reduction in the percentage of eosinophils expressing CRTh2 (**Figure 12A**). A similar trend was observed at day 7, although this lacked statistical significance (**Figure 12A**). In contrast, results pertaining to the abundance of CRTh2 (*i.e.,* MFI of CRTh2) were significant. Both time points revealed a significant decrease in eosinophil CRTh2 MFI when calcitriol was added as a treatment, with a less drastic decrease by day 7 (**Figure 12B**). Overall, IL-5 treatment yielded the highest CRTh2 expressions where calcitriol induced a reduction.

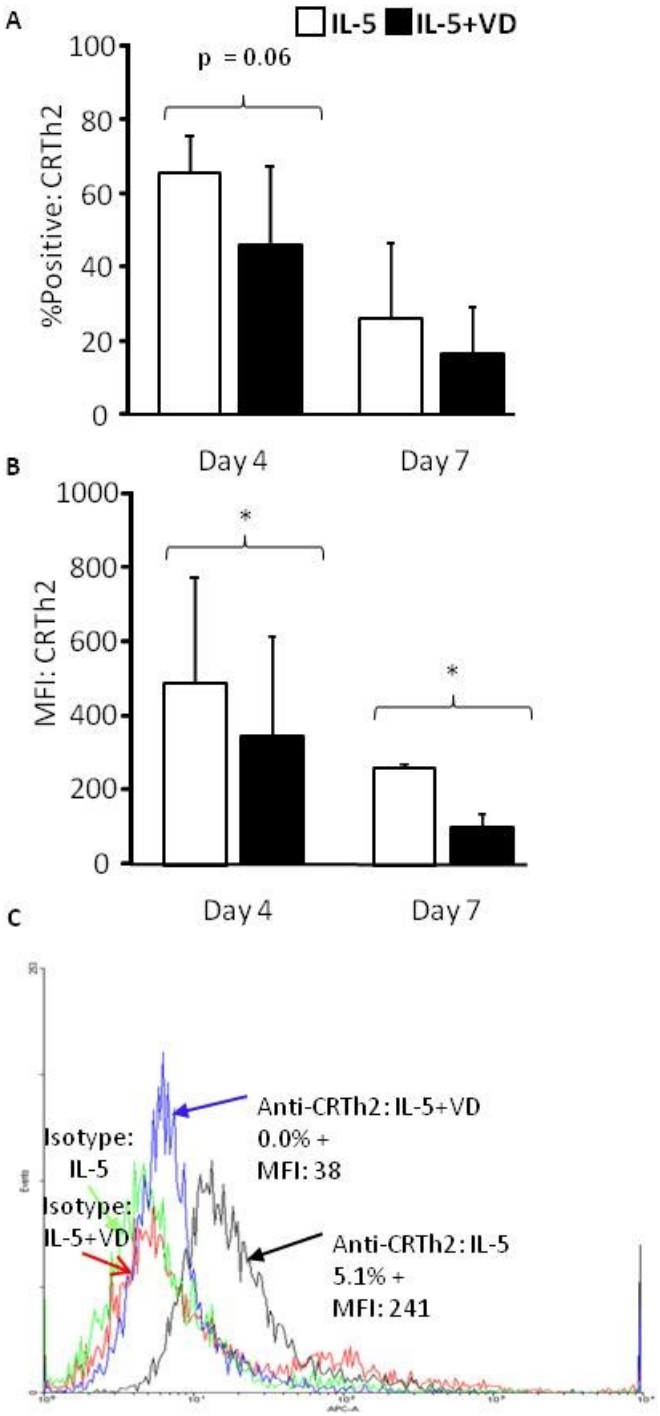


Figure 12: Calcitriol significantly decreases CRTh2 surface expression on eosinophils *ex vivo* (days 4 & 7; *$p < 0.05$; n = 3). (A) Percent CRTh2 positive eosinophils in response to the addition of calcitriol (10 nM) ($p = 0.06$). (B) MFI of CRTh2 positive eosinophils treated with calcitriol (10 nM) (* $p < 0.05$). (C) Representative histogram of CRTh2 staining.

4.2.1.3 CCR10 Expression

Eosinophil expression of CCR10 remained below 10% of total eosinophil population (**Figure 13A**). Furthermore, eosinophils followed the trend of decreasing CCR10 expression upon calcitriol addition, both at population (**Figure 13A**) and cellular levels (**Figure 13B**). Also, MFI of CCR10 was significantly different between treatment groups at both time points (**Figure 13B**). Such a significant difference was not achieved through measurement of percent CCR10 positive eosinophils. Notably, the amount of CCR10 expressing eosinophils is almost significantly lower when calcitriol is added ($p = 0.08$), after only three completed experiments (**Figure 13A**). Overall, eosinophils treated solely with IL-5 demonstrated the highest expression of CCR10 on days 4 and 7 (**Figure 13**).

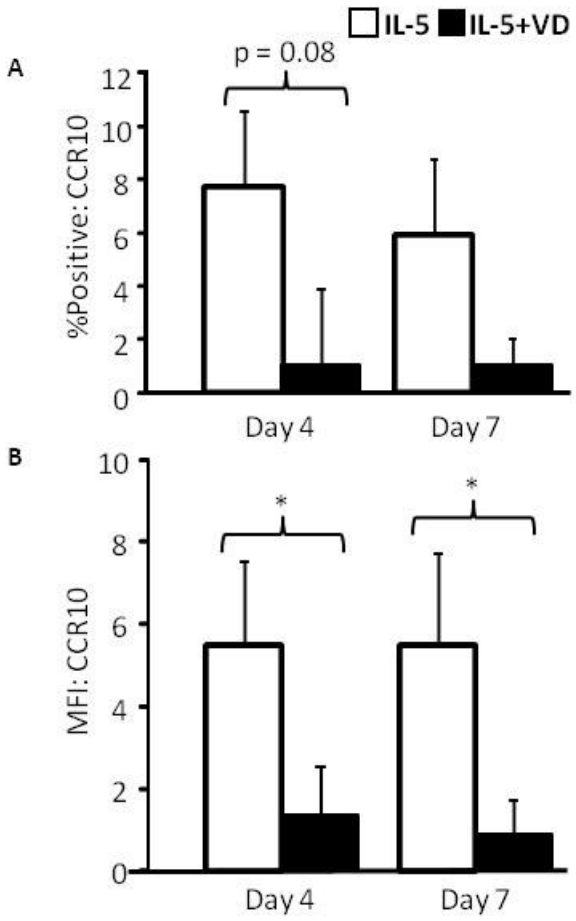


Figure 13: Calcitriol decreases CCR10 surface expression on eosinophils *ex vivo* (days 4 & 7; * $p < 0.05$; n = 3). (A) Percent CCR10 positive eosinophils in response to the addition of calcitriol (10 nM) ($p = 0.08$). (B) MFI of CCR10 positive eosinophils treated with calcitriol (10 nM) (*$p < 0.05$).

4.2.1.4 CCR3 Expression

When eosinophil CCR3 expression was analyzed in relation to calcitriol, no general trends existed. There was no observable pattern at the population level (*i.e.,* percent positive cells) (**Figure 14A**) or the cellular level (*i.e.,* MFI) (**Figure 14B**). CCR3 expression was not significantly different between IL-5 *versus* IL-5+VD treatment groups at both tested time points (*i.e.,* days 4 and 7), in percent positive cells (**Figure 14A**) as well as MFI (**Figure 14B**). Still, it should be noted that a non-significant decrease in MFI of CCR3 positive eosinophils was observed at day 7 (**Figure 1B**). It is apparent that CCR3 expression was low (*i.e.,* only approx. 20% of eosinophils were expressing CCR3) (**Figure 14A**). In general, the expression of CCR3 seemed to be random, with great variation between experiments, regardless of calcitriol treatment.

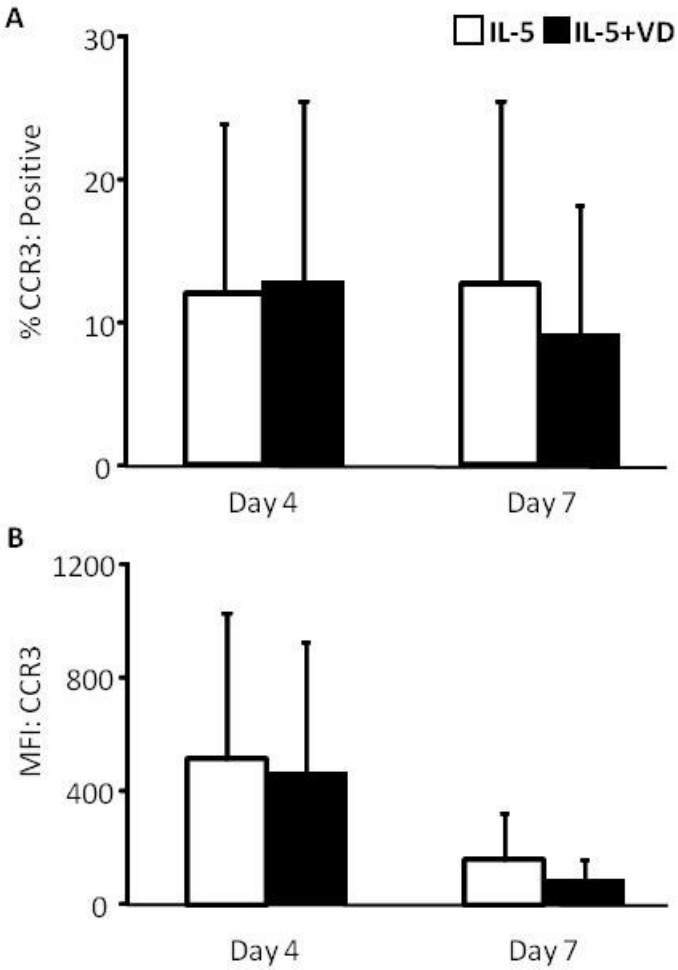


Figure 14: Calcitriol does not modulate CCR3 surface expression on eosinophils *ex vivo* (days 4 & 7; n = 3). (A) Percent CCR3 positive eosinophils in response to the addition of calcitriol (10 nM). (B) MFI of CCR3 positive eosinophils treated with calcitriol (10 nM).

4.2.1.5 CCR4 Expression

After repeating experiments twice, the general trend was a decrease in CCR4 expression (both at the population and cellular levels) with time. There was no specific pattern related to calcitriol treatment (**Figure 15**). In general, CCR4 detection was low (*i.e.,* not more than 6% of eosinophils in all experiments expressed CCR4) (**Figure 15A**). Nevertheless, all assays resulted in a time-dependant decrease in percent positive eosinophils, regardless of calcitriol (**Figure 15A**). The only increase in CCR4 expression was observed in MFI of one IL-5 treated experiment, while all other experiments depicted a decrease in MFI (**Figure 15B**).

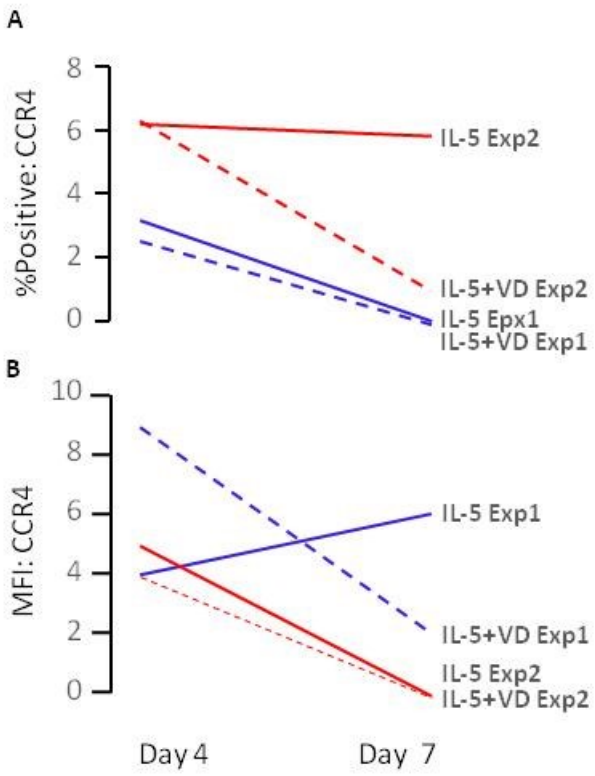


Figure 15: Calcitriol does not affect CCR4 surface expression on eosinophils *ex vivo* (days 4 & 7; n = 2). (A) Percent CCR4 positive eosinophils in response to the addition of calcitriol (10 nM). (B) MFI of CCR4 positive eosinophils treated with calcitriol (10 nM).

4.2.2 Corticosteroid-Induced Eosinophil Death

When dexamethasone-treated groups were compared to their corresponding controls there were no significant differences (**Figure 16**). Unexpectedly, dexamethasone did not execute direct killing of human eosinophils, until day 3. The level of dead eosinophils was higher, yet not significant, in IL-5+Dex and IL-5+VD+Dex when compared to IL-5 and IL-5+VD, respectively (**Figure 16**). On day 3, VD+Dex appears to have less dead eosinophils than Dex only. This difference is not significant since the graph is a representation of two independent experiments. (**Figure 16**). Consequently, calcitriol did not have an effect on dexamethasone efficacy.

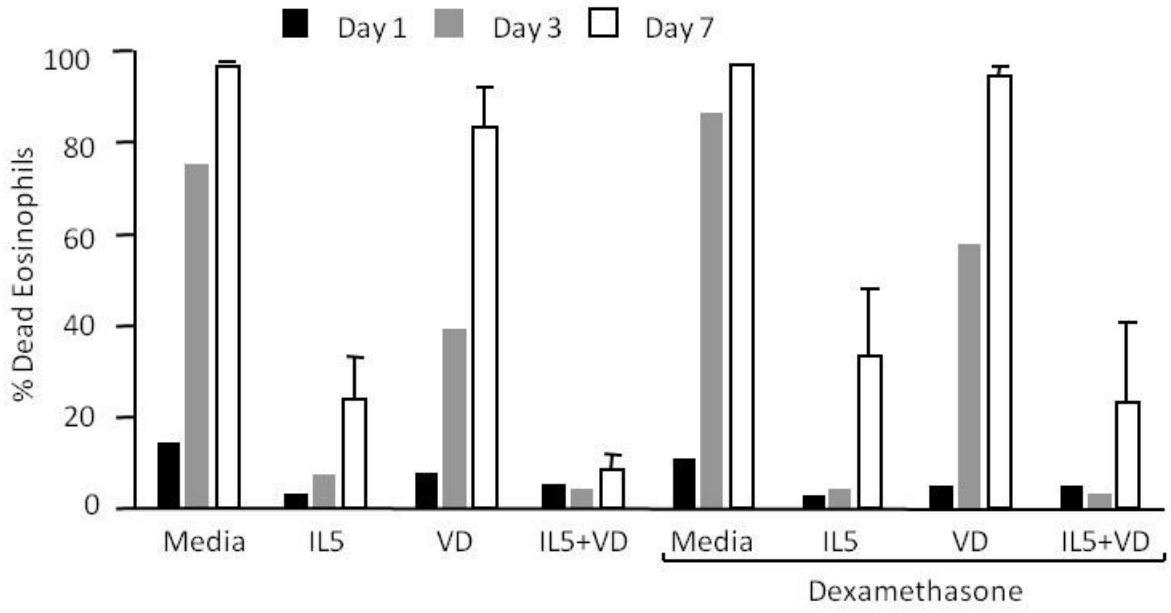


Figure 16: Calcitriol does not improve nor prevent killing of eosinophils in the presence of dexamethasone (1 μM) *ex vivo* (days 1, 3 & 7; n = 2, n = 3, respectively).

4.2.3 Eosinophil Killing with RSV

When RSV containing treatments are compared with their respective negative as well as UV-irradiated RSV controls, there are no differences in amounts of dead eosinophil, at both time points (**Figure 17**). In other words, all treatment sets (*i.e.,* without RSV, with active RSV and with UV-inactivated RSV) resembled each other. Eosinophil death levels at days 3 and 7 were not affected by RSV and calcitriol exposures (**Figure 17**).

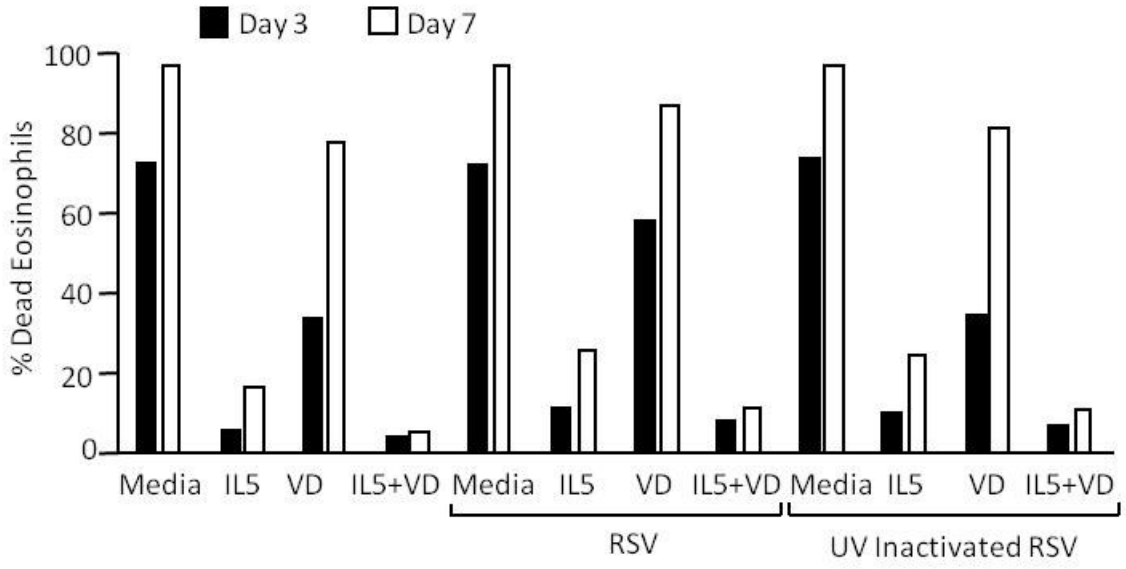


Figure 17: RSV (4.73 x 10^4 PFU/mL) does not directly kill eosinophils and addition of RSV to calcitriol does not alter eosinophil killing (days 3 & 7; n = 2)

CHAPTER 5: Discussion and Conclusions

5.1 General Discussion

5.1.1 Calcitriol Decreased CRTh2 and CCR10 Expression While Having no Effect on IL-5R, CCR3 and CCR4 Expression

Our investigation of CRTh2 expression suggests an anti-inflammatory role for calcitriol in respect to eosinophils. Since CRTh2 mediates eosinophil recruitment to PGD_2 secreting tissues during inflammatory responses, modulation of its expression is of high pertinence for therapeutic approaches. We were able to show that calcitriol specifically decreases CRTh2 expression on mature eosinophils from atopic donors. Although calcitriol seems to reduce the amount of CRTh2 positive eosinophils, consequences were significant at cellular level. Thus, calcitriol seems to render eosinophils less sensitive to PGD_2. Reduction in PGD_2 responsiveness would likely reduce eosinophil recruitment to lung, since CRTh2 antagonists have been demonstrated to improve eosinophil migration to lung tissue [108, 109]. Since our analysis is representative of three independent experiments only, testing calcitriol modulation of eosinophil CRTh2 expression should be repeated. But, regardless of 'n' value, current results are significant.

In our study, we also confirmed that peripheral blood eosinophils from atopic donors express CCR10. Furthermore, our data suggests that only a subpopulation of eosinophils actually express CCR10. Since CCR10 is primarily a mucosa homing receptor, its expression may be specific to a subpopulation of eosinophils which serve as first infiltrators and responders in tissues. This idea is plausible based on reports that eosinophil infiltration precedes lymphocyte influx in allergic mouse models [165]. In respect to vitamin D, calcitriol-induced reduction of CCR10 expression agrees with the perspective that vitamin D is anti-inflammatory. Theoretically, lower CCR10 expression would result in reduced homing to mucosal tissues, such as pulmonary mucosa. In general, further investigation of CCR10 in eosinophils is needed to determine specific CCR10 signaling functions on eosinophil biology as well as implications in airway inflammatory pathogenesis concerning eosinophilia.

Calcitriol treatment had no effect on IL-5R expression on peripheral blood eosinophils. The detection of IL-5Rα was extremely low, suggesting general issues with antibody specificity and/or optimization-related problems. Moreover, IL-5Rα expression did not show consistency between experiments. This may be indicative of genetic variation between blood donors. Similarly,

IL-5Rβ expression was more subtle than expected, suggesting antibody detection concerns. Also, the distribution percentage of eosinophils positive for IL-5Rβ strongly differed from the pattern observed in IL-5Rβ MFI. More specifically, calcitriol addition seemed to decrease IL-5Rβ expression compared to IL-5 treatment, at day 4. Calcitriol may simply lose this effect by day 7. Decreasing IL-5Rβ expression on eosinophils is a plausible mechanism by which calcitriol may modulate eosinophil function in an anti-inflammatory manner. Such a change in cell surface expression would render eosinophils less sensitive to respective ligands: IL-5, IL-3 and GM-CSF. Overall, our investigation of IL-5R expression modulation through calcitriol remains indecisive. These trial experiments must be repeated in order to conclude whether VD exerts a direct effect on eosinophil IL-5R expression. Although calcitriol's effect on other leukocyte receptors have been explored, vitamin D modulation of the IL-5R remains unknown. One study, which found that calcitriol inhibited IL-5 production (as well as eosinophilia) in experimental asthma, may suggest that IL-5R upregulation is not a probable mechanism for calcitriol modulation [179].

Detection of CCR3 on eosinophils was also lower than expected. It has been well-established that eosinophils highly express CCR3. Consequently, it is often used as an eosinophil marker in inflammation [43, 101, 102]. Low CCR3 detection could simply be an optimization-issue. Alternatively, low CCR3 expression in our experimental design may be a result of eosinophil purification leading to decreased receptor expression. Certain manipulations, such as EDTA treatment, which was used as an anti-coagulant, may cause shedding of cell surface receptors. CCR3 levels at baseline (*i.e.,* CCR3 expression of freshly purified eosinophils) are not available in order to comment on this issue. Also, *ex vivo* incubation could discourage CCR3 expression due to limited cellular factors (*i.e.,* eosinophils in this study are exclusively supplemented with FBS). Regardless, these experiments should be repeated in order to conclude whether calcitriol modulates eosinophil CCR3 expression.

Similar to CCR3 experiments, CCR4 analysis was generally inconclusive. We report no apparent trend after two experiments. Evidently, experiments concerning CCR4 expression in relation to calcitriol must be repeated to obtain a higher 'n' value in order to confirm any finding. Interestingly, a recent study did report that calcitriol treatment increases CCR4 expression on eosinophils *ex vivo*. The contradiction with our results may lie in experimental design differences. For example, Hiraguchi and associates obtained peripheral blood eosinophils from healthy donors,

used a 10-fold higher concentration of calcitriol (*i.e.,* 100 nM) and acquired CCR4 expression levels at different time points [42]. Repeating these experiments in order to determine the effect of calcitriol on CCR4 expression in eosinophils is greatly needed due to the lack of scientific literature on the subject. Moreover, eosinophil CCR4 involvement in airway inflammation warrants further investigation due to therapeutic potential [112].

We conclude that the addition of calcitriol to IL-5-treated eosinophils induces a reduction in CRTh2 and CCR10 expression. Presumably, a reduction in such chemokine receptors would discourage eosinophil recruitment to inflamed tissues, such as eosinophil lung infiltration observed in asthma. As such, vitamin D may be anti-inflammatory in regards to dampening eosinophil recruitment abilities which would result in less tissue damage. Experiments pertaining to CRTh2 and CCR10 expression should be repeated in order to confirm the latter findings as well as elaborate a causative mechanism. In contrast, this study cannot conclude calcitriol effects on eosinophil IL-5R, CCR3 and CCR4 expression. In general, a sample size must be equal to or greater than n = 4 to properly execute any statistical test, including a power calculation. Therefore, an experiment must be repeated at least four times to confirm or determine statistical significance. It is also possible that inconsistent results within these experiments are due to genetic differences between blood donors resulting in different responses. Further investigation is needed concerning the latter receptors in order to clarify this matter. Additionally, future assays should include baseline levels of expression, as a reference, as well as earlier time points. Calcitriol modulation of eosinophil cell surface receptors may occur earlier than time points investigated in this study.

5.1.2 Calcitriol did not Modulate Dexamethasone Killing of Eosinophils

Our results show that calcitriol does not directly affect dexamethasone killing of eosinophils. Although cross-talk between these molecules is possible, it may not occur at the cellular level. Thus far, reports which link vitamin D deficiency with poor steroid response have only investigated the matter *in vivo* [44], where influencing variables are not easily distinguished. Since our study only reflects a few experiments, assays looking at combined effects of calcitriol and dexamethasone must be repeated. Our results were also subjected to delayed dexamethasone efficacy, which may be reflective of an inadequate dexamethasone concentration. The dexamethasone concentration used is believed to be a standard, yet it appears to be non-optimal in our experimental scheme. As mentioned, it has previously been shown that dexamethasone (1 μM)

can induce eosinophil death within 24 hours *in vitro* [98]. Future investigation of calcitriol and dexamethasone cross-talk should include a dexamethasone dose response in order to determine optimal corticosteroid concentration exclusive to this study. Beyond repeating experiments, it may be of value to include earlier time points as dexamethasone signaling may occur more rapidly than anticipated.

5.1.3 Calcitriol did not Influence Eosinophil Cytolytic Response to RSV

Using our experimental design, RSV did not directly cause eosinophil cytolytic death. At large, RSV-free and inactivated RSV (*i.e.,* UV) groups were unexpectedly very similar to active RSV treated groups, in eosinophil death percentage. This may be an indication that the active RSV used did not function properly. This could be due to poor quality of RSV stock used. Alternatively, it may simply demonstrate that RSV cannot directly infect eosinophils and cause cytolytic death in this cell type. More than fifteen years ago, Kimpen *et al.* [180, 181] demonstrated that RSV is successfully taken up by human peripheral blood eosinophils. Although RSV virions were visualized within eosinophil phagolysosomal compartments, Kimpen's team was unable to show RSV replication. Nevertheless, they revealed that RSV induced eosinophil activation through PMD *in vitro* [180, 181] and *in vivo* [182]. This particular finding was extended upon by Davoine *et al.* [162] who demonstrated that RSV-induced eosinophil degranulation necessitates interactions with antigen presenting cells as well as CD4+ T cells. Specifically, eosinophil degranulation upon exposure to RSV relies on a Th2 adaptive immune response in comparison to an innate mechanism. Hence, the eosinophil response to RSV is a complex interaction, which depends on the involvement of other immune cells. Additionally, calcitriol had no effect on eosinophil death in relation to RSV exposure. Therefore, current evidence suggesting that vitamin D plays a protective role against respiratory infections may be of a multifaceted nature, involving many immune and non-immune cell types. Although this kind of assay should be repeated to confirm our interpretation, these preliminary results highly discourage the idea that the link between vitamin D and respiratory infections are due to direct interaction with eosinophils.

5.2 Summary of Major Findings

We demonstrated that calcitriol, on its own, can sustain human peripheral blood eosinophil viability, *ex vivo*. This effect was lost after 3 days. In contrast, calcitriol was able to potentiate pro-

survival effects of anti-apoptotic cytokines, IL-5 and IFN-γ, in a long-term manner. Specifically, calcitriol had an additive effect starting at 4 days, which was followed by a synergistic effect with the latter cytokines beginning at 7 days. Higher survival of eosinophils correlated with decreased eosinophil necrosis. Furthermore, we were able to associate eosinophil necrosis levels with amounts of cell debris. We confirmed that cell debris formed by eosinophil necrotic death contained granules as well as the cytotoxic mediator EPX. We expanded on this finding by showing a significant reduction in spontaneous EPX release in eosinophils treated with IL-5 and calcitriol. As a whole, eosinophil survival inversely correlated with pro-inflammatory mediator release. Additionally, we revealed that calcitriol has the capacity to modulate cell surface receptor expression in eosinophils. Specifically, we showed important reductions in CRTh2 as well as CCR10 expression upon addition of calcitriol. Other examined receptors (*i.e.,* and IL-5R, CCR3 and CCR4) require further investigation for appropriate conclusions.

5.3 Eosinophil Fates in Inflammation: Apoptosis, Necrosis and Autophagy

In this study, we demonstrated a synergistic effect when calcitriol was combined with an anti-apoptotic cytokine. The nature of the response in combined treatments is indicative that two separate pathways, related to eosinophil survival, were targeted. Even if this result hints that calcitriol modulates a different pathway than apoptosis, further investigations, focusing on death signaling molecules, are needed to clarify a signaling mechanism. Intracellular death as well as survival signaling pathways are highly intertwined yet result in distinct cellular outcomes.

Apoptosis is the classical PCD pathway described in eosinophils. Apoptosis is a caspase-dependent pathway, where, caspase-8 becomes activated and cleaved to become an effector molecule [72]. Apoptotic eosinophils and their apoptotic bodies are actively phagocytosed by other immune cells, such as macrophages, to avoid granule release, which allows resolution [75]. When apoptosis is inhibited, as in eosinophil exposure to anti-apoptotic cytokine IL-5, Bcl-2 is known to be upregulated in order to counter apoptotic effects [57, 63, 183]. To date, the only known mechanism for increasing eosinophil survival is through inhibition of apoptosis [57, 65]. Our work shown here provides the first evidence of eosinophil necrosis inhibition to sustain eosinophil survival (**Figure 6**).

Although eosinophil apoptosis has been thoroughly studied, other cell death/survival pathways may just be as crucial on eosinophil biology. Interestingly, a study demonstrated that most immune cells are more prone to die by necrosis [79]. As reviewed by Han and co-workers [78], there are many reports suggesting that necrosis in the immune system is a backup death pathway, which competes with apoptosis, in order to stimulate specific immune responses [78]. In contrast to conventional thought, recent research in cell biology has revealed that necrosis (recently renamed 'necroptosis' as to imply its regulated nature) is a PCD pathway. It is distinct from apoptosis by the formation of RIP 1 and 3 protein complex, known as necrosome, which inhibits caspase-8 in order to initiate downstream cytolytic effects [78, 80]. Furthermore, it is extrinsically initiated through cell surface receptors, similarly to apoptosis. For example, activation of the tumor necrosis factor receptor (TNFR) and Fas receptor (FasR) can induce both apoptosis and necroptosis, depending on environment cues [65, 78]. Due to extensive cross-talk between apoptosis and necrosis, it is suggested that necrosis occurs following a prolonged apoptotic state, particularly *in vitro*. As such, many studies refer to necrosis as secondary necrosis [184]. This is very relevant to *in vitro*/*ex vivo* studies where apoptotic cells are not removed rapidly from the environment by phagocytic cells. Whether peripheral blood eosinophils in our study were directly induced to die by necroptosis or secondary necrosis was not addressed. Regardless of necrosis initiation, the reduction of necrotic eosinophils through calcitriol modulation appears to be an anti-inflammatory mechanism. It is plausible that during eosinophilia, as observed in asthma, an accumulation of apoptotic eosinophils overwhelms resolving immune cells leading to necrotic eosinophil death which promotes further inflammation. As mentioned, based on current results pertaining to calcitriol and eosinophil death, our data indicates that calcitriol modulates a separate pathway than apoptosis. Besides necrosis, autophagy is another candidate pathway as it directly promotes cell survival [3, 84, 88]. It is recognized by the formation of autophagosome fusion to lysosome for cytoplasmic material degradation [71]. Autophagy is commonly described by redistribution of LC3 fusion proteins in vesicular structures, which include autophagosomes as well as autolysosomes for degradation [71]. Also, phosphorylation of Ulk-1, leading to interaction with mTOR, in mammalian cells has been shown to directly induce autophagy [89-91]. To complicate matters further, autophagy is known to cross-talk specifically with necrosis, primarily through intracellular ROS production [84]. As a whole, death and survival signaling pathways remain to be properly studied in eosinophils in light of pathologic and therapeutic relevance.

5.3.1 Proposed Mechanism

Overall, our data suggests that calcitriol directly impacts eosinophil biological functions in an anti-inflammatory manner by preventing necrotic release of granule protein. As depicted in **Figure 18**, a mechanism is suggested in order to explain the effects of calcitriol on eosinophils. As mentioned, lung epithelial cells have been shown to constitutively convert calcifediol precursor into active calcitriol *in vitro* [15]. In normal conditions, it is implied that this localized function would create a calcitriol-rich microenvironment. Presumably, vitamin D deficiency would cause a lack in vitamin D metabolites which serve as precursors, resulting in lower calcitriol production. In our study, we showed that calcitriol affects two separate aspects of eosinophil biology:

#1. Inhibition of Eosinophil Necrosis/Cytolysis

Recruited eosinophils residing in lung tissue have three major 'cell fate' options: Autophagy, Apoptosis and Necrosis. Autophagy has been characterized in many immune cells, but is not yet known in eosinophils. When eosinophils receive abundant survival signals, autophagy is proposed to be activated. This would result in viable eosinophils which rely on activation factors (*i.e.,* cytokines, chemokines and cell-cell interactions) to execute specific functions. In the case where death signals overwhelm eosinophils, cells choose to die either by apoptosis or necrosis. Apoptosis remains the preferred death process in eosinophils and promotes anti-inflammatory outcomes. Apoptotic bodies are formed to contain and restrict intracellular components, such as granules and their cytotoxic proteins. These cellular compartments can be eliminated through phagocytosis of apoptotic bodies by macrophages leading to proper resolution of eosinophilic inflammation. In contrast, eosinophils can die by necrosis, resulting in cytolysis. Necrosis is considered a pro-inflammatory process since cytolytic death allows intracellular components to be expelled into tissue. Eosinophil cytotoxic mediators, such as MBP, EPX and EDN, are released and granules are deposited into tissues. Cytotoxic mediators cause direct epithelial damage and activation of pulmonary immune cells, causing production of inflammatory mediators, such as PGD_2. Mature eosinophils, as well as other immune cells, from surrounding areas are thus stimulated to infiltrate the damaged tissue. Calcitriol seems to modulate eosinophil demise in order to prevent necrosis and/or it may encourage autophagy (*i.e.,* survival). Our data indicates, however, VD did not increase apoptosis, but this needs to be further investigated. This

vitamin D metabolite may actually balance death and survival signals in order to avoid pro-inflammatory cell death. Likely, lower calcitriol levels (during vitamin D deficiency) in the lung may result in more eosinophil necrosis allowing inflammation to be established.

#2. Downregulation of Recruitment Receptors

Mature eosinophils from surrounding tissues and blood are recruited to lung tissues in inflammatory situations. Allergic immune responses as well as tissue damage stimulates the production of various inflammatory factors, such as chemokines. Chemokines mediate the recruitment of cells expressing their receptors. For example, PGD_2 is a chemoattractant molecule produced during airway inflammation which recruits eosinophils.[106] Thus, calcitriol-modulated CRTh2 and CCR10 expression reduces eosinophil responsiveness to respective receptor ligands which may lead to decreased eosinophil recruitment. In this way, calcitriol would avoid further tissue damage and, consequently, reduce inflammation amplification. The latter effects would expectedly encourage faster resolution. Seemingly, vitamin D deficiency, causing lower mucosal calcitriol concentrations, would lack such an anti-inflammatory mechanism in lungs.

5.4 Major Conclusions

We conclude that calcitriol may be a significant factor in airway inflammation through its direct anti-inflammatory effects on eosinophil biology. In the presence of IL-5, it diminishes eosinophil necrosis and decreases receptor expression. Thus, we propose that calcitriol helps to avoid eosinophil cytotoxic mediator release while discouraging amplification of pulmonary inflammatory responses through immune cell infiltration. Both distinct mechanisms would aim to diminish the propagation of eosinophil-driven inflammation.

Due to the descriptive nature of this study, functional mechanisms may be proposed but remain to be fully described. Further studies are needed in order to determine how calcitriol modulates: (1) death and/or survival pathways to avoid necrotic death, and (2) receptor expression to dampen eosinophil recruitment.

5.5 Future Directions

5.5.1 Calcitriol Modulating Eosinophil Death Signaling Pathways

Although not extensively studied, eosinophil cytolysis remains a clinically relevant observation and a major factor in allergic airway inflammation [75] as well as eosinophilic esophagitis [142]. Our results demonstrate that calcitriol reduces eosinophil granule and mediator release through decreased necrosis. Although findings may imply that calcitriol serves as a necrosis inhibitor, further studies are needed to evaluate calcitriol modulation of intrinsic death signals. The study of apoptosis, necrosis and/or autophagy is often complicated by cross-talk between these pathways. Therefore it is essential to select discriminatory signaling molecules used as specific pathway markers. To study the effect of calcitriol on eosinophil apoptosis, expression of caspase-8 and Bcl-2 should be analyzed. If caspase-8 is cleaved or phosphorylated, it indicates apoptosis induction. In contrast, Bcl-2 is an anti-apoptotic molecule which would be upregulated if apoptosis is being inhibited. With respect to programmed necrosis, calcitriol may modulate RIP1 as well as RIP3 expression. Furthermore, RIP3 phosphorylation would indicate necroptosis activation. Finally, to ensure autophagy is not a target of calcitriol, one may evaluate expression and phosphorylation of factors Ulk-1 and mTOR. Analysis of LC3 vesicles could serve as verification that autophagy is being induced. Overall, survival states and death outcomes are

classically defined by morphological characteristics [71]. Therefore, morphology assessment should be used to confirm signaling pathway induction with cell death outcome.

Eosinophils also rely on certain cell-cell interactions to execute functions [43, 50, 57]. Pertaining to eosinophil cytolysis, death can be induced extrinsically. Therefore, it is pertinent to also investigate calcitriol modulation on extrinsic death signals, such as TNF and FasL. Cell surface expression of TFNR and FasR could be assessed in response to calcitriol treatment. Furthermore, eosinophil death should be measured using Annexin-V-PI double stain (as executed in this study) in response to the respective receptors ligands, TNF and FasL, following calcitriol treatment. This would allow to determine if calcitriol not only modulates intrinsic death signaling pathways, but possibly influences susceptibility to death induced by other immune cells as an additional resolution mechanism.

5.5.2 Calcitriol Altering Eosinophil Recruitment

As discussed, experiments investigating eosinophil cell surface expression in response to calcitriol must be repeated in order to have statistical credibility. It is also important to verify chemokine receptor expression of peripheral blood eosinophils at baseline (*i.e.,* before treatments) as a reference point. It would be more relevant to show changes in receptor expression from baseline. Furthermore, changes in chemokine receptor expression should be supported by functional migration assays. Specifically, eosinophil migration patterns following calcitriol treatment would be analyzed in response to a chemoattractant, such as PGD_2. This would allow to confirm that downregulation of a receptor results in lower responsiveness to its ligand.

Additionally, eosinophils are known to secrete a variety of cytokines. Since eosinophils are multi-faceted immune cells, involved in homeostasis as well as pathology, they have the capacity to release pro-inflammatory cytokines (including: IL-4 and IL-6) and anti-inflammatory cytokines (including: TGF-β and IL-10). Notably, some eosinophil-produced cytokines do not only influence other immune cells, but alter tissue structure [165]. As a result, eosinophils have both the capacity to create microenvironments which either promote inflammation or induce resolution. Hence, it would be of value to investigate whether calcitriol modulates eosinophil cytokine aptitude. Such data would offer information on the potential for eosinophils to alter the lung environment when sufficient vitamin D is present.

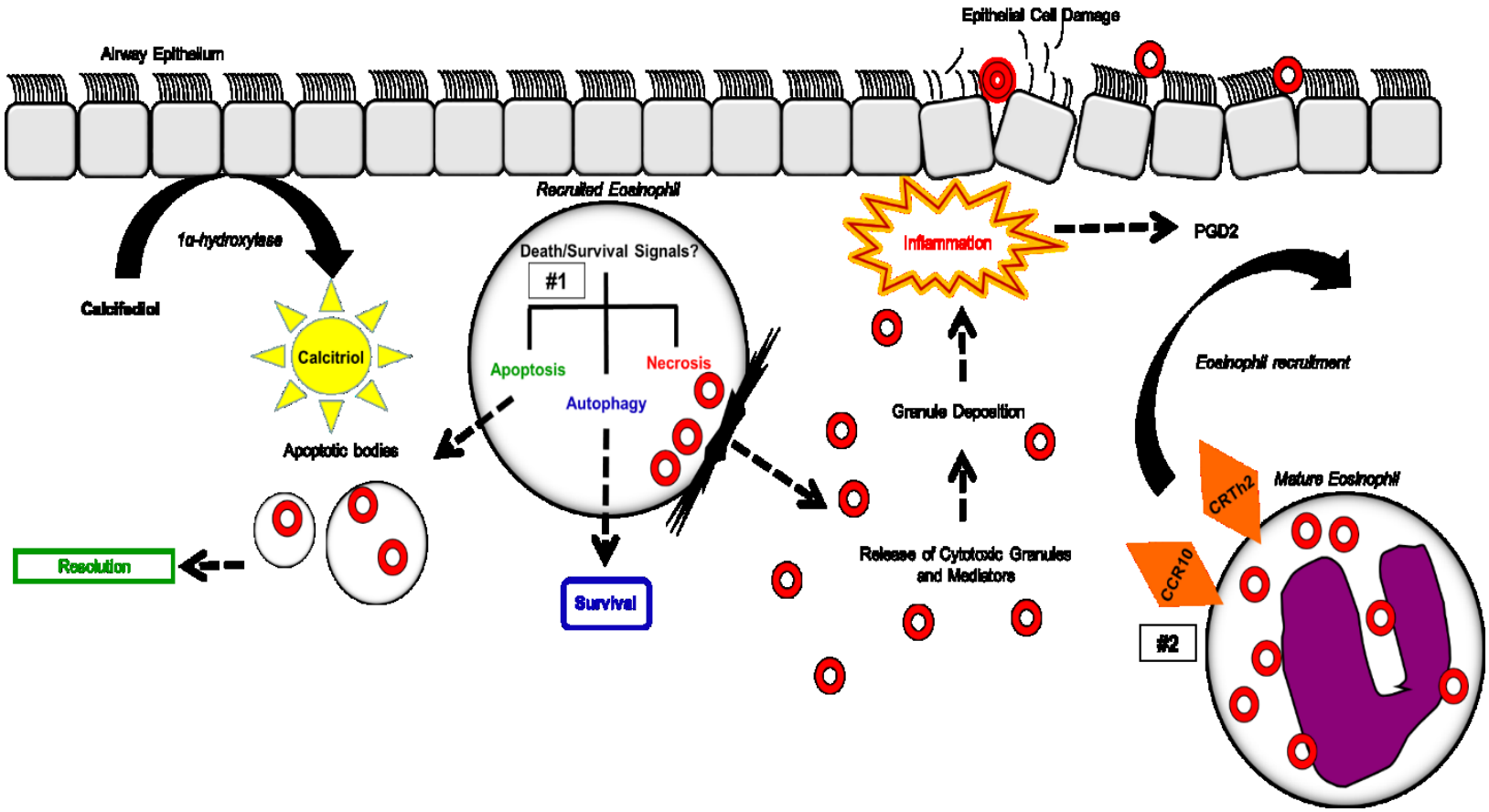


Figure 18: Proposed mechanism for effects of calcitriol on eosinophil inflammatory functions.

#1. Calcitriol impacts death signaling pathways to prevent eosinophil necrosis (*i.e.,* cytolytic death).

#2. Calcitriol decreases eosinophil cell surface receptor expression to discourage recruitment.

REFERENCES

1. Holick, M. F. (2007) Vitamin D deficiency. N Engl J Med 357, 266-81.
2. Bouillon, R., Carmeliet, G., Verlinden, L., van Etten, E., Verstuyf, A., Luderer, H. F., Lieben, L., Mathieu, C., Demay, M. (2008) Vitamin D and human health: lessons from vitamin D receptor null mice. Endocr Rev 29, 726-76.
3. Picotto, G., Liaudat, A. C., Bohl, L., Tolosa de Talamoni, N. (2012) Molecular aspects of vitamin D anticancer activity. Cancer Invest 30, 604-14.
4. Valdivielso, J. M. and Fernandez, E. (2006) Vitamin D receptor polymorphisms and diseases. Clin Chim Acta 371, 1-12.
5. Baeke, F., Takiishi, T., Korf, H., Gysemans, C., Mathieu, C. (2010) Vitamin D: modulator of the immune system. Curr Opin Pharmacol 10, 482-96.
6. Berg, J. P. (1999) Vitamin D-binding protein prevents vitamin D deficiency and presents vitamin D for its renal activation. Eur J Endocrinol 141, 321-2.
7. Berg, J. P. and Haug, E. (1999) Vitamin D: a hormonal regulator of the cAMP signaling pathway. Crit Rev Biochem Mol Biol 34, 315-23.
8. Haussler, M. R., Mangelsdorf, D. J., Komm, B. S., Terpening, C. M., Yamaoka, K., Allegretto, E. A., Baker, A. R., Shine, J., McDonnell, D. P., Hughes, M., et al. (1988) Molecular biology of the vitamin D hormone. Recent Prog Horm Res 44, 263-305.
9. Molnar, F. (2014) Structural considerations of vitamin D signaling. Front Physiol 5, 191.
10. Norman, A. W. (2008) From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. Am J Clin Nutr 88, 491S-499S.
11. Sutton, A. L. and MacDonald, P. N. (2003) Vitamin D: more than a "bone-a-fide" hormone. Mol Endocrinol 17, 777-91.
12. Akbar, N. A. and Zacharek, M. A. (2011) Vitamin D: immunomodulation of asthma, allergic rhinitis, and chronic rhinosinusitis. Curr Opin Otolaryngol Head Neck Surg 19, 224-8.
13. Ponchon, G., Kennan, A. L., DeLuca, H. F. (1969) "Activation" of vitamin D by the liver. J Clin Invest 48, 2032-7.
14. Szekely, J. I. and Pataki, A. (2012) Effects of vitamin D on immune disorders with special regard to asthma, COPD and autoimmune diseases: a short review. Expert Rev Respir Med 6, 683-704.

15. Hansdottir, S., Monick, M. M., Hinde, S. L., Lovan, N., Look, D. C., Hunninghake, G. W. (2008) Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. J Immunol 181, 7090-9.
16. Hansdottir, S. and Monick, M. M. (2011) Vitamin D effects on lung immunity and respiratory diseases. Vitam Horm 86, 217-37.
17. Sandhu, M. S. and Casale, T. B. (2010) The role of vitamin D in asthma. Ann Allergy Asthma Immunol 105, 191-9; quiz 200-2, 217.
18. Bozzetto, S., Carraro, S., Giordano, G., Boner, A., Baraldi, E. (2012) Asthma, allergy and respiratory infections: the vitamin D hypothesis. Allergy 67, 10-7.
19. Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J., O'Malley, B. W. (1988) Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci U S A 85, 3294-8.
20. MacDonald, P. N., Dowd, D. R., Nakajima, S., Galligan, M. A., Reeder, M. C., Haussler, C. A., Ozato, K., Haussler, M. R. (1993) Retinoid X receptors stimulate and 9-cis retinoic acid inhibits 1,25-dihydroxyvitamin D3-activated expression of the rat osteocalcin gene. Mol Cell Biol 13, 5907-17.
21. Wang, T. T., Tavera-Mendoza, L. E., Laperriere, D., Libby, E., MacLeod, N. B., Nagai, Y., Bourdeau, V., Konstorum, A., Lallemant, B., Zhang, R., Mader, S., White, J. H. (2005) Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. Mol Endocrinol 19, 2685-95.
22. Cantorna, M. T. (2010) Mechanisms underlying the effect of vitamin D on the immune system. Proc Nutr Soc 69, 286-9.
23. Cantorna, M. T., Zhao, J., Yang, L. (2012) Vitamin D, invariant natural killer T-cells and experimental autoimmune disease. Proc Nutr Soc 71, 62-6.
24. Yu, S., Zhao, J., Cantorna, M. T. (2011) Invariant NKT cell defects in vitamin D receptor knockout mice prevents experimental lung inflammation. J Immunol 187, 4907-12.
25. Wittke, A., Weaver, V., Mahon, B. D., August, A., Cantorna, M. T. (2004) Vitamin D receptor-deficient mice fail to develop experimental allergic asthma. J Immunol 173, 3432-6.

26. Berndt, A., Savage, H. S., Stearns, T. M., Paigen, B. (2011) Genetic analysis of lung function in inbred mice suggests vitamin D receptor as a candidate gene. Mol Genet Genomics 286, 237-46.
27. Zosky, G. R., Berry, L. J., Elliot, J. G., James, A. L., Gorman, S., Hart, P. H. (2011) Vitamin D deficiency causes deficits in lung function and alters lung structure. Am J Respir Crit Care Med 183, 1336-43.
28. Poon, A. H., Laprise, C., Lemire, M., Montpetit, A., Sinnett, D., Schurr, E., Hudson, T. J. (2004) Association of vitamin D receptor genetic variants with susceptibility to asthma and atopy. Am J Respir Crit Care Med 170, 967-73.
29. Raby, B. A., Lazarus, R., Silverman, E. K., Lake, S., Lange, C., Wjst, M., Weiss, S. T. (2004) Association of vitamin D receptor gene polymorphisms with childhood and adult asthma. Am J Respir Crit Care Med 170, 1057-65.
30. Du, R., Litonjua, A. A., Tantisira, K. G., Lasky-Su, J., Sunyaev, S. R., Klanderman, B. J., Celedon, J. C., Avila, L., Soto-Quiros, M. E., Weiss, S. T. (2012) Genome-wide association study reveals class I MHC-restricted T cell-associated molecule gene (CRTAM) variants interact with vitamin D levels to affect asthma exacerbations. J Allergy Clin Immunol 129, 368-73, 373 e1-5.
31. Wjst, M. (2005) Variants in the vitamin D receptor gene and asthma. BMC Genet 6, 2.
32. Saadi, A., Gao, G., Li, H., Wei, C., Gong, Y., Liu, Q. (2009) Association study between vitamin D receptor gene polymorphisms and asthma in the Chinese Han population: a case-control study. BMC Med Genet 10, 71.
33. Rudders, S. A., Espinola, J. A., Camargo, C. A., Jr. (2010) North-south differences in US emergency department visits for acute allergic reactions. Ann Allergy Asthma Immunol 104, 413-6.
34. Morales, E., Romieu, I., Guerra, S., Ballester, F., Rebagliato, M., Vioque, J., Tardon, A., Rodriguez Delhi, C., Arranz, L., Torrent, M., Espada, M., Basterrechea, M., Sunyer, J. (2012) Maternal vitamin D status in pregnancy and risk of lower respiratory tract infections, wheezing, and asthma in offspring. Epidemiology 23, 64-71.
35. Paul, G., Brehm, J. M., Alcorn, J. F., Holguin, F., Aujla, S. J., Celedon, J. C. (2012) Vitamin D and asthma. Am J Respir Crit Care Med 185, 124-32.

36. Hart, P. H., Gorman, S., Finlay-Jones, J. J. (2011) Modulation of the immune system by UV radiation: more than just the effects of vitamin D? Nat Rev Immunol 11, 584-96.
37. Hewison, M. (2010) Vitamin D and the intracrinology of innate immunity. Mol Cell Endocrinol 321, 103-11.
38. Hewison, M. (2011) Antibacterial effects of vitamin D. Nat Rev Endocrinol 7, 337-45.
39. Baeke, F., Korf, H., Overbergh, L., Verstuyf, A., Thorrez, L., Van Lommel, L., Waer, M., Schuit, F., Gysemans, C., Mathieu, C. (2011) The vitamin D analog, TX527, promotes a human CD4+CD25highCD127low regulatory T cell profile and induces a migratory signature specific for homing to sites of inflammation. J Immunol 186, 132-42.
40. van der Aar, A. M., Sibiryak, D. S., Bakdash, G., van Capel, T. M., van der Kleij, H. P., Opstelten, D. J., Teunissen, M. B., Kapsenberg, M. L., de Jong, E. C. (2011) Vitamin D3 targets epidermal and dermal dendritic cells for induction of distinct regulatory T cells. J Allergy Clin Immunol 127, 1532-40 e7.
41. Khoo, A. L., Chai, L. Y., Koenen, H. J., Oosting, M., Steinmeyer, A., Zuegel, U., Joosten, I., Netea, M. G., van der Ven, A. J. (2011) Vitamin D(3) down-regulates proinflammatory cytokine response to Mycobacterium tuberculosis through pattern recognition receptors while inducing protective cathelicidin production. Cytokine 55, 294-300.
42. Hiraguchi, Y., Tanida, H., Sugimoto, M., Hosoki, K., Nagao, M., Tokuda, R., Fujisawa, T. (2012) 1,25-Dihydroxyvitamin D3 upregulates functional C-x-C chemokine receptor type 4 expression in human eosinophils. Int Arch Allergy Immunol 158 Suppl 1, 51-7.
43. Fulkerson, P. C. and Rothenberg, M. E. (2013) Targeting eosinophils in allergy, inflammation and beyond. Nat Rev Drug Discov 12, 117-29.
44. Sutherland, E. R., Goleva, E., Jackson, L. P., Stevens, A. D., Leung, D. Y. (2010) Vitamin D levels, lung function, and steroid response in adult asthma. Am J Respir Crit Care Med 181, 699-704.
45. Brostoff, J. and Hall, T. (1998) Hypersentivity-Type I. In Immunology (I. M. Roitt, J. Brostoff, and D. Male, eds), Mosby, London.
46. Lacy, P. and Moqbel, R. (2001) Immune effector functions of eosinophils in allergic airway inflammation. Current opinion in allergy and clinical immunology 1, 79-84.
47. Erle, D. J. and Sheppard, D. (2014) The cell biology of asthma. J Cell Biol 205, 621-631.

48. Akuthota, P., Xenakis, J. J., Weller, P. F. (2011) Eosinophils: offenders or general bystanders in allergic airway disease and pulmonary immunity? J Innate Immun 3, 113-9.
49. Holgate, S. T. (2008) Pathogenesis of asthma. Clin Exp Allergy 38, 872-97.
50. Wegmann, M. (2011) Targeting eosinophil biology in asthma therapy. Am J Respir Cell Mol Biol 45, 667-74.
51. Lee, J. J., Dimina, D., Macias, M. P., Ochkur, S. I., McGarry, M. P., O'Neill, K. R., Protheroe, C., Pero, R., Nguyen, T., Cormier, S. A., Lenkiewicz, E., Colbert, D., Rinaldi, L., Ackerman, S. J., Irvin, C. G., Lee, N. A. (2004) Defining a link with asthma in mice congenitally deficient in eosinophils. Science 305, 1773-6.
52. Kupczyk, M., Ten Brinke, A., Sterk, P. J., Bel, E. H., Papi, A., Chanez, P., Nizankowska-Mogilnicka, E., Gjomarkaj, M., Gaga, M., Brusselle, G., Dahlen, B., Dahlen, S. E. (2014) Frequent exacerbators - a distinct phenotype of severe asthma. Clin Exp Allergy 44, 212-21.
53. Castro, M., Mathur, S., Hargreave, F., Boulet, L. P., Xie, F., Young, J., Wilkins, H. J., Henkel, T., Nair, P. (2011) Reslizumab for poorly controlled, eosinophilic asthma: a randomized, placebo-controlled study. Am J Respir Crit Care Med 184, 1125-32.
54. Haldar, P., Brightling, C. E., Hargadon, B., Gupta, S., Monteiro, W., Sousa, A., Marshall, R. P., Bradding, P., Green, R. H., Wardlaw, A. J., Pavord, I. D. (2009) Mepolizumab and exacerbations of refractory eosinophilic asthma. N Engl J Med 360, 973-84.
55. Simon, H. U., Yousefi, S., Schranz, C., Schapowal, A., Bachert, C., Blaser, K. (1997) Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. J Immunol 158, 3902-8.
56. Simon, H. U. (1997) Molecular mechanisms of defective eosinophil apoptosis in diseases associated with eosinophilia. Int Arch Allergy Immunol 113, 206-8.
57. Park, Y. M. and Bochner, B. S. (2010) Eosinophil survival and apoptosis in health and disease. Allergy Asthma Immunol Res 2, 87-101.
58. Rothenberg, M. E. (1998) Eosinophilia. N Engl J Med 338, 1592-600.
59. Kankaanranta, H., Moilanen, E., Zhang, X. (2005) Pharmacological regulation of human eosinophil apoptosis. Curr Drug Targets Inflamm Allergy 4, 433-45.
60. Rosenberg, H. F., Phipps, S., Foster, P. S. (2007) Eosinophil trafficking in allergy and asthma. J Allergy Clin Immunol 119, 1303-10; quiz 1311-2.

61. Corren, J. (2011) Anti-interleukin-5 antibody therapy in asthma and allergies. Curr Opin Allergy Clin Immunol 11, 565-70.
62. Hercus, T. R., Dhagat, U., Kan, W. L., Broughton, S. E., Nero, T. L., Perugini, M., Sandow, J. J., D'Andrea, R. J., Ekert, P. G., Hughes, T., Parker, M. W., Lopez, A. F. (2013) Signalling by the betac family of cytokines. Cytokine Growth Factor Rev 24, 189-201.
63. Simon, H. U. (2009) Cell death in allergic diseases. Apoptosis 14, 439-46.
64. Egan, R. W., Athwahl, D., Chou, C. C., Chapman, R. W., Emtage, S., Jenh, C. H., Kung, T. T., Mauser, P. J., Murgolo, N. J., Bodmer, M. W. (1997) Pulmonary biology of anti-interleukin 5 antibodies. Mem Inst Oswaldo Cruz 92 Suppl 2, 69-73.
65. Geering, B., Stoeckle, C., Conus, S., Simon, H. U. (2013) Living and dying for inflammation: neutrophils, eosinophils, basophils. Trends Immunol 34, 398-409.
66. Ochiai, K., Kagami, M., Matsumura, R., Tomioka, H. (1997) IL-5 but not interferon-gamma (IFN-gamma) inhibits eosinophil apoptosis by up-regulation of bcl-2 expression. Clin Exp Immunol 107, 198-204.
67. Letuve, S., Druilhe, A., Grandsaigne, M., Aubier, M., Pretolani, M. (2001) Involvement of caspases and of mitochondria in Fas ligation-induced eosinophil apoptosis: modulation by interleukin-5 and interferon-gamma. J Leukoc Biol 70, 767-75.
68. Kankaanranta, H., Lindsay, M. A., Giembycz, M. A., Zhang, X., Moilanen, E., Barnes, P. J. (2000) Delayed eosinophil apoptosis in asthma. J Allergy Clin Immunol 106, 77-83.
69. 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.
70. Nair, P., Pizzichini, M. M., Kjarsgaard, M., Inman, M. D., Efthimiadis, A., Pizzichini, E., Hargreave, F. E., O'Byrne, P. M. (2009) Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. N Engl J Med 360, 985-93.
71. Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., Blagosklonny, M. V., El-Deiry, W. S., Golstein, P., Green, D. R., Hengartner, M., Knight, R. A., Kumar, S., Lipton, S. A., Malorni, W., Nunez, G., Peter, M. E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B., Melino, G. (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16, 3-11.

72. Simon, H. U. and Blaser, K. (1995) Inhibition of programmed eosinophil death: a key pathogenic event for eosinophilia? Immunol Today 16, 53-5.
73. Takatsu, K. (2011) Interleukin-5 and IL-5 receptor in health and diseases. Proc Jpn Acad Ser B Phys Biol Sci 87, 463-85.
74. Yamaguchi, Y., Suda, T., Ohta, S., Tominaga, K., Miura, Y., Kasahara, T. (1991) Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. Blood 78, 2542-7.
75. Erjefalt, J. S. and Persson, C. G. (2000) New aspects of degranulation and fates of airway mucosal eosinophils. Am J Respir Crit Care Med 161, 2074-85.
76. Anderson, G. P. (1996) Resolution of chronic inflammation by therapeutic induction of apoptosis. Trends Pharmacol Sci 17, 438-42.
77. Ward, C., Dransfield, I., Chilvers, E. R., Haslett, C., Rossi, A. G. (1999) Pharmacological manipulation of granulocyte apoptosis: potential therapeutic targets. Trends Pharmacol Sci 20, 503-9.
78. Han, J., Zhong, C. Q., Zhang, D. W. (2011) Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nat Immunol 12, 1143-9.
79. Hitomi, J., Christofferson, D. E., Ng, A., Yao, J., Degterev, A., Xavier, R. J., Yuan, J. (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135, 1311-23.
80. Challa, S. and Chan, F. K. (2010) Going up in flames: necrotic cell injury and inflammatory diseases. Cell Mol Life Sci 67, 3241-53.
81. Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, A., Valitutti, S., Bodmer, J. L., Schneider, P., Seed, B., Tschopp, J. (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1, 489-95.
82. Xu, Y., Huang, S., Liu, Z. G., Han, J. (2006) Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. J Biol Chem 281, 8788-95.
83. Zong, W. X., Ditsworth, D., Bauer, D. E., Wang, Z. Q., Thompson, C. B. (2004) Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 18, 1272-82.

84. Lu, J. V. and Walsh, C. M. (2012) Programmed necrosis and autophagy in immune function. Immunol Rev 249, 205-17.
85. Kaczmarek, A., Vandenabeele, P., Krysko, D. V. (2013) Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity 38, 209-23.
86. Nicotera, P. and Melino, G. (2004) Regulation of the apoptosis-necrosis switch. Oncogene 23, 2757-65.
87. Ethier, C. and Davoine, F. (2013) A gap in cell death knowledge: is necroptosis of eosinophils involved in allergic airway inflammation. OA Inflammation 1.
88. Chang, Y. P., Chen, C. L., Chen, S. O., Lin, Y. S., Tsai, C. C., Huang, W. C., Wang, C. Y., Hsieh, C. Y., Choi, P. C., Lin, C. F. (2011) Autophagy facilitates an IFN-gamma response and signal transduction. Microbes Infect 13, 888-94.
89. Hosokawa, N., Hara, T., Kaizuka, T., Kishi, C., Takamura, A., Miura, Y., Iemura, S., Natsume, T., Takehana, K., Yamada, N., Guan, J. L., Oshiro, N., Mizushima, N. (2009) Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell 20, 1981-91.
90. Jung, C. H., Jun, C. B., Ro, S. H., Kim, Y. M., Otto, N. M., Cao, J., Kundu, M., Kim, D. H. (2009) ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell 20, 1992-2003.
91. Kim, J., Kundu, M., Viollet, B., Guan, K. L. (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 13, 132-41.
92. Walsh, G. M., Sexton, D. W., Blaylock, M. G. (2003) Corticosteroids, eosinophils and bronchial epithelial cells: new insights into the resolution of inflammation in asthma. J Endocrinol 178, 37-43.
93. Kawabori, S., Soda, K., Perdue, M. H., Bienenstock, J. (1991) The dynamics of intestinal eosinophil depletion in rats treated with dexamethasone. Lab Invest 64, 224-33.
94. Druilhe, A., Wallaert, B., Tsicopoulos, A., Lapa e Silva, J. R., Tillie-Leblond, I., Tonnel, A. B., Pretolani, M. (1998) Apoptosis, proliferation, and expression of Bcl-2, Fas, and Fas ligand in bronchial biopsies from asthmatics. Am J Respir Cell Mol Biol 19, 747-57.
95. Foresi, A., Teodoro, C., Leone, C., Pelucchi, A., D'Ippolito, R., Chetta, A., Olivieri, D. (2000) Eosinophil apoptosis in induced sputum from patients with seasonal allergic rhinitis

and with asymptomatic and symptomatic asthma. Ann Allergy Asthma Immunol 84, 411-6.

96. Woolley, K. L., Gibson, P. G., Carty, K., Wilson, A. J., Twaddell, S. H., Woolley, M. J. (1996) Eosinophil apoptosis and the resolution of airway inflammation in asthma. Am J Respir Crit Care Med 154, 237-43.

97. Druilhe, A., Letuve, S., Pretolani, M. (2003) Glucocorticoid-induced apoptosis in human eosinophils: mechanisms of action. Apoptosis 8, 481-95.

98. Zhang, J. P., Wong, C. K., Lam, C. W. (2000) Role of caspases in dexamethasone-induced apoptosis and activation of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase in human eosinophils. Clin Exp Immunol 122, 20-7.

99. Wang, W., Li, J. J., Foster, P. S., Hansbro, P. M., Yang, M. (2010) Potential therapeutic targets for steroid-resistant asthma. Curr Drug Targets 11, 957-70.

100. Zhang, Y., Leung, D. Y., Goleva, E. (2013) Vitamin D enhances glucocorticoid action in human monocytes: involvement of granulocyte-macrophage colony-stimulating factor and mediator complex subunit 14. J Biol Chem 288, 14544-53.

101. Fulkerson, P. C., Fischetti, C. A., Rothenberg, M. E. (2006) Eosinophils and CCR3 regulate interleukin-13 transgene-induced pulmonary remodeling. Am J Pathol 169, 2117-26.

102. Fulkerson, P. C., Fischetti, C. A., McBride, M. L., Hassman, L. M., Hogan, S. P., Rothenberg, M. E. (2006) A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. Proc Natl Acad Sci U S A 103, 16418-23.

103. Wegmann, M., Goggel, R., Sel, S., Erb, K. J., Kalkbrenner, F., Renz, H., Garn, H. (2007) Effects of a low-molecular-weight CCR-3 antagonist on chronic experimental asthma. Am J Respir Cell Mol Biol 36, 61-7.

104. Komai, M., Tanaka, H., Nagao, K., Ishizaki, M., Kajiwara, D., Miura, T., Ohashi, H., Haba, T., Kawakami, K., Sawa, E., Yoshie, O., Inagaki, N., Nagai, H. (2010) A novel CC-chemokine receptor 3 antagonist, Ki19003, inhibits airway eosinophilia and subepithelial/peribronchial fibrosis induced by repeated antigen challenge in mice. J Pharmacol Sci 112, 203-13.

105. Ying, S., Robinson, D. S., Meng, Q., Rottman, J., Kennedy, R., Ringler, D. J., Mackay, C. R., Daugherty, B. L., Springer, M. S., Durham, S. R., Williams, T. J., Kay, A. B. (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, 3507-16.

106. Hirai, H., Tanaka, K., Yoshie, O., Ogawa, K., Kenmotsu, K., Takamori, Y., Ichimasa, M., Sugamura, K., Nakamura, M., Takano, S., Nagata, K. (2001) Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med 193, 255-61.

107. Kagawa, S., Fukunaga, K., Oguma, T., Suzuki, Y., Shiomi, T., Sayama, K., Kimura, T., Hirai, H., Nagata, K., Nakamura, M., Asano, K. (2011) Role of prostaglandin D2 receptor CRTH2 in sustained eosinophil accumulation in the airways of mice with chronic asthma. Int Arch Allergy Immunol 155 Suppl 1, 6-11.

108. Sugimoto, H., Shichijo, M., Iino, T., Manabe, Y., Watanabe, A., Shimazaki, M., Gantner, F., Bacon, K. B. (2003) An orally bioavailable small molecule antagonist of CRTH2, ramatroban (BAY u3405), inhibits prostaglandin D2-induced eosinophil migration in vitro. J Pharmacol Exp Ther 305, 347-52.

109. Schuligoi, R., Sturm, E., Luschnig, P., Konya, V., Philipose, S., Sedej, M., Waldhoer, M., Peskar, B. A., Heinemann, A. (2010) CRTH2 and D-type prostanoid receptor antagonists as novel therapeutic agents for inflammatory diseases. Pharmacology 85, 372-82.

110. Barnes, N., Pavord, I., Chuchalin, A., Bell, J., Hunter, M., Lewis, T., Parker, D., Payton, M., Collins, L. P., Pettipher, R., Steiner, J., Perkins, C. M. (2012) A randomized, double-blind, placebo-controlled study of the CRTH2 antagonist OC000459 in moderate persistent asthma. Clin Exp Allergy 42, 38-48.

111. Honjo, A., Ogawa, H., Azuma, M., Tezuka, T., Sone, S., Biragyn, A., Nishioka, Y. (2013) Targeted reduction of CCR4(+) cells is sufficient to suppress allergic airway inflammation. Respir Investig 51, 241-9.

112. Garcia, G., Godot, V., Humbert, M. (2005) New chemokine targets for asthma therapy. Curr Allergy Asthma Rep 5, 155-60.

113. Xiong, N., Fu, Y., Hu, S., Xia, M., Yang, J. (2012) CCR10 and its ligands in regulation of epithelial immunity and diseases. Protein Cell 3, 571-80.

114. Homey, B., Alenius, H., Muller, A., Soto, H., Bowman, E. P., Yuan, W., McEvoy, L., Lauerma, A. I., Assmann, T., Bunemann, E., Lehto, M., Wolff, H., Yen, D., Marxhausen,

H., To, W., Sedgwick, J., Ruzicka, T., Lehmann, P., Zlotnik, A. (2002) CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. Nat Med 8, 157-65.

115. Kunkel, E. J., Kim, C. H., Lazarus, N. H., Vierra, M. A., Soler, D., Bowman, E. P., Butcher, E. C. (2003) CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. J Clin Invest 111, 1001-10.
116. Gleich, G. J. (2000) Mechanisms of eosinophil-associated inflammation. J Allergy Clin Immunol 105, 651-63.
117. Melo, R. C. and Weller, P. F. (2010) Piecemeal degranulation in human eosinophils: a distinct secretion mechanism underlying inflammatory responses. Histol Histopathol 25, 1341-54.
118. Erjefalt, J. S., Greiff, L., Andersson, M., Adelroth, E., Jeffery, P. K., Persson, C. G. (2001) Degranulation patterns of eosinophil granulocytes as determinants of eosinophil driven disease. Thorax 56, 341-4.
119. Erjefalt, J. S., Greiff, L., Andersson, M., Matsson, E., Petersen, H., Linden, M., Ansari, T., Jeffery, P. K., Persson, C. G. (1999) Allergen-induced eosinophil cytolysis is a primary mechanism for granule protein release in human upper airways. Am J Respir Crit Care Med 160, 304-12.
120. Melo, R. C., Liu, L., Xenakis, J. J., Spencer, L. A. (2013) Eosinophil-derived cytokines in health and disease: unraveling novel mechanisms of selective secretion. Allergy 68, 274-84.
121. Lacy, P., Logan, M. R., Bablitz, B., Moqbel, R. (2001) Fusion protein vesicle-associated membrane protein 2 is implicated in IFN-gamma-induced piecemeal degranulation in human eosinophils from atopic individuals. J Allergy Clin Immunol 107, 671-8.
122. Logan, M. R., Lacy, P., Bablitz, B., Moqbel, R. (2002) Expression of eosinophil target SNAREs as potential cognate receptors for vesicle-associated membrane protein-2 in exocytosis. J Allergy Clin Immunol 109, 299-306.
123. Logan, M. R., Odemuyiwa, S. O., Moqbel, R. (2003) Understanding exocytosis in immune and inflammatory cells: the molecular basis of mediator secretion. J Allergy Clin Immunol 111, 923-32; quiz 933.

124. Bankers-Fulbright, J. L., Kephart, G. M., Bartemes, K. R., Kita, H., O'Grady, S. M. (2004) Platelet-activating factor stimulates cytoplasmic alkalinization and granule acidification in human eosinophils. J Cell Sci 117, 5749-57.
125. Horie, S. and Kita, H. (1994) CD11b/CD18 (Mac-1) is required for degranulation of human eosinophils induced by human recombinant granulocyte-macrophage colony-stimulating factor and platelet-activating factor. J Immunol 152, 5457-67.
126. Kroegel, C., Yukawa, T., Dent, G., Venge, P., Chung, K. F., Barnes, P. J. (1989) Stimulation of degranulation from human eosinophils by platelet-activating factor. J Immunol 142, 3518-26.
127. Takizawa, T., Kato, M., Kimura, H., Suzuki, M., Tachibana, A., Obinata, H., Izumi, T., Tokuyama, K., Morikawa, A. (2002) Inhibition of protein kinases A and C demonstrates dual modes of response in human eosinophils stimulated with platelet-activating factor. J Allergy Clin Immunol 110, 241-8.
128. Takizawa, T., Kato, M., Suzuki, M., Tachibana, A., Motegi, Y., Fujiu, T., Kimura, H., Arakawa, H., Mochizuki, H., Tokuyama, K., Morikawa, A. (2003) Distinct isoforms of protein kinase C are involved in human eosinophil functions induced by platelet-activating factor. Int Arch Allergy Immunol 131 Suppl 1, 15-9.
129. Wardlaw, A. J., Moqbel, R., Cromwell, O., Kay, A. B. (1986) Platelet-activating factor. A potent chemotactic and chemokinetic factor for human eosinophils. J Clin Invest 78, 1701-6.
130. Zoratti, E. M., Sedgwick, J. B., Vrtis, R. R., Busse, W. W. (1991) The effect of platelet-activating factor on the generation of superoxide anion in human eosinophils and neutrophils. J Allergy Clin Immunol 88, 749-58.
131. Kimani, G., Tonnesen, M. G., Henson, P. M. (1988) Stimulation of eosinophil adherence to human vascular endothelial cells in vitro by platelet-activating factor. J Immunol 140, 3161-6.
132. Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T., et al. (1991) Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. Nature 349, 342-6.
133. Ishii, S., Kuwaki, T., Nagase, T., Maki, K., Tashiro, F., Sunaga, S., Cao, W. H., Kume, K., Fukuchi, Y., Ikuta, K., Miyazaki, J., Kumada, M., Shimizu, T. (1998) Impaired

anaphylactic responses with intact sensitivity to endotoxin in mice lacking a platelet-activating factor receptor. J Exp Med 187, 1779-88.

134. Ishii, S. and Shimizu, T. (2000) Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. Prog Lipid Res 39, 41-82.

135. Korth, R. M. (1996) Specific high affinity binding of platelet activating factor to intact human blood neutrophils and eosinophils. Int Arch Allergy Immunol 110, 124-31.

136. Ukena, D., Krogel, C., Dent, G., Yukawa, T., Sybrecht, G., Barnes, P. J. (1989) PAF-receptors on eosinophils: identification with a novel ligand, [3H]WEB 2086. Biochem Pharmacol 38, 1702-5.

137. Erjefalt, J. S., Andersson, M., Greiff, L., Korsgren, M., Gizycki, M., Jeffery, P. K., Persson, G. A. (1998) Cytolysis and piecemeal degranulation as distinct modes of activation of airway mucosal eosinophils. J Allergy Clin Immunol 102, 286-94.

138. Schwartz, R. H. (2003) T cell anergy. Annu Rev Immunol 21, 305-34.

139. Persson, C. G. and Erjefalt, J. S. (1997) Eosinophil lysis and free granules: an in vivo paradigm for cell activation and drug development. Trends Pharmacol Sci 18, 117-23.

140. Frigas, E., Loegering, D. A., Solley, G. O., Farrow, G. M., Gleich, G. J. (1981) Elevated levels of the eosinophil granule major basic protein in the sputum of patients with bronchial asthma. Mayo Clin Proc 56, 345-53.

141. Filley, W. V., Holley, K. E., Kephart, G. M., Gleich, G. J. (1982) Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. Lancet 2, 11-6.

142. Saffari, H., Hoffman, L. H., Peterson, K. A., Fang, J. C., Leiferman, K. M., Pease, L. F., 3rd, Gleich, G. J. (2014) Electron microscopy elucidates eosinophil degranulation patterns in patients with eosinophilic esophagitis. J Allergy Clin Immunol 133, 1728-1734 e1.

143. Rosenberg, H. F., Dyer, K. D., Domachowske, J. B. (2009) Eosinophils and their interactions with respiratory virus pathogens. Immunol Res 43, 128-37.

144. Rosenberg, H. F. and Domachowske, J. B. (2001) Eosinophils, eosinophil ribonucleases, and their role in host defense against respiratory virus pathogens. J Leukoc Biol 70, 691-8.

145. Rosenberg, H. F., Dyer, K. D., Domachowske, J. B. (2009) Respiratory viruses and eosinophils: exploring the connections. Antiviral Res 83, 1-9.

146. Kim, C. K., Koh, J. Y., Han, T. H., Kim do, K., Kim, B. I., Koh, Y. Y. (2006) Increased levels of BAL cysteinyl leukotrienesinacute [corrected] RSV bronchiolitis. Acta Paediatr 95, 479-85.

147. Piedimonte, G. (2013) Respiratory syncytial virus and asthma: speed-dating or long-term relationship? Curr Opin Pediatr 25, 344-9.

148. Cannell, J. J., Vieth, R., Umhau, J. C., Holick, M. F., Grant, W. B., Madronich, S., Garland, C. F., Giovannucci, E. (2006) Epidemic influenza and vitamin D. Epidemiol Infect 134, 1129-40.

149. Cannell, J. J., Zasloff, M., Garland, C. F., Scragg, R., Giovannucci, E. (2008) On the epidemiology of influenza. Virol J 5, 29.

150. Hansdottir, S., Monick, M. M., Lovan, N., Powers, L., Gerke, A., Hunninghake, G. W. (2010) Vitamin D decreases respiratory syncytial virus induction of NF-kappaB-linked chemokines and cytokines in airway epithelium while maintaining the antiviral state. J Immunol 184, 965-74.

151. Davoine, F., Lavigne, S., Chakir, J., Ferland, C., Boulay, M. E., Laviolette, M. (2002) Expression of FcgammaRIII (CD16) on human peripheral blood eosinophils increases in allergic conditions. J Allergy Clin Immunol 109, 463-9.

152. Ogawa, K., Hashida, R., Miyagawa, M., Kagaya, S., Sugita, Y., Matsumoto, K., Katsunuma, T., Akasawa, A., Tsujimoto, G., Saito, H. (2003) Analysis of gene expression in peripheral blood eosinophils from patients with atopic dermatitis and in vitro cytokine-stimulated blood eosinophils. Clin Exp Immunol 131, 436-45.

153. Bloom, J. W., Chacko, J., Lohman, I. C., Halonen, M., Martinez, F. D., Miesfeld, R. L. (2004) Differential control of eosinophil survival by glucocorticoids. Apoptosis 9, 97-104.

154. Reutelingsperger, C. P. and van Heerde, W. L. (1997) Annexin V, the regulator of phosphatidylserine-catalyzed inflammation and coagulation during apoptosis. Cell Mol Life Sci 53, 527-32.

155. Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184, 39-51.

156. Yang, M. Y., Chuang, H., Chen, R. F., Yang, K. D. (2002) Reversible phosphatidylserine expression on blood granulocytes related to membrane perturbation but not DNA strand breaks. J Leukoc Biol 71, 231-7.
157. Lecoeur, H. (2002) Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases. Exp Cell Res 277, 1-14.
158. Ageberg, M. and Lindmark, A. (2003) Characterisation of the biosynthesis and processing of the neutrophil granule membrane protein CD63 in myeloid cells. Clin Lab Haematol 25, 297-306.
159. Mahmudi-Azer, S., Downey, G. P., Moqbel, R. (2002) Translocation of the tetraspanin CD63 in association with human eosinophil mediator release. Blood 99, 4039-47.
160. Kim, J. D., Willetts, L., Ochkur, S., Srivastava, N., Hamburg, R., Shayeganpour, A., Seabra, M. C., Lee, J. J., Moqbel, R., Lacy, P. (2013) An essential role for Rab27a GTPase in eosinophil exocytosis. J Leukoc Biol 94, 1265-74.
161. Adamko, D. J., Wu, Y., Gleich, G. J., Lacy, P., Moqbel, R. (2004) The induction of eosinophil peroxidase release: improved methods of measurement and stimulation. J Immunol Methods 291, 101-8.
162. Davoine, F., Cao, M., Wu, Y., Ajamian, F., Ilarraza, R., Kokaji, A. I., Moqbel, R., Adamko, D. J. (2008) Virus-induced eosinophil mediator release requires antigen-presenting and CD4+ T cells. J Allergy Clin Immunol 122, 69-77, 77 e1-2.
163. Martin, L. B., Kita, H., Leiferman, K. M., Gleich, G. J. (1996) Eosinophils in allergy: role in disease, degranulation, and cytokines. Int Arch Allergy Immunol 109, 207-15.
164. Kroegel, C., Warner, J. A., Virchow, J. C., Jr., Matthys, H. (1994) Pulmonary immune cells in health and disease: the eosinophil leucocyte (Part II). Eur Respir J 7, 743-60.
165. Kita, H. (2011) Eosinophils: multifaceted biological properties and roles in health and disease. Immunol Rev 242, 161-77.
166. Persson, C. G. and Erjefalt, J. S. (1997) "Ultimate activation" of eosinophils in vivo: lysis and release of clusters of free eosinophil granules (Cfegs). Thorax 52, 569-74.
167. Tumes, D. J., Cormie, J., Calvert, M. G., Stewart, K., Nassenstein, C., Braun, A., Foster, P. S., Dent, L. A. (2007) Strain-dependent resistance to allergen-induced lung pathophysiology in mice correlates with rate of apoptosis of lung-derived eosinophils. J Leukoc Biol 81, 1362-73.

168. Ohnmacht, C., Pullner, A., van Rooijen, N., Voehringer, D. (2007) Analysis of eosinophil turnover in vivo reveals their active recruitment to and prolonged survival in the peritoneal cavity. J Immunol 179, 4766-74.
169. Erjefalt, J. S., Sundler, F., Persson, C. G. (1996) Eosinophils, neutrophils, and venular gaps in the airway mucosa at epithelial removal-restitution. Am J Respir Crit Care Med 153, 1666-74.
170. Greiff, L., Erjefalt, J. S., Andersson, M., Svensson, C., Persson, C. G. (1998) Generation of clusters of free eosinophil granules (Cfegs) in seasonal allergic rhinitis. Allergy 53, 200-3.
171. Tavera-Mendoza, L. E. and White, J. H. (2007) Cell defenses and the sunshine vitamin. Sci Am 297, 62-5, 68-70, 72.
172. Bosse, Y., Lemire, M., Poon, A. H., Daley, D., He, J. Q., Sandford, A., White, J. H., James, A. L., Musk, A. W., Palmer, L. J., Raby, B. A., Weiss, S. T., Kozyrskyj, A. L., Becker, A., Hudson, T. J., Laprise, C. (2009) Asthma and genes encoding components of the vitamin D pathway. Respir Res 10, 98.
173. Bosse, Y., Maghni, K., Hudson, T. J. (2007) 1alpha,25-dihydroxy-vitamin D3 stimulation of bronchial smooth muscle cells induces autocrine, contractility, and remodeling processes. Physiol Genomics 29, 161-8.
174. Wjst, M. (2005) Variants in the vitamin D receptor gene and asthma. BMC Genet 6, 2.
175. Melo, R. C., Dvorak, A. M., Weller, P. F. (2010) Contributions of Electron Microscopy to Understand Secretion of Immune Mediators by Human Eosinophils. Microsc Microanal, 1-8.
176. Hallett, J. M., Leitch, A. E., Riley, N. A., Duffin, R., Haslett, C., Rossi, A. G. (2008) Novel pharmacological strategies for driving inflammatory cell apoptosis and enhancing the resolution of inflammation. Trends in pharmacological sciences 29, 250-7.
177. Kodama, T., Matsuyama, T., Miyata, S., Nishimura, H., Nishioka, Y., Kitada, O., Sugita, M. (1998) Kinetics of apoptosis in the lung of mice with allergic airway inflammation. Clin Exp Allergy 28, 1435-43.
178. Dusso, A., Gonzalez, E. A., Martin, K. J. (2011) Vitamin D in chronic kidney disease. Best Pract Res Clin Endocrinol Metab 25, 647-55.

179. Lemire, J. M., Adams, J. S., Kermani-Arab, V., Bakke, A. C., Sakai, R., Jordan, S. C. (1985) 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity in vitro. J Immunol 134, 3032-5.

180. Kimpen, J. L., Garofalo, R., Welliver, R. C., Fujihara, K., Ogra, P. L. (1996) An ultrastructural study of the interaction of human eosinophils with respiratory syncytial virus. Pediatr Allergy Immunol 7, 48-53.

181. Kimpen, J. L., Garofalo, R., Welliver, R. C., Ogra, P. L. (1992) Activation of human eosinophils in vitro by respiratory syncytial virus. Pediatr Res 32, 160-4.

182. Garofalo, R., Kimpen, J. L., Welliver, R. C., Ogra, P. L. (1992) Eosinophil degranulation in the respiratory tract during naturally acquired respiratory syncytial virus infection. J Pediatr 120, 28-32.

183. Simon, H. and Alam, R. (1999) Regulation of eosinophil apoptosis: transduction of survival and death signals. Int Arch Allergy Immunol 118, 7-14.

184. Silva, M. T. (2010) Secondary necrosis: the natural outcome of the complete apoptotic program. FEBS Lett 584, 4491-9.

APPENDIX 1

A gap in cell death knowledge: Is necroptosis of eosinophils involved in allergic airway inflammation?

Introductory Comments

My supervisor, Dr. Francis Davoine, and I had the opportunity to write a brief review article for the Open Access Inflammation, related to our field of research. Since our team directly studies the eosinophil granulocyte in asthma and allergy, at a basic science level, we decided to focus on this cell type. We constructed a review focused on new ideas and concepts in relation to eosinophil functions. Since aspects of my graduate research project touched upon eosinophil death, we decided to link new discoveries in cell death signaling pathways (specifically apoptosis and necrosis) with unexplained observations of eosinophils in allergic inflammatory disorders. We also included hints of the newly suggested, yet controversial, anti-viral role of eosinophils.

Writing such a review, outside the scope of the vitamin D hypothesis, allowed me to deepen my knowledge on cutting-edge cell death advances as well as broaden my knowledge of eosinophil immunological roles.

Publication Reference:

Ethier C, Davoine F. A gap in cell death knowledge: Is necroptosis of eosinophils involved in allergic airway inflammation? OA Inflammation 2013 Aug 01;1(1):9.

OA Inflammation
Open Access

Critical Review

Cell Biology

A gap in cell death knowledge: is necroptosis of eosinophils involved in allergic airway inflammation?

C Ethier[1], F Davoine[1,2]*

Abstract

Introduction

Eosinophils are continually targeted in allergic airway inflammatory disease therapies. The presence of their granules in airway tissues remains unexplained, yet implies the involvement of necrosis. The latter's definition has evolved into a highly regulated and distinct signalling pathway leading to physiological inflammation, known as necroptosis. Even though necroptosis is a recently introduced concept, we currently recognize its role as an alternative mechanism in the absence of apoptosis. Furthermore, necroptosis seems to act as a host-defence strategy against viral infections, which consequently associates itself with eosinophils' role in airway viral clearance. The aim of this review is to discuss if necroptosis of eosinophils is involved in allergic airway inflammation.

Conclusion

The investigation of necroptosis in eosinophil is currently an area which lacks research, yet harbours great promise for drug development.

Introduction

Allergic airway inflammatory diseases remain prevalent in many populations today. Their management remains a burden on health care systems; moreover, the diseases affect the patients' quality of life. Although therapies triggering eosinophils, in particular corticosteroids, have greatly improved allergic and asthmatic symptoms in airways, disease management is not optimal in every case[1]. Human studies within this field continue to unveil unexplained phenomena involving eosinophils in persisting inflammation. With recent studies suggesting that the regulation of cell death pathways is implicated in the progression of allergic inflammation[2,3], one may wonder if necrosis may be the missing link in therapeutic approaches. Physiological necrosis was first observed over a decade ago[4], yet we recently started to understand its regulated mechanisms within the immune system as well as its pathological relevance[4–7]. The purpose of this review is to bridge our knowledge of eosinophil biology in allergic airway disease with cutting-edge discoveries in programmed cell death (PCD) pathways, in order to incite research towards better alternatives for treating eosinophilic airway inflammation. Even though concepts discussed in this review may be applicable to other eosinophilic pathologies, this paper will be limited to the area of eosinophilic allergic airway inflammation.

Discussion

The authors have referenced some of their own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.

The ever-changing concept of 'necrosis'

Necrosis is largely characterized by morphological traits, such as an increase in cell volume, swelling of organelles, among loss of plasma membrane integrity, among other traits. The term 'necrosis' was classically understood as an unregulated form of cell death that induces inflammation. Stemming from this perspective, necrosis is commonly known as the opposite of apoptosis, the classical PCD pathway which promotes the resolution of inflammation[8]. Nowadays, the concept of necrosis has adopted some complexity as well as a new name, necroptosis, as to indicate its regulated nature[4,8,9]. Based on current scientific literature, necrosis may be subdivided into three distinct definitions: cytolysis, secondary necrosis and necroptosis. Cytolysis is a general term used to describe cells which have died through lysis, thus expelling their intracellular contents due to non-specific external factors which have compromised the integrity of the plasma membrane. Similarly, secondary necrosis also fits the latter description, yet is said to occur within a cell that has first been in the apoptotic state. In contrast, necroptosis is a newer concept which describes a highly regulated death signalling pathway specific for this pro-inflammatory process[8]. It is now known that necroptosis can be initiated, similarly to apoptosis, by 'extrinsic stimulus', such as tumour necrosis factor (TNF) and Fas ligand (FasL), or 'intrinsic stimulus', such as DNA damage, depending on the cell's state[5,10–12]. Pertaining to host-defence, it has also been shown that necroptosis can be specifically triggered through recognition of pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs) and sensors of DNA as well as RNA. Considering its involvement in inflammation, necroptosis is beginning to be studied

* Corresponding author
Email: fdavoine@ualberta.ca

[1] Department of Medicine, University of Alberta
[2] FaculténSaint-Jean, University of Alberta 8406, rue Marie-Anne-Gaboury (91 ST), Edmonton, AB, Canada, T6C3V5

FOR CITATION PURPOSES: *Ethier C, Davoine F.* A gap in cell death knowledge: is necroptosis of eosinophils involved in allergic airway inflammation? OA Inflammation 2013 Aug 01;1(1):9.

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

OA Inflammation
Open Access

Critical Review

in some immune cells, such as T cells[4,7]. Even though eosinophils have been well established as effector cells in allergic inflammatory reactions, necroptosis remains a mystery within this cell type.

Eosinophils in allergic airway inflammation: revealing hints of necroptosis

Over the years, eosinophils have been shown to play a variety of roles ranging from homeostasis, pathogen clearance and pathology. Its pro-inflammatory abilities are largely related to its granules and granule contents. In addition to a plethora of immunological and physiological molecules (i.e. chemokines, cytokines and growth factors), eosinophil granules contain cationic proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPX)[13–16]. Combined with traditional intracellular adjuvants, also known as damage-associated molecular patterns (DAMPs) (i.e. HMGB1, heat shock proteins, DNA and RNA), these cytotoxic molecules render eosinophils extremely potent inducers of inflammation in the event of their cytolysis. In fact, cytolysis is now recognized as a separate mode of degranulation in eosinophils[17–19].

Apart from the basic biology of eosinophils, their abnormal recruitment and infiltration into lung tissues, which lead to airway inflammation and tissue damage, have become hallmarks of asthma. Although the exact mechanism is not yet fully described, various researchers previously proposed that the increased total amount of eosinophils observed in asthmatics caused improper airway eosinophilia. More recently, studies in this field have shown that delayed-apoptosis or insufficient apoptosis of eosinophils is an important factor in the pathology of asthma[20–22]. As previously mentioned, immune cells involved in allergic inflammation tend to lack proper cell death regulation.

Moreover, studies on airway tissues, even as old as the study by Parrot and Leyden in the 1800s, report the presence of free extracellular eosinophil granules (Cfegs). Cfegs can equally be found in human whole-mount airway preparations[17,23–25]. Although the study of Cfegs in wild-type mouse models have not been reported due to lack of degranulation and cytolysis, eosinophil cytolysis has been observed in rats, dogs, primates and guinea pigs. In fact, cytolysis of mucosal tissue eosinophils was induced within 1 h of an allergen challenge in guinea pigs. These findings suggest a necrotic type of eosinophil cell death rather than apoptotic, where intracellular components would remain in apoptotic bodies for proper clearance[26]. In support of this, a recently developed IL-5/human eotaxin-2 double transgenic mouse model demonstrates eosinophil-dependent inflammation as well as granule deposition[27]. It has also been reported that 'asthma-like' insults, such as airway inflammation, induces rapid death of eosinophils, which causes granule deposition in these tissues[18]. Likewise, eosinophil cytolysis has been often observed and imaged in various tissues subdued to eosinophilic inflammation[17,24]. Interestingly, apoptosis has been viewed as the primary mode of demise for eosinophils[13,28–32], although classic studies, such as work done by Kodama and co-workers, fail to demonstrate apoptotic eosinophils in these tissues[31]. In addition, the ambiguous outcomes in asthma symptom management from various anti-interleukin-5 (anti-IL-5) therapies, which specifically target the eosinophil apoptotic pathway, could be interpreted as supporting that an alternative PCD pathway is playing a role in airway inflammatory pathologies[33–35].

Overall, eosinophils are distinct immune cells that are highly susceptible to dying in the absence of pro-survival cytokines[13,14,16,29,32,36,37], which validates that death signalling pathways are at the heart of their biology and, consequently, our understanding of eosinophilic pathologies. Therefore, deepening our understanding of PCD pathways, primarily necroptosis in relation to apoptosis, is necessary in improving eosinophil-targeting therapies.

Cross-talk between apoptosis and necroptosis

While the subject of eosinophil cytolysis and necrosis has been largely pushed aside within scientific literature[17], eosinophil apoptosis has received major attention. It is currently accepted that apoptosis is an important component for the resolution of inflammation. Thus, most popular drugs targeting eosinophil seek to increase the initiation of apoptosis (e.g. corticosteroids and anti-IL-5 therapies)[17,28,38,39]. In fact, most of our knowledge of eosinophil apoptosis stems from studies on the role of IL-5 in eosinophilpoiesis and eosinophil survival[32,35,40]. Extensive knowledge of eosinophil apoptosis can indeed help us understand eosinophil necroptosis. We now know that apoptosis and necroptosis share many components for signalling complexes in which receptor interacting serine/threonine-protein kinase 3 (RIPK3) recruitment and activation causes it to complex with RIPK1, which specifically determines the cell's fate[4,6–8,41]. In fact, it has been shown in pancreatic tissues that a switch from apoptosis to necroptosis can occur through induction of RIPK3[4,42]. Also, overexpression of RIPK3 explicitly induces necroptosis[43]. Interestingly, the inhibition of caspase-8 (casp8), a molecule necessary for the extrinsic apoptotic pathway, results in increased expression of RIPK3[44]. This finding may be suggestive of a cause–effect relationship between delayed apoptosis and increased necroptosis, which would

FOR CITATION PURPOSES: *Ethier C, Davoine F.* A gap in cell death knowledge: is necroptosis of eosinophils involved in allergic airway inflammation? OA Inflammation 2013 Aug 01;1(1):9.

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

OA Inflammation
Open Access

Critical Review

explain the presence of improper apoptosis in airway inflammatory pathologies. Also, there is increasing evidence that both PCD pathways may inhibit each other[4,6,41].

In general, our familiarity with apoptosis–necroptosis cross-talk remains basic, yet expansion in this field of research is very promising. So far, we understand that necroptosis may serve as a backup PCD pathway in the event of caspase-dependent apoptosis inhibition[4].

The role of necroptosis within the immune system

The role of necroptosis as an immunological process is currently gaining more interest. Although there is still much to learn about necroptosis, examining its inflammatory function during immune responses as a whole can help us clarify its mechanisms in relation to pathology. Even if necroptosis is a pro-inflammatory event, there is increasing evidence that such a cytolytic death may be a natural process for immune cells[6]. When various mouse tissues were screened for the expression of necroptosis-related genes using zVAD.fmk, a necroptosis inducer and non-specific caspase inhibitor, clusters showing increased expression were found particularly in immune as well as neuronal cells. More specifically, out of 119 mouse tissue samples, 83% were primary immune cells. Furthermore, necrostatin-1 (Nec-1), an inhibitor of necroptosis, was able to block zVAD.fmk-induced death as well as spontaneous death in macrophages[9]. Although the effects of zVAD.fmk and Nec-1 were not directly studied using eosinophils, one may extrapolate that eosinophils be intrinsically more prone to necroptosis as well.

The marked presence of necroptosis in immune cells may be explained through immune cell activation. More specifically, necroptosis is implicated in certain immune cellular processes, such as mitochondrial and lysosomal alterations leading to production of reactive oxygen species (ROS), nitric oxide (NO) and other similar compounds[8,41]. In fact, Hitomi and colleagues were able to link RIPK1-dependant necroptosis to increased ROS production by which they suggest that necroptosis may be driven by ROS production in T cells[9]. Overall, the production of these compounds in immune cells might explain why necroptosis may be more prominent in these cell types.

It is presently well known that allergic responses are inappropriate events which consist of immune cells mimicking a host-defence strategy. Because necroptosis is pro-inflammatory in nature, one may wonder the primary function of having such a regulated mechanism.Therefore, the function of necroptosis may be explained through pathogen clearance. There is a marked association between viral infections, such as vaccinia virus, herpes virus and cowpox, and 'necrosis-like' cell death[4,45,46]. Also, necroptosis seems to be intrinsically initiated by viruses that block apoptosis through virally encoded caspase inhibitors[4]. Specifically, the inhibition of casp8 is a popular strategy for such viruses to silence the apoptotic pathway in host cells[47,48]. In this way, necroptosis may be an evolutionary back-up signalling pathway to clear pathogens in the absence of apoptosis. More convincingly, cytomegalovirus (CMV) actually possesses a gene which encodes a necroptosis inhibitor[49]. It seems this virus has already evolved a mechanism in order to counter the necroptotic host-defence strategy. Such a virulence factor is suggestive that necroptosis is an important pathway for immune defence and should be studied more closely. The latter explanation may pertain to allergic airway disease since eosinophils are known to promote viral clearance in the lung through ligand binding of various TLRs[13,50]. Indeed, certain viruses, such as respiratory syncytial virus (RSV), have been shown to induce eosinophil degranulation[51]. As previously alluded, viral-specific pathogen-recognition receptors (PRRs) may directly trigger necroptosis. Furthermore, it has been well established that respiratory viruses, including RSV, are associated with asthma exacerbations[52]. Taking into account both facts as well as the knowledge of viruses with anti-apoptotic abilities, one may assume that RSV could trigger eosinophil recruitment into lungs as well as cause the switch from apoptosis to necroptosis, in order to trigger potent inflammation as a mechanism of viral clearance. Such an explanation would be in line with worsening of asthma symptoms during respiratory viral infections, such as asthma exacerbations.

If major findings in the necroptotic signalling pathway were to be merged with our knowledge of eosinophil functions related to allergic airway inflammatory diseases, a conceptual mechanism can take shape, as depicted in Figure 1. Theoretically, eosinophils which are recruited to airway tissues may be triggered by viral particles directly through PRRs, or indirectly by cytokines, such as through the TNFα receptor, or interactions with other immune cells, through the Fas receptor, in order to induce necroptosis. The activation of eosinophils causes ROS production, which may further induce necroptosis. It may also be possible that direct viral infection of eosinophils causes a state of anti-apoptosis through the inhibition of casp8, which results in an upregulation of RIPK3. The latter would form a complex with other death signalling proteins, including RIPK1, rendering it a necroptotic signalling complex. In the context of allergy, an allergen may initiate a very similar cascade of events. Finally, the induction of necroptosis would result in plasma membrane disruption

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

FOR CITATION PURPOSES: *Ethier C, Davoine F.* A gap in cell death knowledge: is necroptosis of eosinophils involved in allergic airway inflammation? OA Inflammation 2013 Aug 01;1(1):9.

OA Inflammation
Open Access

Critical Review

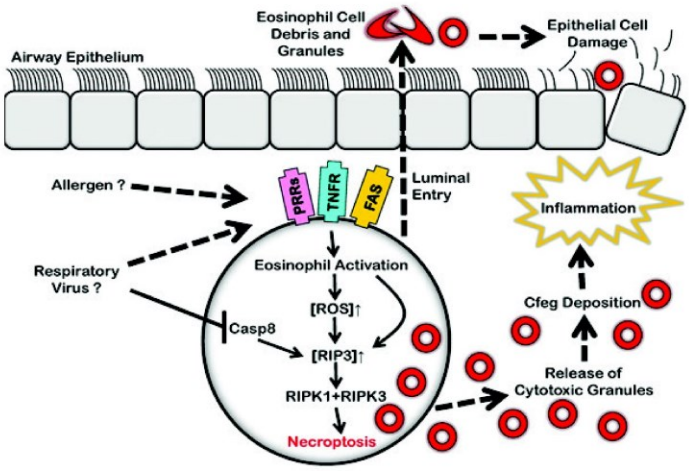


Figure 1: Conceptual mechanism of eosinophil granule release through necroptosis. Eosinophils become activated due to various stimuli, such as PAMPs, DAMPs, TNFα and FasL, which induce a cascade of intracellular events leading to increased RIPK3 expression ROS production may increase upon immune cell activation. Complexing of RIPK1 and RIPK3, results in specific induction of necroptosis, which leads to eosinophil granule release.

causing intracellular components to leak out. Some researchers have previously suggested that eosinophil cellular debris might be expelled from the lung tissue through luminal entry, where eosinophils may be further expelled from the body by mucociliary movement and coughing. Cfegs would be deposited within the lung tissue, which could extend the inflammation. In order to confirm this regulated pro-inflammatory mechanism, various supporting evidence is needed. Although the presence of Cfegs in airway tissues is not a new observation, advancements in this area of research were discouraged by lack of techniques in PCD pathways as well as general tissue-handling methods. In addition, it remains unclear if necroptosis of immune cells, such as eosinophils, is intrinsically predominant or is the cause of their interactions with pathogens, allergens and/or other immune cells.

Conclusion

Presently, the topic of eosinophil PCD pathways is a field with many unanswered questions although it is a very promising area for drug development. More specifically, there is no direct evidence that necroptosis of eosinophils is a factor in allergic airway inflammation, even though current knowledge strongly implies that necroptosis may be the missing link in understanding how eosinophils potently promote inflammation during allergic responses. More than 25 years ago, Fukuda and team had noted that eosinophils may be programmed to lyse upon stimulation under specific conditions. Although their research efforts alluded to necroptosis, it cannot be denied that this question was asked prior to its time. It is very reasonable to postulate that necroptosis is gravely influencing the pro-inflammatory capabilities of eosinophils through controlling the release of their intracellular cytotoxic elements as an independent degranulation mode.

Abbreviations list

DAMPs, damage-associated molecular patterns; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPX, eosinophil peroxidase; FasL, Fas ligand; MBP, major basic protein; PAMP, pathogen-associated molecular pattern; PCD, programmed cell death; PRR, pathogen-recognition receptor; ROS, reactive oxygen species; RSV, respiratory syncytial virus; TNF, tumour necrosis factor.

Acknowledgement

We would like to thank Dr Paige Lacy for her help in perfecting this review.

References

1. Wegmann M. Targeting eosinophil biology in asthma therapy. Am J Respir Cell Mol Biol. 2011 Oct;45(4):667–74.
2. Tumes DJ, Cormie J, Calvert MG, Stewart K, Nassenstein C, Braun A, et al. Strain-dependent resistance to allergen-induced lung pathophysiology in mice correlates with rate of apoptosis of lung-derived eosinophils. J Leukoc Biol. 2007 Jun;81(6):1362–73.
3. Ohnmacht C, Pullner A, van Rooijen N, Voehringer D. Analysis of eosinophil turnover in vivo reveals their active recruitment to and prolonged survival in the peritoneal cavity. J Immunol. 2007 Oct;179(7):4766–74.
4. Han J, Zhong CQ, Zhang DW. Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nat Immunol. 2011 Dec;12(12):1143–9.
5. Challa S, Chan FK. Going up in flames: necrotic cell injury and inflammatory diseases. Cell Mol Life Sci. 2010 Oct;67(19):3241–53.
6. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity. 2013 Feb;38(2):209–23.
7. Lu JV, Walsh CM. Programmed necrosis and autophagy in immune function. Immunol Rev. 2012 Sep;249(1):205–17.
8. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH,

FOR CITATION PURPOSES: *Ethier C, Davoine F.* A gap in cell death knowledge: is necroptosis of eosinophils involved in allergic airway inflammation? OA Inflammation 2013 Aug 01;1(1):9.

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

OA Inflammation
Open Access

Critical Review

et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ. 2009 Jan;16(1):3–11.
9. Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, et al. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell. 2008 Dec;135(7):1311–23.
10. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol. 2000 Dec;1(6):489–95.
11. Xu Y, Huang S, Liu ZG, Han J. Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. J Biol Chem. 2006 Mar;281 (13):8788–95.
12. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev. 2004 Jun;18(11):1272–82.
13. Fulkerson PC, Rothenberg ME. Targeting eosinophils in allergy, inflammation and beyond. Nat Rev Drug Discov. 2013 Feb;12(2):117–29.
14. Martin LB, Kita H, Leiferman KM, Gleich GJ. Eosinophils in allergy: role in disease, degranulation, and cytokines. Int Arch Allergy Immunol. 1996 Mar; 109(3):207–15.
15. Kroegel C. The immunologic role of eosinophilic granulocytes within the scope of inflammatory lung diseases. Pneumologie. 1994 Feb;48(2):74–84.
16. Kroegel C, Warner JA, Virchow JC, Jr, Matthys H. Pulmonary immune cells in health and disease: the eosinophil leucocyte (Part II). Eur Respir J. 1994 Apr;7(4):743–60.
17. Erjefalt JS, Persson CG. New aspects of degranulation and fates of airway mucosal eosinophils. Am J Respir Crit Care Med. 2000 Jun;161 (6):2074–85.
18. Persson CG, Erjefalt JS. Eosinophil lysis and free granules: an in vivo paradigm for cell activation and drug development. Trends Pharmacol Sci. 1997 Apr;18(4):117–23.
19. Persson CG, Erjefalt JS. "Ultimate activation" of eosinophils in vivo: lysis and release of clusters of free eosinophil granules (Cfegs). Thorax. 1997 Jun;52(6):569–74.
20. Kankaanranta H, Moilanen E, Zhang X. Pharmacological regulation of human eosinophil apoptosis. Curr Drug Targets Inflamm Allergy. 2005 Aug;4(4):433–45.
21. Simon HU. Molecular mechanisms of defective eosinophil apoptosis in diseases associated with eosinophilia. Int Arch Allergy Immunol. 1997 May–Jul; 113(1–3):206–8.
22. Simon HU, Yousefi S, Schranz C, Schapowal A, Bachert C, Blaser K. Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. J Immunol. 1997 Apr;158 (8):3902–8.
23. Erjefalt JS, Sundler F, Persson CG. Eosinophils, neutrophils, and venular gaps in the airway mucosa at epithelial removal-restitution. Am J Respir Crit Care Med. 1996 May;153(5):1666–74.
24. Erjefalt JS, Andersson M, Greiff L, Korsgren M, Gizycki M, Jeffery PK, et al. Cytolysis and piecemeal degranulation as distinct modes of activation of airway mucosal eosinophils. J Allergy Clin Immunol. 1998 Aug;102(2):286–94.
25. Greiff L, Erjefalt JS, Andersson M, Svensson C, Persson CG. Generation of clusters of free eosinophil granules (Cfegs) in seasonal allergic rhinitis. Allergy. 1998 Feb;53(2):200–3.
26. Rothenberg ME. Eosinophilia. N Engl J Med. 1998 May;338(22):1592–600.
27. Ochkur SI, Jacobsen EA, Protheroe CA, Biechele TL, Pero RS, McGarry MP, et al. Coexpression of IL-5 and eotaxin-2 in mice creates an eosinophil-dependent model of respiratory inflammation with characteristics of severe asthma. J Immunol. 2007 Jun;178(12):7879–89.
28. Anderson GP. Resolution of chronic inflammation by therapeutic induction of apoptosis. Trends Pharmacol Sci. 1996 Dec;17(12):438–42.
29. Ilmarinen P, Kankaanranta H. Eosinophil apoptosis as a therapeutic target in allergic asthma. Basic Clin Pharmacol Toxicol. 2013 Oct;114(1):109–17.
30. Jacobsen EA, Ochkur SI, Lee NA, Lee JJ. Eosinophils and asthma. Curr Allergy Asthma Rep. 2007 Apr;7(1):18–26.
31. Kodama T, Matsuyama T, Miyata S, Nishimura H, Nishioka Y, Kitada O, et al. Kinetics of apoptosis in the lung of mice with allergic airway inflammation. Clin Exp Allergy. 1998 Nov;28(11): 1435–43.
32. Park YM, Bochner BS. Eosinophil survival and apoptosis in health and disease. Allergy Asthma Immunol Res. 2010 Apr;2(2):87–101.
33. Corren J. Anti-interleukin-5 antibody therapy in asthma and allergies. Curr Opin Allergy Clin Immunol. 2011 Dec; 11(6):565–70.
34. Corren J. Inhibition of interleukin-5 for the treatment of eosinophilic diseases. Discov Med. 2012 Apr;13(71):305–12.
35. Takatsu K. Interleukin-5 and IL-5 receptor in health and diseases. Proc Jpn Acad Ser B Phys Biol Sci. 2011;87(8):463–85.
36. Kita H. Eosinophils: multifaceted biological properties and roles in health and disease. Immunol Rev. 2011 Jul; 242(1):161–77.
37. Kroegel C, Virchow JC, Jr, Luttmann W, Walker C, Warner JA. Pulmonary immune cells in health and disease: the eosinophil leucocyte (Part I). Eur Respir J. 1994 Mar;7(3):519–43.
38. Simon HU, Blaser K. Inhibition of programmed eosinophil death: a key pathogenic event for eosinophilia? Immunol Today. 1995 Feb;16(2):53–5.
39. Ward C, Dransfield I, Chilvers ER, Haslett C, Rossi AG. Pharmacological manipulation of granulocyte apoptosis: potential therapeutic targets. Trends Pharmacol Sci. 1999 Dec;20(12):503–9.
40. Yamaguchi Y, Suda T, Ohta S, Tominaga K, Miura Y, Kasahara T. Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. Blood. 1991 Nov;78(10):2542–7.
41. Nicotera P, Melino G. Regulation of the apoptosis-necrosis switch. Oncogene. 2004 Apr;23(16):2757–65.
42. He S, Wang L, Miao L, Wang T, Du F, Zhao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell. 2009 Jun; 137(6):1100–11.
43. Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science. 2009 Jul;325 (5938):332–6.
44. Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, et al. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular

FOR CITATION PURPOSES: *Ethier C, Davoine F.* A gap in cell death knowledge: is necroptosis of eosinophils involved in allergic airway inflammation? OA Inflammation 2013 Aug 01;1(1):9.

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

OA Inflammation
Open Access

Critical Review

cell death complex differentially regulated by cFLIP isoforms. Mol Cell. 2011 Aug;43(3):449–63.
45. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell. 2009 Jun;137(6): 1112–23.
46. Ray CA, Pickup DJ. The mode of death of pig kidney cells infected with cowpox virus is governed by the expression of the crmA gene. Virology. 1996 Mar;217(1):384–91.
47. Galluzzi L, Kepp O, Morselli E, Vitale I, Senovilla L, Pinti M, et al. Viral strategies for the evasion of immunogenic cell death. J Intern Med. 2010 May; 267(5):526–42.
48. Lamkanfi M, Dixit VM. Manipulation of host cell death pathways during microbial infections. Cell Host Microbe. 2010 Jul;8(1):44–54.
49. Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe. 2010 Apr;7(4):302–13.
50. Phipps S, Lam CE, Mahalingam S, Newhouse M, Ramirez R, Rosenberg HF, et al. Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. Blood. 2007 Sep;110(5):1578–86.
51. Davoine F, Cao M, Wu Y, Ajamian F, Ilarraza R, Kokaji AI, et al. Virus-induced eosinophil mediator release requires antigen-presenting and CD4+ T cells. J Allergy Clin Immunol. 2008 Jul;122(1): 69–77, e1–2.
52. Tsukagoshi H, Ishioka T, Noda M, Kozawa K, Kimura H. Molecular epidemiology of respiratory viruses in virus-induced asthma. Front Microbiol. 2013 Sep;4:278.

FOR CITATION PURPOSES: *Ethier C, Davoine F.* A gap in cell death knowledge: is necroptosis of eosinophils involved in allergic airway inflammation? OA Inflammation 2013 Aug 01;1(1):9.

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

APPENDIX 2

Other Accomplishments

Appendix Preface

Since my research focused on eosinophil biology, the ability to isolate and purify primary human eosinophils became a necessity. I had the pleasure of receiving training from Ying Wu, an experienced laboratory technician and member of the Airway Inflammation Team within the Pulmonary Research Group at the University of Alberta, regarding eosinophil isolations and experimental handling. I also volunteered to be trained in phlebotomy, at research level, through Alberta Health Services Phlebotomy Department at the University of Alberta Hospital. I took the initiative in order to be more independent in the laboratory. During my graduate studies, both skill sets allowed me to make eosinophil handling in the Airway Inflammation Team laboratory my specialty. With on-going technical practice, I have been able to optimize eosinophil isolations in total number and purity as well as developed a confidence in venous blood extraction from blood donors. For these reasons, I have become a dynamic member of the Airway Inflammation Team. I have been able to share my technical skills in order to aid to laboratory colleagues' projects. Furthermore, I was recruited due to my practical skills in eosinophil handling and technical knowledge in eosinophil isolations for the following publications:

1. **Eosinophils in human oral squamous carcinoma; role of prostaglandin D2.** (Journal of Inflammation)

For this study, I isolated primary eosinophils from peripheral blood in order to test the effect of HQL-79 drug (a PGD2 synthase inhibitor) on eosinophil viability. This experiment was done in response to reviewer comments suggesting that HQL-79 may decrease eosinophil survival, therefore impacting overall results. I was able to confirm that HQL-79 did not induce death of eosinophils, by verifying viability levels through flow cytometry using Annexin-V-PI double stain. Hence, we were able to refute the expressed concern which allowed the manuscript's publication.

Publication Reference:

Davoine F., Sim A., Tang C., Fisher S., **Ethier C.**, Puttagunta L., Wu Y., McGaw W.T., Yu D., Cameron L., Adamko D.J., Moqbel R. (2013) Eosinophils in human oral squamous carcinoma; role of prostaglandin D2. J Inflamm (Lond) 10, 1-7

2. **Identification of human eosinophils in whole blood by flow cytometry** (Springer Protocols Manuscript - Humana Press)

Due to the familiarity I developed during my graduate work in eosinophil isolation from human blood, I contributed to a scientific protocol describing how to discriminately stain human eosinophils in whole blood by flow cytometry. I was able to share my observations and experiences in eosinophil manipulation as well as acquisition of eosinophils through flow cytometry. Therefore, I was heavily involved in the writing process of the following technical manuscript, alongside Dr. F. Davoine and Dr. P. Lacy. This effort did not only offer me another soon-to-be publication, but allowed me to view my research project and eosinophil handling *ex vivo* from a more technical and analytical perspective. Consequently, my involvement on this scientific communication deepened my overall knowledge on eosinophil research.

Publication Reference:

In Press

Identification of human eosinophils in whole blood by flow cytometry

Caroline Ethier[1], Paige Lacy[1] and Francis Davoine [1,2]

Origin: [1]Pulmonary Research Group and [2]Campus Saint-Jean, University of Alberta, Edmonton, Alberta, Canada.

Summary

Identification of eosinophils in whole blood samples by flow cytometry is often problematic. There are usually only a low number and percentage of cells that may be detected, and it may be difficult to discriminate eosinophils from other granulocytes. Here, we propose a simple approach using the eosinophil's intrinsic autofluorescence properties, combined with detection of CCR3 expression, to reliably identify eosinophils in a mixed leukocyte population.

Key words: Flow cytometry, eosinophils, granulocytes, white blood cell differential, CCR3, CD193.

1. Introduction

The identification of eosinophil granulocytes in whole blood samples represents a challenge. These cells are part of a rare population, and are usually difficult to discriminate from other granulocytes simply by the measurement of light scattering properties. Isolation of circulating eosinophils requires large quantities of blood (50-100 ml) as well as lengthy manipulation procedures. Moreover, many of the techniques used for eosinophil isolation may activate granulocytes and/or deplete important subpopulations, such hypodense or CD16-positive subpopulations. For example, CD16 expression on eosinophils can vary in pathological conditions such as asthma, or by cytokine stimulation [1-6]. This can be circumvented through whole blood staining of eosinophils in combination with standard flow cytometry equipment using simple gating strategies and specific eosinophil markers. The use of intrinsic physical and optical characteristics of eosinophils in order to identify these cells in whole blood has been elegantly demonstrated by Lavigne et al. in 1997[7]. They reported the use of depolarized orthogonal light scattering to allow better discrimination of neutrophils from eosinophils. Unfortunately, it may not be possible or practical to modify side scatter settings in most flow cytometry facilities with a depolarized light filter, which poses a major

technical limitation. Here, we describe the use of simple physical-optical attributes of light scattering, autofluorescence, and specific receptor expression of CCR3 to reliably identify human eosinophils in a mixed leukocyte population from whole venous blood samples.

2. Materials

1. Sterile distilled water must be used to prepare all solutions. No sodium azide should be added to any reagents.
2. Lysis Buffer: Ammonium chloride-potassium bicarbonate lysing solution. Add 800 ml of water to a glass beaker. Weigh 8.29 g of NH_4Cl, 1 g of $KHCO_3$, 0.0372 g of Na_2EDTA and transfer to the beaker. Mix and adjust the pH to 7.35 with HCl. Transfer to a 1 L graduated glass cylinder and make up to 1 L with distilled water. Store at room temperature and use within 4 weeks.
3. Phosphate-buffered saline (PBS): Add 800 ml of distilled water to a glass beaker. Weigh 8 g of NaCl, 1.15 g of Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g of KCl and transfer to the beaker, and dissolve thoroughly with stirring. Adjust pH to 7.35 with HCl. Transfer to a graduated glass cylinder and make up to 1 L. Keep at room temperature (see Note 1).
4. 10X Phosphate-buffered saline (10X PBS): Add ~70 ml of water to a glass beaker. Weigh 8 g of NaCl, 1.15 g of Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g of KCl and transfer to the beaker, and dissolve thoroughly with stirring. Transfer to a graduated glass cylinder and make up to 100 ml. Keep at room temperature (see Note 1).
5. Blocking Buffer: 5% goat serum in PBS. Add 0.5 mL of goat serum in 10 ml of PBS. Mix well and keep at 4°C.
6. Flow Buffer: 5% bovine serum albumin in PBS. Weigh 50 g of bovine serum albumin and add to 1 L PBS in a beaker. Mix well and keep at 4°C. Prepare fresh on the day of use, preferentially, and discard within a week of preparation as this is easily contaminated by microbial growth.
7. Paraformaldehyde (4%): Warm 80 ml of PBS to 65°C in a beaker using a microwave (i.e., not boiling!) (see Note 2). Weigh 4 g of paraformaldehyde and add to the warm water under the fume hood. Add a magnetic stirrer and mix for 10 minutes until the solution is fully suspended and cloudy. Then add 1 drop of NaOH (4 M) to the solution. The solution will

turn almost instantly clear. If not, add another drop of NaOH and repeat until the solution is absolutely clear (see Note 3). Cool down the solution for 30 minutes in an ice bucket under the fume hood. When the solution is ice cold adjust pH to 7.3 with HCl. Then using a graduated glass cylinder, add PBS to make up to 100 ml. Aliquot the solutions in 15 ml tubes and keep at -20°C.(see Note 4)

3. Methods

3.1. Staining of whole blood leukocytes

1. Venous blood is usually collected in Vacutainer™ collection tubes containing anti-coagulant (see Note 5).
2. To 5 mL of blood, add 10 ml of Lysis Solution in a 50 ml Falcon conical tube (see Note 6).
3. Incubate 10 min at room temperature on a rocking plate (gentle mixing at 30 rpm). Add PBS (room temperature) to 50 ml to stop the reaction and centrifuge at 200 g for 7 minutes.
4. Remove the supernatant by aspiration. Recap the tube and resuspend the pellet of cells by flicking the bottom of the tube with your fingers (see Note 7). Then add 5 ml Blocking Buffer, and count cells using a hemocytometer. If there are still some red blood cells, this won't cause a problem for staining and flow cytometry acquisition.
5. Adjust the cell concentration to 107 cells/mL with Blocking Buffer. Mix gently by manually flicking the cell suspension without using a pipette or vortexing. This is important for maintaining cell integrity.
6. Add 100 uL of cell suspension to a flow cytometry tube (5 ml snap cap tubes) for each sample (see Note 8). Typically, you will need one tube for an isotype control (e.g., IgG1 if the test antibody is an IgG1 antibody), and a second tube for the test control (i.e., the antibody of interest which is IgG1 in this example) where you wish to carry out single staining. If performing multiple marker labeling with antibodies conjugated to different fluorescent dyes, you will need to generate a multiple isotype control for each antibody used by combining isotype control antibodies in one tube. It is also recommended to include an additional tube as an unstained autofluorescence control that lacks antibodies.

7. For a demonstration experiment, we used conjugated antibodies against human CD4-FITC, CD45-FITC, CD8-PE, CD3-PERCP, CCR3-APC and CD25-APC. See Table 1 for the staining protocol.
8. Add antibodies according to your staining plan (e.g., Table 1). Since monoclonal antibodies may come from different sources and production batches, refer to the manufacturer's instructions for optimal concentrations required. Add the antibodies directly in the cell suspension at the bottom of respective tubes. Mix gently without vortexing.
9. Incubate antibodies with cell suspensions. Ideally, incubate on ice for 20 min protected from light.
10. After incubation, add 4 ml Flow Buffer to each tube and centrifuge at 200 g for 5 min at 4°C (see Note 9).
11. Remove supernatants and discard liquid directly into a beaker containing a small volume of bleach for biohazard control. The last remaining drop of liquid at the tube collar should be wiped using absorbent tissue paper (see Note 10).
12. Resuspend each pellet by gentle tube flicking.
13. Add 100 uL of paraformaldehyde (4%) solution, mix each tube by gentle agitation, and keep on ice for 10 min.
14. After fixation, add 300 uL of Flow Buffer (see Note 11).
15. Keep in dark at 0-4°C until ready for flow cytometry acquisition.

3.2. Distinguishing eosinophils from other blood leukocytes by flow cytometry

1. Since there are a wide variety of flow cytometers available, we will not discuss specific instrument settings here, but instead will provide strategies to obtain the desired results. Results shown as an example in this section were obtained using a BD FACSCanto™ II Flow Cytometer.
2. To begin acquisition, we recommend performing a baseline setting of the instrument's photomultipliers using Tube #1 (autofluorescence, Table 1). Using forward scattering (FS) and side scattering (SSC), adjust the voltage settings of the photomultipliers to visualize the entire cell population. In our example, we used a log scale for SSC to spread the scale and allow better grouping of different populations depicted on flow cytometry density plots. Increase the voltage and gain as much as necessary for FS and SS properties of your

cell population to ensure you visualize the entire population. Too low a voltage setting will result in grouping all cells in the lower left corner of the density plot, and render it impossible to discriminate intact cells from debris and erythrocytes. In Figure 1, a representative flow cytometry density plot of side scatter (SSC-log) and forward scatter (FS-Linear) is shown in a whole blood sample. Granulocytes, monocytes and lymphocytes are easily discriminated based on their light scattering properties using the argon 488 nm laser. However, it is usually difficult to clearly distinguish eosinophils from neutrophils in the granulocyte population using only forward and side scattering characteristics.

3. Next, examine the FITC-related fluorescence in the cell population by acquisition of data using the argon 488 laser. Neutrophils are less autofluorescent than eosinophils when excited by the argon laser, allowing discrimination of these two cell types. Figure 2A shows a typical FITC isotype control result. This density plot reveals a small population of cells (eosinophils) exhibiting high SSC-Log along with higher autofluorescence. Using different antibodies against CD4, CD3, CD8, CD45 and CD25 receptors conjugated to common fluorophores (FITC, PE, PERCP, APC), it is possible to distinguish a population of CD4+ cells (Figure 2B). CD45 is present on all leukocytes, to varying intensities, as depicted in Figure 2C. This density plot depicts CD45+ cells shifted to the right side of the graph, while CD45- erythrocytes and debris remain in their original position on the left side of the density plot. In Figures 2A, B and C, autofluorescent eosinophils are clearly discernible from neutrophils.
4. Eosinophil autofluorescence may also be detected with other commonly used photomultipliers for PE (585 nm) and PERCP (670 nm) when excited with 488 nm argon laser. Figure 3A and C show eosinophil autofluorescence observed with isotype controls for PE and PERCP, respectively. Staining for CD8-PE and CD3-PERCP (Figure 3 B & D) as well as CD4-FITC (Figure 2B) did not affect the autofluorescence of eosinophils.
5. To confirm the identity of eosinophils, a specific marker is required. Good surface markers for flow cytometry identification of human eosinophils include VLA-4 (CD49)[8], CRTH2 (CD294)[9] and CCR3 (CD193)[10]. In our example, we confirmed eosinophil identification by autofluorescence using a far red wavelength HeNe (633 nm) laser. In contrast to the use of the 488 nm argon laser to detect eosinophil autofluorescence, eosinophils fail to autofluoresce distinctly from neutrophils when excited with the 633 nm

laser, as seen in Figure 4A using the isotype control for APC (660 nm). This allows a clear identification of eosinophils using anti-CCR3-APC in Figure 4B. The CCR3+ population of cells with high SSC-log (eosinophils) was back-gated on autofluorescent cells in Figures 2A, 3A and 3C to confirm their identification as eosinophils, shown in Figure 5 for PE autofluorescence. Figure 4C shows an additional control (staining with anti-CD25-APC, the IL-2 receptor) indicating the absence of autofluorescent high SSC-log cells, as well as confirming the absence of non-specific binding in the isotype control in Figure 4A and CCR3-APC (Figure 4B).

6. Using autofluorescence-based identification strategies for eosinophils, it is possible to collect highly purified eosinophils by sorting using flow cytometry. Sorting should be carried out only on unlabeled cells to ensure that they are not activated for additional measurements.

4. Notes

1. You can also use ready-made PBS and 10X PBS from laboratory suppliers. To prevent clumping of leukocytes, use PBS without Mg2+ and Ca2+; these cations contribute to coagulation. PBS must be prepared fresh on the day of use, and must be discarded if unused as it is prone to microbial contamination.
2. Wear a mask and gloves when weighing paraformaldehyde. Cover the weigh boat with another weigh boat of similar size to avoid dispersion of paraformaldehyde powder when transporting to the fume hood. Keep the solution under the fume hood until it is ice cold. We highly recommend adding PBS at this stage, because buffered paraformaldehyde is superior to paraformaldehyde prepared in water. The reason for this is that paraformaldehyde becomes formaldehyde in solution, which then breaks down into formic acid very readily if the solution is not buffered. Even if it is frozen at -20 C, thawing paraformaldehyde in water increases formic acid development. Formic acid degrades cells and does not fix them appropriately.
3. Paraformaldehyde is very difficult to solubilize unless you warm the solution and increase its pH. Usually one or two drops of NaOH (4 M) is sufficient to dissolve paraformaldehyde powder.

4. Paraformaldehyde solution will stay stable for at least one year when stored at -20°C. It is highly recommended to use PBS to dissolve paraformaldehyde, because buffered paraformaldehyde is superior to paraformaldehyde prepared in water. The reason for this is that when paraformaldehyde becomes formaldehyde in solution, it rapidly produces formic acid if the solution is not buffered. Even if frozen at -20°C, thawing paraformaldehyde in water will result in formic acid generation. Formic acid does not fix cells and instead degrades them. Once paraformaldehyde is prepared in this manner, aliquot the solution in 15 ml conical tubes, and thaw the minimum amount necessary for daily use. If after thawing the solution is slightly cloudy, place the hermetically closed tube in lukewarm water (50-60°C) for 10 minutes until clear again. Then place tube in an ice bucket to cool it down.
5. VacutainerTM blood collection tubes may contain EDTA (purple cap) or heparin (green cap) to prevent coagulation. EDTA is the preferred anti-coagulant to use when working with granulocytes.
6. All these manipulations should be done in a biosafety laminar flow cabinet in a Level 2 laboratory, and in compliance with your institution's biosafety regulations regarding handling of human samples.
7. Flicking the tubes: Hold one well-closed tube by the cap and tap the bottom side with one finger to cause the cell pellet to gently breakdown. If this flicking does not work you can carefully use a 2 ml pipette and, by gentle up-down aspiration, break up the pellet.
8. Carefully pipette 100 uL cell suspensions without up and down aspirations, since shear stress through the pipette tip may cause cells to disintegrate. Slowly dispense cell suspensions directly to the bottom of the tube. The resulting number of cells per tube should be 106/100 uL. Keep tubes in an open tube rack and place over crushed ice in a large ice box.
9. This is a washing step to remove unbound antibodies. You should be able to see a similar small pellet of cells at the bottom of each tube. Some remaining red blood cells may give the pellet a pink color.
10. Our experience suggests that pouring out supernatants (direct dispensation) is less likely to cause loss of cells, and may be less time-consuming than aspiration of supernatants directly from tubes. With top to bottom aspiration of supernatant liquid, there is more of a risk of

turbulence and inadvertent loss of cells. Perform this step in one gentle motion without shaking the tube. There is usually about 50 uL of liquid remaining in the tube after direct dispensation. This also prevents exposure of cells to air, which causes degradation of cell integrity.

11. Fixation of labeled cells with paraformaldehyde will stabilize antibodies on cell surfaces and prevent further degradation of cells. The final concentration of paraformaldehyde will be a little less than 1%, which is sufficient to preserve cell integrity and staining for at least 72 h.

Acknowledgements

This work was supported by funding from Agriculture Life and Environmental Science (ALES) and Campus Saint-Jean research funds from University of Alberta to FD.

References

1. Hartnell, A., Moqbel, R., Walsh, G. M., Bradley, B., Kay, A. B. (1990) Fc gamma and CD11/CD18 receptor expression on normal density and low density human eosinophils. Immunology 69, 264-70.

2. Davoine, F., Labonte, I., Ferland, C., Mazer, B., Chakir, J., Laviolette, M. (2004) Role and modulation of CD16 expression on eosinophils by cytokines and immune complexes. International archives of allergy and immunology 134, 165-72.

3. Davoine, F., Lavigne, S., Chakir, J., Ferland, C., Boulay, M. E., Laviolette, M. (2002) Expression of FcgammaRIII (CD16) on human peripheral blood eosinophils increases in allergic conditions. J Allergy Clin Immunol 109, 463-9.

4. Valerius, T., Repp, R., Kalden, J. R., Platzer, E. (1990) Effects of IFN on human eosinophils in comparison with other cytokines. A novel class of eosinophil activators with delayed onset of action. Journal of immunology 145, 2950-8.

5. Zhu, X., Hamann, K. J., Munoz, N. M., Rubio, N., Mayer, D., Hernrreiter, A., Leff, A. R. (1998) Intracellular expression of Fc gamma RIII (CD16) and its mobilization by chemoattractants in human eosinophils. Journal of immunology 161, 2574-9.

6. Hartnell, A., Kay, A. B., Wardlaw, A. J. (1992) IFN-gamma induces expression of Fc gamma RIII (CD16) on human eosinophils. Journal of immunology 148, 1471-8.

7. Lavigne, S., Bosse, M., Boulet, L. P., Laviolette, M. (1997) Identification and analysis of eosinophils by flow cytometry using the depolarized side scatter-saponin method. Cytometry 29, 197-203.

8. Thurau, A. M., Schylz, U., Wolf, V., Krug, N., Schauer, U. (1996) Identification of eosinophils by flow cytometry. Cytometry 23, 150-8.

9. Faucher, J. L., Lacronique-Gazaille, C., Frebet, E., Trimoreau, F., Donnard, M., Bordessoule, D., Lacombe, F., Feuillard, J. (2007) "6 markers/5 colors" extended white blood cell differential by flow cytometry. Cytometry A 71, 934-44.

10. Ponath, P. D., Qin, S., Post, T. W., Wang, J., Wu, L., Gerard, N. P., Newman, W., Gerard, C., Mackay, C. R. (1996) Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. The Journal of experimental medicine 183, 2437-48.

Figure legends

Figure 1: A typical 488 nm light scattering density plot of whole blood after lysis of erythrocytes with ammonium chloride solution.

Figure 2: Density plots of 488 nm argon laser excitation and green (530 nm) emission of whole blood leukocytes. (A) Emission from isotype control (mouse IgG) coupled with FITC (non-specific staining control). (B) Staining of CD4+ T cells with anti-CD4-FITC. (C) Staining of total leukocytes with anti-CD45-FITC.

Figure 3: Density plots of 488 nm argon laser excitation and orange (585 nm) and red (670 nm) emission of whole blood leukocytes. (A & C) Isotype controls for PE and PERCP, respectively. (B & D) Specific staining for CD8+ T cells (CD8-PE) and all T cells (CD3-PERCP). Autofluorescent eosinophil cell populations are circled in each density plot.

Figure 4: Density plots of 633 nm HeNe laser excitation and red (660 nm) emission of whole blood leukocytes. (A) Isotype control emission (mouse IgG coupled with APC). (B) Leukocytes stained with anti-CCR3-APC to confirm eosinophils. (C) Staining for anti-CD25-APC to detect cells expressing IL-2 receptor. Rectangles in density plots show positive staining of high SSC-Log (SSC-A) for CCR3 in (B) and CD25+ T cells in (C).

Figure 5: Confirmation of specific eosinophil granulocytes autofluorescence. The red events in panels (C & D) are coming from the sort rectangle gate in (A & B) respectively. CCR3+ cells in (B) are showing in red in panel (D) confirming that high SS/CCR3+ cells are also high SS/high autofluorescent cells.

Figure 1

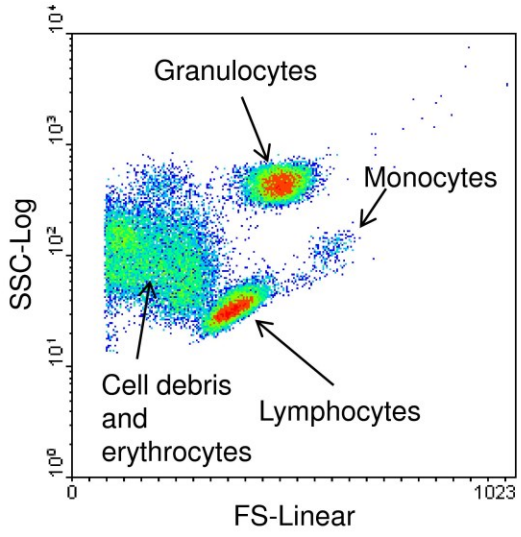

Granulocytes
Monocytes
Cell debris
and
erythrocytes
Lymphocytes
SSC-Log
FS-Linear
0
1023

Figure 2

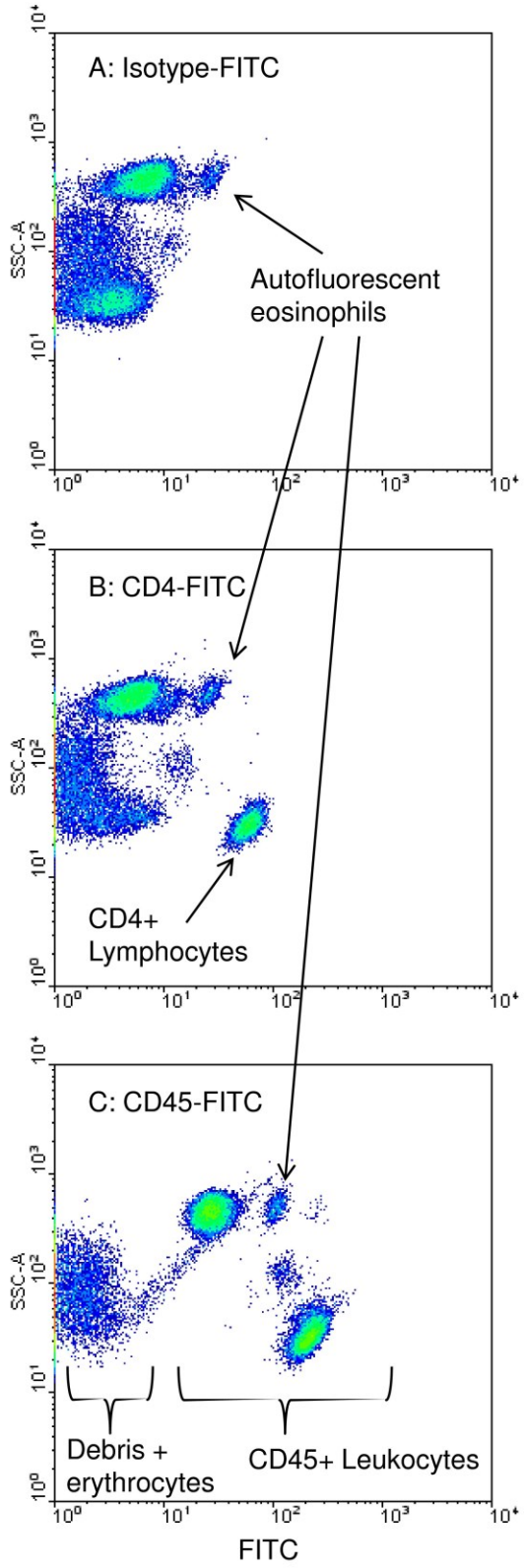

A: Isotype-FITC
Autofluorescent
eosinophils
B: CD4-FITC
CD4+
Lymphocytes
C: CD45-FITC
Debris +
erythrocytes
CD45+ Leukocytes
SSC-A
FITC

Figure 3

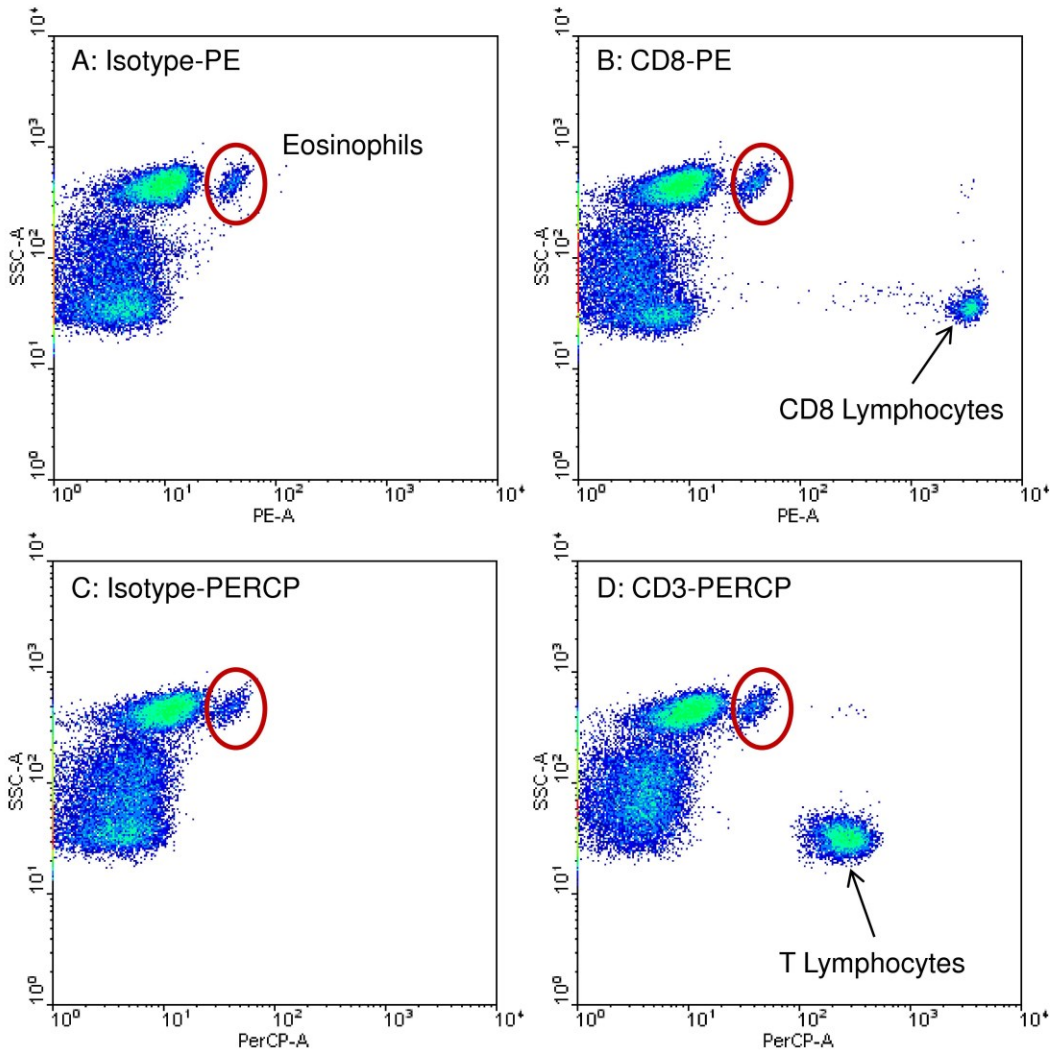

A: Isotype-PE
Eosinophils
B: CD8-PE
CD8 Lymphocytes
C: Isotype-PERCP
D: CD3-PERCP
T Lymphocytes
SSC-A
PE-A
PerCP-A

Figure 4

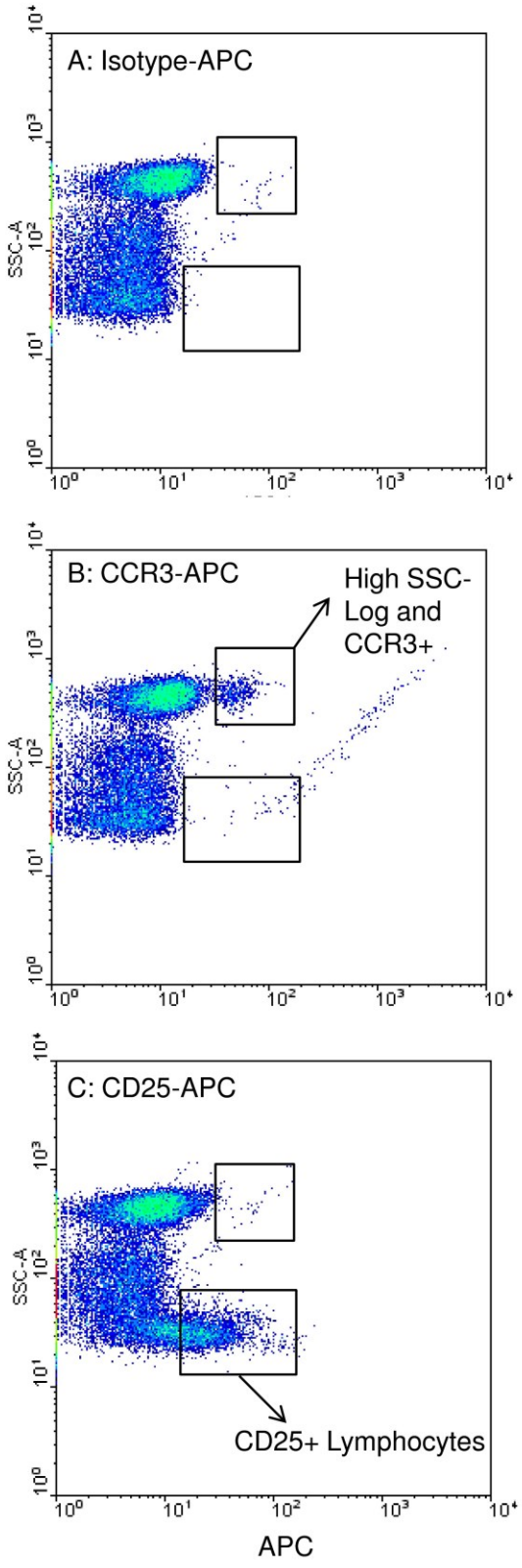
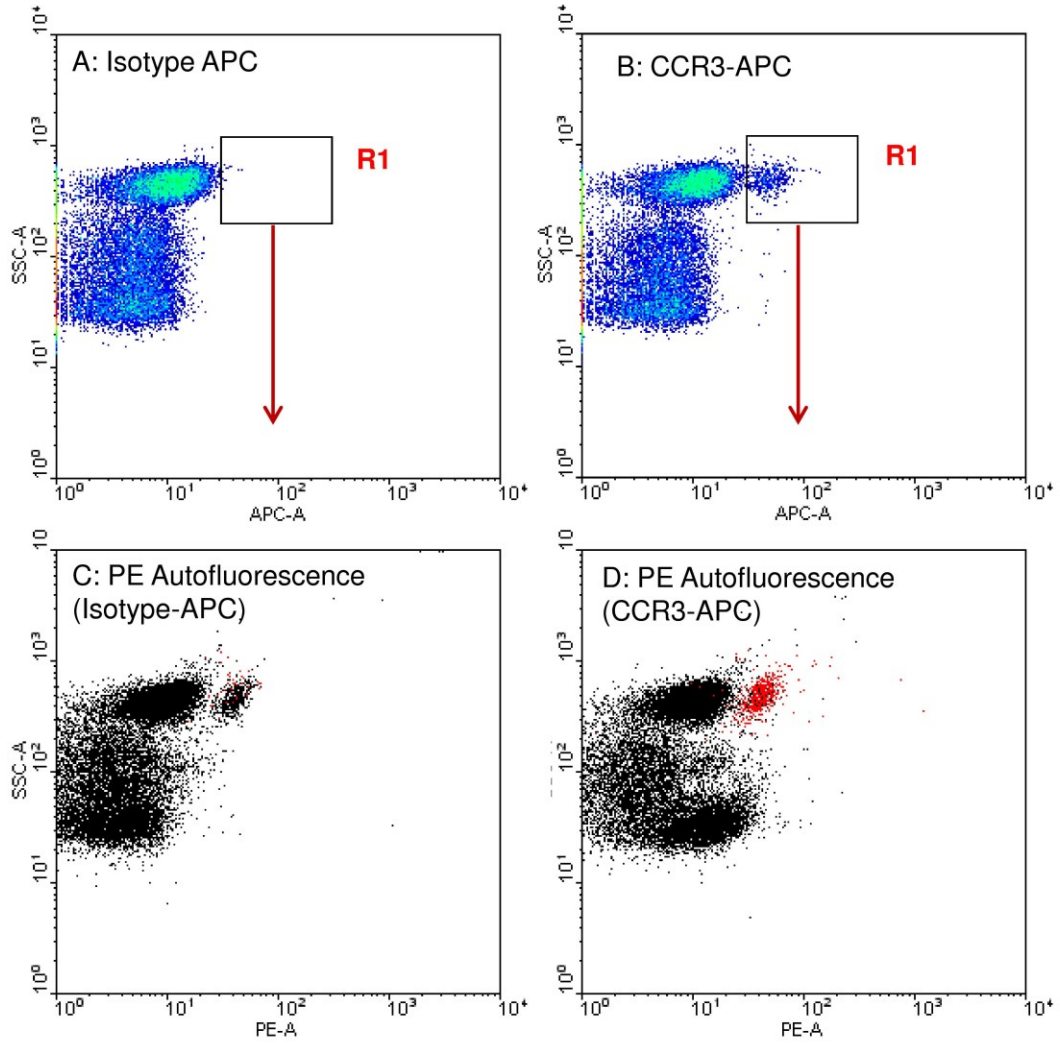

A: Isotype-APC
B: CCR3-APC
High SSC-Log and CCR3+
C: CD25-APC
CD25+ Lymphocytes
SSC-A
APC

Figure 5


A: Isotype APC
R1
SSC-A
APC-A
B: CCR3-APC
R1
SSC-A
APC-A
C: PE Autofluorescence
(Isotype-APC)
SSC-A
PE-A
D: PE Autofluorescence
(CCR3-APC)
PE-A