THE ROLE OF NEUTROPHILS IN SEVERE ASTHMA

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By

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

DESCRIPTIVE NOTE

MASTER OF SCIENCE (2012) McMaster University

Medical Sciences Hamilton, Ontario

TITLE: The Role of Neutrophils in Severe Asthma

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NUMBER OF PAGES: xvi, 119

ABSTRACT

Various studies have shown an association between neutrophilic airway inflammation and severe asthma, but have failed to establish a causal relationship. In these studies airway neutrophilia could be due to high steroid doses, an airway infection , an epiphenomenon of severe asthma or a combination of these. We have examined the role of neutrophils in severe asthma in patients on optimum steroid doses with controlled eosinophilic airway inflammation and chemotactic activity of IL-17 as one potential mechanism of neutrophil recruitment to the airway.

We examined the number, viability and activity of neutrophils in blood and sputum of three groups of asthma subjects divided on the basis of asthma severity. We also compared direct migration of blood neutrophils towards IL-17 between non-asthmatics and severe asthma subjects.

Viability and survival at 24 hours was measured by examining apoptotic and non-apoptotic cells. Activation was examined by measuring the production of hydrogen peroxide and the expression of primary and secondary granule proteins . In migration study, migration of neutrophils towards IL-17 was measured.

Blood neutrophils were increased in severe asthma subjects as compared to moderate and mild asthma subjects. There was no difference in sputum neutrophil numbers. There was no difference in viability, although blood neutrophil 24 hour survival was increased in severe asthma subjects as compared to moderate asthma subjects. There was no difference in the level of activation amongst the three groups. IL-17 was not a chemotactic stimulus for neutrophils.

The study results show that sputum neutrophil numbers and activation are not increased in severe asthma as compared to less severe asthma. Therefore, the study results do not support a causal relationship between airway neutrophilia and severe asthma. Airway neutrophilia observed in previous studies might be due to airway infections or high doses of steroids taken by study subjects.

ACKNOWLEDGEMENTS

I would like to extend special thanks to my supervisor, Dr.Nair, for his tremendous support during the time that I have spent as a graduate student at McMaster University. His encouragement and guidance has helped me develop an understanding of various aspects of asthma.

I owe my deepest gratitude to Katherine Radford, whose patience and help has made this thesis possible. I would also like to extend my thanks to Melanie Kjarsgaard and Shauna Denis for helping me with my project, including subject recruitment. I am very grateful to Ann Efthimiadis for running an efficient sputum lab which has helped me with my experiments. It was also a pleasure to work with Katri Tuomala, Earla Smith and Mary Ellen DeRushie in the sputum lab.

My committee members, Dr.Gerard Cox and Dr.Mark Larche are thanked for their intellectual input and support. It was an honor to have Dr.Frederick Hargreave as my committee member whose kindness, intellect and mentorship will always be remembered.

Finally, a very special thanks to my family for their patience and understanding during this time.

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LIST OF ABBREVIATIONS

AHR – Airway hyperresponsiveness

ANOVA - Analysis of variance

A1- Anti apoptotic protein A1

ATP – Adenosine triphosphate

BAL – Bronchoalveolar lavage

BALF - Bronchoalveolar lavage fluid

BSA – Bovine serum albumin

CD - Cluster of differentiation

COPD – Chronic obstructive pulmonary disease

C5a - Complement fragment

CXCL – CXC chemokines

CXCL1- Growth-related oncogene (GRO alpha)

CXCL5 - Epithelial cell neutrophil-activating protein-78

CXCL6 – Granulocyte chemotactic protein -2

CXCL8 – Interleukin 8

CXCL10 - Interferon-gamma–inducible protein10

CXCL12 - Stromal cell-derived factor (SDF)-1 alpha/beta

DTT – Dithiothreitol

ELISA - Enzyme-linked immunosorbent assay

FEV_1 – Forced expiratory volume in 1 second

FVC – Forced vital capacity

fMLP - N-formyl-methionine- leucine-phenylalanine

FCS – Fetal calf serum

H_2O_2 - Hydrogen peroxide

hCAP-18 - Human cathelicidin antimicrobial protein

ICAM- Intercellular adhesion molecule

ICS – Inhaled corticosteroid

IL-4 – Interleukin 4

IL-5 – Interleukin 5

IL-6 – Interleukin 6

IL-8 - Interleukin 8

IL-17 – Interleukin 17

IL-21 – Interleukin 21

IL-23 – Interleukin 23

IP-10 - Interferon-gamma–inducible protein10

IFN-γ – Interferon gamma

GATA-3 - Trans-acting T-cell-specific transcription factor

G-CSF – Granulocyte colony stimulating factor

GM-CSF - Granulocyte monocyte colony stimulating factor

HBSS - Hank's Buffered Salt Solution

LPS - Lipopolysaccharide

MAPK - Mitogen-activated protein kinase

Mcl-1 - Myeloid cell leukemia-1

MMP - Matrix metalloproteinase

MPO - Myeloperoxidase

NADPH - Nicotinamide adenine dinucleotide phosphate

NF-κB - Nuclear Factor Kappa B

NGAL - Neutrophil gelatinase associated lipocalin

Nramp-1 - Natural resistance-associated macrophage protein 1

PAMPs - Pathogen-associated molecular patterns

PBS - Phosphate buffered saline

PI 3K - Phosphatidylinositol-3 Kinase

PMA - Phorbol myristate acetate

PMSF - Phenylmethylsulfonyl fluoride

PRRs - Pattern recognition receptors

ROS – Reactive oxygen species

RORγt - Retinoic acid-related orphan receptor (ROR) γt

RPMI - Roswell Park Memorial Institute (culture medium)

SOD - Superoxide dismutase

SPSS - Statistical Packages for the Social Sciences

STAT - Signal Transducer and Activator of Transcription

Th cells – T helper cells

TLRs - Toll like receptors

TNF-α – Tumour necrosis factor alpha

TGFβ - Transforming growth factor-beta

VCAM - Vascular cell adhesion molecule

CHAPTER 1

INTRODUCTION

1.1. Asthma and Asthma Severity

Asthma is a chronic disease characterized by inflammation of the airways and airway hyperresponsiveness. Airway inflammation plays a central role as it is a cause of symptoms, variable airflow limitation and chronic airflow limitation. The symptoms of asthma include cough, chest tightness, wheezing, dyspnea and sputum production (Hargreave and Nair 2006). Almost 300 million people worldwide suffer from asthma (Turato G, 2007).

Although asthma is usually well controlled with inhaled corticosteroids and long acting β_2 agonists, a minority of patients have uncontrolled asthma in spite of these treatments. These patients with severe asthma suffer greater morbidity, are at higher risk of dying due to fatal asthma and also consume a large proportion of health resources than other patients with asthma (Macedo et al. 2009).

1.2. Airway Inflammation in Severe Asthma

The cellular components of airway inflammation in asthma can be measured by sputum cell counts. It can be of different types and can result from different causes. It can be eosinophilic in response to allergens, chemical sensitizers and steroid reduction. It can be neutrophilic in response to viral or bacterial infections, cigarette smoking or pollution. Both eosinophilic and neutrophilic inflammatory responses can be present at the same time depending on the causal factors (Hargreave and Nair 2009).

There has been a lot of interest with regards to the role of neutrophilic inflammation in the pathophysiology of severe asthma. Neutrophils have been found to be increased in sputum and tracheal aspirates of patients with severe asthma (Uddin M et al. 2010), asthma exacerbations (Fahy *et al.* 1995) and acute severe asthma (Ordonez *et al*. 2000). Neutrophils have also been found to be increased in the transverse sections of airways of patients with fatal asthma (Carrol *et al*. 1996). Although these studies have shown an association of neutrophils with severe asthma, they have failed to show a causal relationship of neutrophilic inflammation with severe asthma because of the cross-sectional nature of these studies. Further, it is difficult to understand if the observed increased in neutrophil number is a true reflection of the biology of asthma severity or due to an airway infection or simply a consequence of high doses of corticosteroids used in these patients which is how asthma severity is defined. High doses of corticosteroids have been shown to induce airway neutrophilia by increasing airway epithelial expression of neutrophil chemokines, IL-8 and IP-10 (Fukakusa *et al*.2005). Finally, most of the studies reported may not have used the optimal doses of corticosteroids to control eosinophilic inflammation. Thus, if higher doses of steroids were necessary to control airway eosinophilia, this could potentially result in even higher number or percentage of neutrophils in the airway by the mechanisms described above.

1.3. Neutrophil Biology

Neutrophils are the most abundant population of white blood cells circulating in blood. Their primary function is phagocytosis in which they identify, ingest and destroy microbes. Neutrophils are spherical cells with a diameter of 12 -15 μm. The nucleus is segmented into three to five connected lobules therefore neutrophils are also called polymorphonuclear leukocytes (Abbas *et al*.2007: 29). The average peripheral blood

neutrophil count is 2.5-7.5 x 10^9/L with a half-life of 6-8 hours in circulation (Cowburn *et al*. 2008).

Circulating neutrophil numbers are determined by their rate of production, release and clearance. Neutrophil production or granulopoiesis takes place in the bone marrow. These neutrophils form the bone marrow reserve. Under homeostatic conditions, G-CSF produced in the bone marrow regulates granulopoiesis and egress or release of neutrophils from the bone marrow into the blood. In contrast, when neutrophil production needs to be increased, for example in chronic inflammatory conditions, G-CSF, GM-CSF and IL-6, produced at the site of inflammation, regulate granulopoiesis.

Clearance of circulating neutrophils that do not enter the site of infection is carried out by macrophages in the liver, spleen and bone marrow. Clearance via bone marrow takes place in an age dependent manner. CXCL12, a chemokine, is produced in the bone marrow and binds to CXCR4 receptors on neutrophils. Circulating senescent neutrophils up-regulate expression of CXCR4. These aging and pre-apoptotic neutrophils traffic back to the bone marrow via CXCR4/CXCL12 axis where they are phagocytosed by bone marrow macrophages.

Macrophages present in the liver and spleen are part of the reticuloendothelial system and are in direct contact with blood. These macrophages recognize, bind and phagocytose circulating apoptotic neutrophils. Macrophages are also involved in clearance of apoptotic neutrophils at sites of inflammation (Rankin, 2010)

Circulating neutrophils migrate to the site of infection within a few hours after the entry of microbes (Abbas *et al*.2007: 29). Microbial interaction with neutrophils triggers an arsenal of antimicrobial measures that leads to efficient killing of microbes. Microbial killing involves phagocytosis, respiratory burst and release of granule contents into the phagosome (Nordenfelt and Tapper 2011).

The neutrophil degranulation and production of respiratory burst is a two stage process. The first stage is priming and the second stage is activation.

1.3.1. Neutrophil priming

The primed circulating neutrophils acquire the capacity to exhibit maximal degranulation and respiratory burst responses when exposed to subsequent activating stimuli. Priming in contrast to activation involves mobilization of secretory vesicles but lacks complete degranulation and production of superoxide. Priming agents also extends the lifespan of neutrophils by inhibiting apoptosis. These include host derived cytokines, chemokines and growth factors and microbial products. Examples are IL-8, IFN-γ, GM-CSF, G-CSF, fMLP and LPS.

These primed neutrophils may become trapped in pulmonary microcirculation and if not induced to migrate by a subsequent activating stimulus (e.g. Infection) can de-prime and be released back into the circulation in a quiescent state. The most important role of priming is to promote maximum antibacterial capacity of neutrophils and clearance of invading microorganisms (Cowburn *et al*. 2008 and Kobayashi *et al*. 2005).

1.3.2. Neutrophil migration to the site of an infection

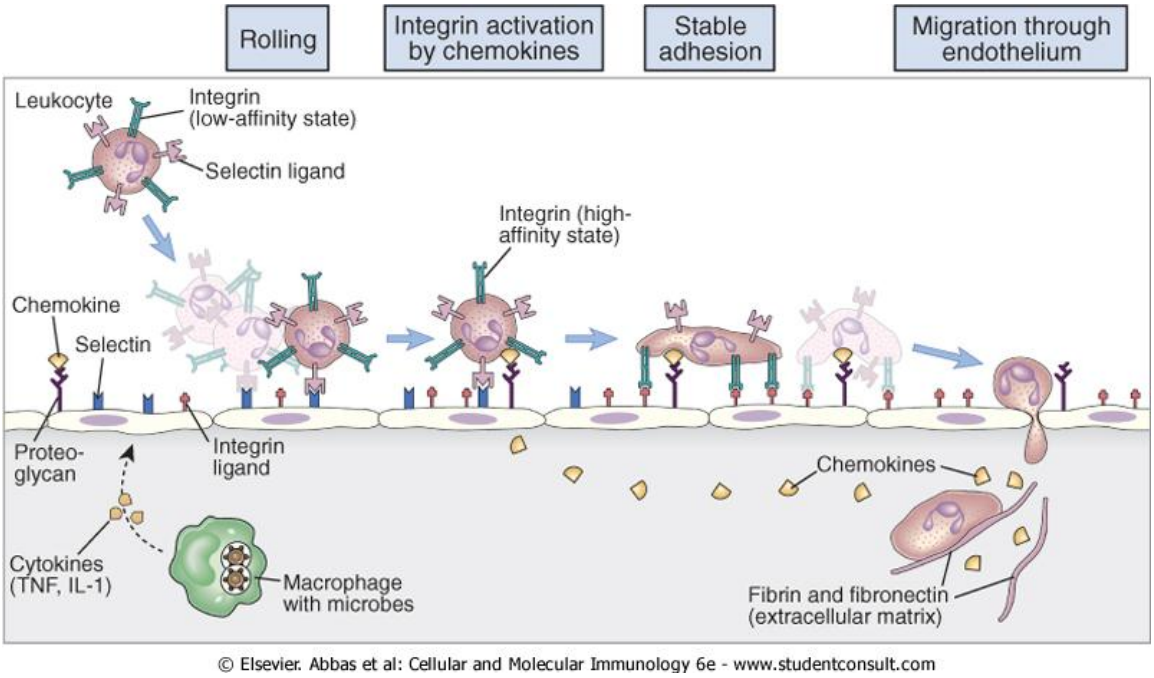

Figure 1. Migration of neutrophils to the sites of infection. This is a multi-step process in which chemoattractants are produced in response to an infection and neutrophils are recruited from blood by binding to adhesion molecules on luminal surface of endothelial cells. The process involves rolling, adhesion and migration of these cells through the endothelial cells. (Reproduced from Abbas *et al*. 2007:31)

The most potent host derived chemoattractants are leukotriene-B_4 (acting via leukotriene- B_4 receptor) and interleukin 8 (IL-8) (acting via receptors CXCR1 and CXCR2). Other mediators like C5a, CXCL1 (growth-related oncogene), CXCL5 (epithelial cell neutrophil-activating protein-78) and elastase-α_1-antitrypsin complexes also contribute to neutrophil recruitment (Cowburn *et al*. 2008). Bacteria products like *N*-formylated peptides also directly recruit neutrophils (Kobayashi *et al*. 2005). Neutrophil elastase can also lead to neutrophil recruitment by inducing IL-8 gene transcription and protein release in lung epithelial cells (Kuwahara *et al*. 2006)

Endothelial cells increase surface expression of proteins called selectins which are P-selectin and E-selectin. Neutrophils express ligands for these selectins at the tips of their microvilli. Endothelial cells at the site of infection express these selectins in response to microbes and cytokines produced by cells like macrophages. Selectins mediate only weak tethering and rolling of neutrophils on the endothelium.

Chemokines produced by tissue macrophages, endothelial cells and other cell types in response to microbial infections also bind to their respective receptors expressed on the surface of neutrophils. Neutrophils also express adhesion molecules called integrins in a low-affinity state. Ligands for integrins are expressed on endothelial surface and are VCAM-1 and ICAM-1. Chemokine receptor signalling results in enhanced affinity of neutrophil integrins to their ligands. The net result of these changes is firm attachment of neutrophils to the endothelium (Abbas *et al*. 2007:30). Firm adhesion is followed by transmigration either between or directly through the endothelial cells and neutrophils finally arrive at the site of extravascular infection to execute microbial killing (Burg and Pillinger 2001)

1.3.3. Microbial killing by neutrophils

Microbial killing by neutrophils involves phagocytosis of microbes which triggers respiratory burst producing reactive oxygen species which are potent microbicidal agents. This is accompanied by degranulation of cytoplasmic granules into the microbe containing phagosome or vacuole thereby enriching it with antibacterial peptides and proteases. (Kobayashi *et al*. 2005).

1.3.3.1 Phagocytosis of microbes by neutrophils

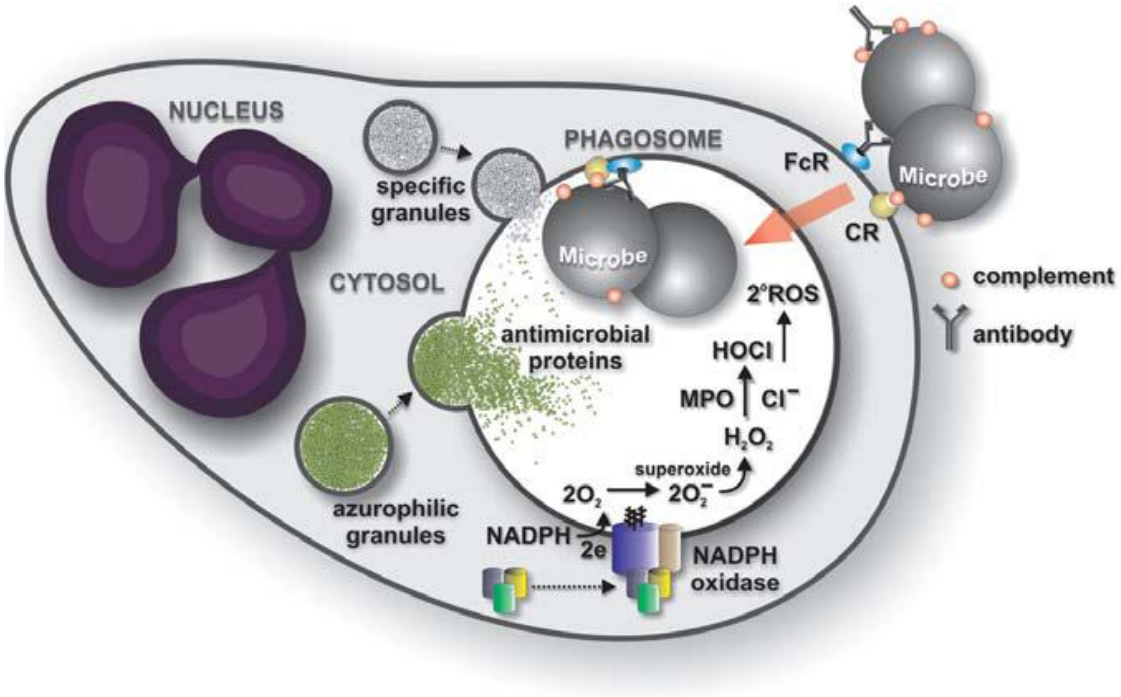

Figure 2. Neutrophil phagocytosis of microbes and activation of microbicidal system at the site of infection. Efficient phagocytosis takes place in the presence of opsonised microbes resulting in the formation of phagosome. Microbicidal system involves degranulation of azurophilic granules and specific granules into the phagosome. Also, production of reactive oxygen species by NADPH-dependent oxidase system promotes efficient killing of ingested microbes. (Reproduced from Kobayashi *et al*. 2005).

In the process of phagocytosis, neutrophils ingest microbes into vesicles. The first step in this process is the recognition of microbes by neutrophils. Neutrophils are constantly exposed to normal cells, which they ignore but specifically ingest microbes. This specificity is due to the expression of receptors that specifically recognise these microbes and are linked to the mechanisms of phagocytosis.(Abbas *et al*. 2007: 36). Neutrophils recognize pathogen derived molecules including peptidoglycan, lipoproteins, lipoteichoic acid, lipopolysaccharide (LPS) and flagellin. These molecules, called pathogen-associated molecular patterns (PAMPs), interact with pattern recognition receptors (PRRs) expressed on the surface of neutrophils. Toll like receptors (TLRs) belong to the family of PRRs.

Binding of PAMPS to TLRs especially TLR2 and 4 leads to activation of signal transduction pathways. This results in enhancement of various neutrophil functions including adhesion, phagocytosis, cell survival, release of cytokines and chemokines, production of reactive oxygen species and degranulation

Although TLRs play an important role in microbial recognition by neutrophils, phagocytosis is more efficient when microbes are coated with serum host proteins called opsonins. These opsonins include complement proteins and antibodies. Activation of compliment leads to deposition of compliment components on microbial surface. These compliment components include CIq, C3b and iC3b. Compliment surface receptors (CRs) expressed on the surface of neutrophils efficiently recognize microbes bound with compliment components. CRs include CIqR, CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18). (Kobayashi *et al*. 2005).

Neutrophils also express Fc receptors that recognize antibodies. These antibodies have an antigen binding site and a Fc region for the Fc receptors on neutrophils. These receptors include CD23 (FcεRI, IgE receptor), CD89 (FcαR, IgA receptor), CD64 (FcγRI,

IgG receptor), CD32 (FcγRIIa, low affinity IgG receptor) and CD16 (FcγRIIIb, low-affinity IgG receptor).(Kobayashi *et al*. 2005 and Abbas *et al*. 2007:36).

During the process of phagocytosis, the plasma membrane of the neutrophil in the region of the receptors extends a cup shaped extension around the microbe. This eventually leads to internalization of the microbe into a phagosome within the neutrophil. Receptors involved in microbial recognition also activate neutrophils to kill ingested microbes. Fusion of the phagosome with the neutrophil granules (lysosomes) leads to the formation of phagolysosome. Most of the microbicidal activity takes place within the phagolysosome (Abbas *et al*. 2007:36)

1.3.3.2. Neutrophilic oxidative burst

Neutrophils kill microbes by using oxygen-dependent and oxygen- independent mechanisms. The process of phagocytosis triggers the production of reactive oxygen species through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. It is a multicomponent enzyme. In unstimulated neutrophils, the components are separated into cytosolic and membrane compartments. The cytosolic compartments are p40*phox,* p47*phox,* p67*phox* and Rac2. Membrane compartments are flavocytochrome b_{558} and Rap 1A, present in specific granules, plasma membranes and secretory vesicles. During phagocytosis the cytosolic components join the membrane components to form active oxidase. This complex is assembled first at the plasma membrane and later at the phagosomal membrane.

The active oxidase at these sites generates superoxide (O_2^-) as it transfers electrons from cytosolic NADPH to molecular oxygen. Within the phagosome, the superoxide anion is dismutated by superoxide dismutase (SOD) to form oxygen and hydrogen

peroxide (H_2O_2). Hydrogen peroxide is converted by enzyme myeloperoxidase (MPO) to other ROS like hypochlorous acid, chloramines, singlet oxygen which are effective microbicidal compounds (Kobayashi *et al*. 2005, Walrand *et al*.2003, Nordenfelt and Tapper, 2011) (Figure 2).

1.3.3.3 Neutrophil granules

Cytoplasmic granules present in the neutrophils play a major role in neutrophil mediated inflammatory responses. These granules and vesicles not only contain antimicrobial proteins, proteases and component of respiratory burst oxidase but also contain receptors of matrix proteins, bacterial products and soluble mediators of inflammation. These cytoplasmic organelles are released in a controlled fashion which transforms passively circulating neutrophils to potent effector cells of innate immunity (Faurschou and Borregaard, 2003).

Neutrophils contain four major types of granules. These are secretory vesicles, tertiary, specific and azurophilic granules. Azurophilic granules (primary granules) are formed in the early stages of neutrophil maturation and have high content of myeloperoxidase (MPO) and are also called peroxidase positive granules. Granules formed in the later stages of granulopoiesis are peroxidase negative and are specific (secondary) and gelatinase (tertiary) granules depending upon their time of appearance and granule contents. Secretory vesicles appear last and are formed by endocytosis and contain plasma proteins.

Azurophilic granules contain antimicrobial proteins like defensins, elastase, cathepsin and proteinase-3. These also contain CD63 (granulophysin) in their membrane. Myeloperoxidase in these granules is involved in the production of reactive oxygen

species (ROS) as mentioned earlier. H_2O_2 produced in the oxidative burst is converted to a powerful antimicrobial agent, hypochlorous acid (HOCL) by MPO.

Specific and gelatinase granules contain several potent antimicrobial proteins like lactoferrin, hCAP-18, neutrophil gelatinase associated lipocalin (NGAL), lysozyme and natural resistance-associated macrophage protein 1 (Nramp-1). These granules also contain metalloproteinases which are capable of degrading extracellular matrix components like collagen, fibronectin, proteoglycans, laminin and gelatin. These play a central role in in degradation of vascular basement membranes and interstitial structures during neutrophil extravasation and migration.

Secretory vesicles contain membrane associated receptors required in the earliest phases of neutrophil-mediated inflammatory response. Their membranes are rich in fMLP receptors, complement receptor 1 (CR1), LPS/lipoteichoic acid receptor CD14, Fcγ receptor CD16, β_2 integrin CD11b/CD18 and metalloprotease leukolysin. All these are incorporated in the plasma membrane of neutrophils after exocytosis. Membrane surface changes induced by incorporation of secretory vesicles allows neutrophils to establish firm contact with activated vascular endothelium (Faurschou and Borregaard 2003)

1.3.3.4. Neutrophil degranulation

Neutrophil recruitment into the inflamed tissues involves a regulated release of its granules that gradually changes the functional state of neutrophils. The neutrophil-endothelial interactions, involving selectins and selectin ligands, promote neutrophil rolling along the endothelium and trigger exocytosis of secretory vesicles from neutrophils. This enriches neutrophil surface membrane with β integrins. As substances contained in the secretory vesicles are plasma derived, exocytosis of secretory vesicles is not associated with release of potentially toxic granule proteins. These integrins engage with endothelial adhesion molecules of the intercellular adhesion molecule family (ICAM). This binding mediates firm adhesion and initiates neutrophil transmigration.

Transmigration involves exocytosis of gelatinase granules releasing collagenolytic metalloproteases which degrade the vascular basement membrane and facilitate neutrophil movement. On interaction with bacteria, neutrophils activate their diverse antimicrobial arsenal by releasing azurophilic and specific granules into the phagocytic vacuole or to the exterior of the cell. Granule substances released target bacteria in different ways. Some granule substances like defensins, lactoferrin and lysozyme disrupt microbial membranes. NGAL, lactoferrin and Nramp interfere with bacterial metabolic pathways that are iron dependent. MPO participates in the generation of reactive oxygen species toxic to bacteria (Faurschou and Borregaard 2003)

1.3.4. Inflammatory mediators released by neutrophils causing tissue damage

Although neutrophils, as professional phagocytes of the innate immune system, kill ingested microorganisms using ROS and cytotoxic contents of its granules, these cytotoxic components can also cause host tissue damage, if released into the surrounding tissues.

Azurophilic granules contain elastase, a serine protease that has important antimicrobial functions. It can also digest key components of extracellular tissue matrix which includes elastin, laminin, fibronectin, collagen type III and IV and core proteins of proteoglycans. It can degrade surfactant proteins A & D. These surfactant proteins are involved in the clearance of apoptotic neutrophils. Degradation of these anti-inflammatory proteins can prolong inflammation. Elastase also stimulates lung epithelial cells to release growth factors and proinflammatory cytokines (Grommes and Soehnlein, 2011 and Dallegri and Ottonello, 1997) and in vitro can induce apoptosis of lung epithelial cells, which can result in the disruption of airway epithelial barrier causing alveolar edema (Moraes *et al.*2006). Neutrophil elastase is also a potent mucin secretagogue for goblet cells and submucosal glands. This may mediate mucus hypersecretion observed in acute severe asthma (Ordonez *et al*. 2000)

Matrix metalloproteinase are present in secondary (MMP-8) and tertiary granules (MMP-2 and MMP-9). Substrates for these enzymes also include elastin and other basement membrane components. These enzymes can influence tissue remodelling, angiogenesis and cell motility through their effects on extracellular matrix. Their role in tissue injury has been demonstrated in patients with Acute Lung Injury (ALI). BAL fluid and plasma of these patients displayed increased levels of MMPs which correlated with clinical severity. Also in a rat model, it has been demonstrated that inhibition of MMP-9 attenuated ventilator induced lung injury. However, there are conflicting results

in the literature. Studies done in MMP-$8^{-/-}$ mice show two fold increases in neutrophils in BAL fluid after intratracheal LPS application. These studies show that MMPs may both have a beneficial and deleterious effects in lung injury as different MMPs may have opposing functions in the modulation of inflammation.(Grommes and Soehnlein, 2011 and Lee WL and Downey GP, 2001)

Defensins (α defensins) are stored in azurophilic granules and have microbicidal function. These have been shown to activate macrophages to release TNF and IFNγ and promote a more pro inflammatory phenotype. α defensins also increase the permeability of epithelial monolayer in vitro and also exert chemotactic effects on T cells, mast cells and dendritic cells.(Grommes and Soehnlein, 2011)

Neutrophils produce ROS during respiratory burst upon activation. Hypochlorous acid (HOCl) is the most toxic oxidant produced during neutrophilic inflammation.
In addition to its strong antimicrobial properties, it can have unwanted effects. It can cause cell injury by inducing depletion of ATP and also cell necrosis. It can also inactivate alpha-1-antitrypsin (AT) which is a specific inhibitor of neutrophil elastase. The inactivation of AT can result in uninhibited activity of elastase, which is digestion of intercellular tissue matrix. (Dallegri and Ottonello, 1997). A recent study has demonstrated that ROS can disrupt tight junctions of the endothelium as a result of phosphorylation of focal adhesion kinase. ROS play a major role in neutrophil mediated tissue injury. (Grommes and Soehnlein, 2011)

1.3.5. Resolution of inflammation and neutrophil apoptosis

Although the neutrophil mediated inflammatory response is crucial for resolution of infection, timely removal of these phagocytes is required as they are armed with potent cytotoxic components like ROS and granule products that can cause tissue injury. This is important for resolution of inflammation and maintaining homeostasis of the human immune system (Kobayashi *et al*. 2005). It is accomplished through apoptosis or programmed cell death of neutrophils, an event that triggers their recognition and phagocytosis by resident macrophages.

There is expression of phosphatidyleserine (membrane phospholipid) on the surface of apoptotic neutrophils which interacts with macrophage receptor CD36. This leads to phagocytic uptake by macrophages and timely removal of neutrophils which helps in resolution of inflammation (Cowburn *et al*. 2008). Other morphological changes observed during apoptosis include chromatin condensation, nucleolar disruption, cytoplasmic contraction and membrane blebbing (Abbas *et al.* 2007:255).

If apoptotic neutrophils are not removed by macrophages, these cells can undergo secondary necrosis resulting in cell disruption and leakage of toxic cellular contents causing tissue injury (Silva, 2008).

Neutrophil Apoptosis

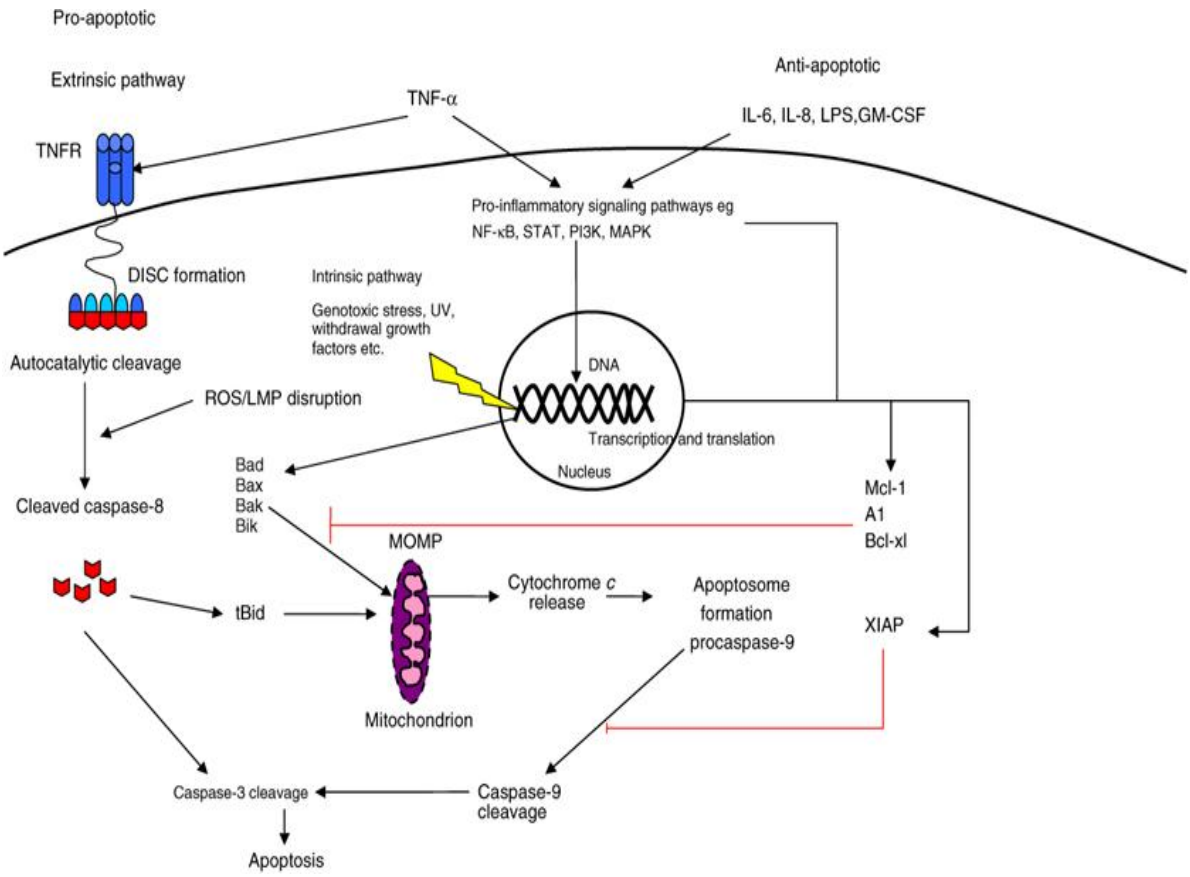

Figure 3. There are two major signalling pathways involved in neutrophil apoptosis. These are the "the intrinsic or mitochondrial pathway" and the "death receptor or extrinsic pathway". These two pathways culminate in the activation of executioner caspases like Caspase 3 of the common death pathway (Reproduced from Leitch *et al*, 2008, with permission from Macmillan Publishers Ltd)

Caspases are a set of proteolytic enzymes that play a key role in induction of apoptosis. These enzymes exist as latent pro-enzymes that are activated in a cascade-like fashion. Apoptotic signals from the mitochondrial and death receptor pathways activate initiator caspases which in turn activate executioner caspases of the common death pathway. Executioner caspases contribute to dismantling and packaging of the dying cell by cleaving specific substrates. Caspase-3 is the best characterized executioner caspase. One of the substrates of caspase-3 is caspase-activated DNase (CAD). CAD can cleave chromosomal DNA into nucleosome sized fragments, characteristic of apoptosis. Its activity is held in check by an inhibitor called ICAD (inhibitor of CAD). Caspase-3 cleaves ICAD resulting in the activation of CAD (Abbas *et al.* 2007:255)

1.3.5.1. The mitochondrial or the intrinsic pathway of apoptosis

In this pathway, there is there is loss of mitochondrial integrity resulting in leakage of death inducing proteins from the mitochondria. Mitochondrial stability is regulated by Bcl-2 family of proteins. These are divided into two groups. 1) Anti-apoptotic proteins include Bcl-$X_{L,}$ A1 and Myeloid cell leukemia-1 (Mcl-1) 2) Pro-apoptotic proteins include Bcl-2-associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak), Bcl-2-associated death promoter (Bad), Bcl-2 homology-3 (BH3)-interacting domain death agonist (Bid), Bcl-2 interacting protein (Bim) and Bcl-2 interacting killer (Bik). (van Raam et al , 2006 and Geering and Simon, 2011)

The mitochondrial outer membrane permeabilization (MOMP) is induced by pro-apoptotic Bcl-2 family of proteins. This leads to release of mitochondrial inducers of apoptosis which are normally sequestered inside these organelles.

These include cytochrome c, Smac/Diablo and Omi/HtrA2. Cytochrome c binds to a protein called apoptosis-activating factor-1 (Apaf-1) to form a complex called apotosome. This complex binds to and activates procaspase-9. Procaspase-9, being a key initiator caspase in the mitochondrial pathway, activates the executioner caspases of the common death pathway.

The activity of caspases in the cytosol is held in check by a family of inhibitors called XIAP or IAPs (inhibitors of apoptosis). These inhibit caspases -3, -8 and -9. Smac/Diablo and Omi/HtrA2 which are mitochondrial inducers of apoptosis, antagonize the activity of IAPs , thereby promoting activity of caspases and apoptosis (Abbas *et al*. 2007:255 and Leitch *et al*, 2008)

1.3.5.2 The death or the extrinsic pathway of apoptosis

This pathway is activated by ligation of external cell membrane death receptors such as the tumour necrosis factor receptor (TNFR), the Fas receptor (FasR) and the TNF-related apoptosis-inducing ligand receptor (TRAILR). The ligand activation of receptors results in clustering of these receptors and association with their internal adaptor proteins. Internal adaptor proteins are TNFR-associated death domain protein and Fas-associated death domain protein. Procaspase 8 molecules assemble at the adaptor proteins forming a death inducing signalling complex (DISC). This results in activation of procaspase 8 to caspase 8 which then activates caspase 3 of the common death pathway.

The death receptor pathway can also interact with mitochondrial pathway to induce apoptosis. This involves cleavage of pro apoptotic protein Bid by caspase -8. This triggers the release of mitochondrial inducers of apoptosis (Leitch *et al* , 2008)

1.3.5.3. Factors modifying apoptosis

A number of factors influence neutrophil apoptosis or survival. For example, in the case of bacterial infections, proinflammatory cytokines like IL-6, IL-8, G-CSF and GM-CSF and bacterial products like LPS delay neutrophil apoptosis by activating pro-inflammatory signalling pathways. These include NF-κB, STAT, PI 3K and MAPK. This results in up regulation of anti-apoptotic proteins of the intrinsic pathway Mcl-1, Bcl-X_L and A1 enhancing neutrophil survival. This suggests that enhanced neutrophil survival is desirable in early stages of inflammation to promote clearance of bacteria. Intrinsic pathway of apoptosis can be activated by genotoxic stress, withdrawal of growth factors and ultraviolet radiation.

Glucocorticoids also enhance neutrophil survival or delay apoptosis by up regulating Mcl-1 in neutrophils.(Kobayashi *et al*. 2005 and Leitch *et al* , 2008). TNF-α has both pro and anti-apoptotic activity. At low doses, it induces survival while it is pro apoptotic at higher doses. Also, neutrophil survival is influenced by the process of phagocytosis. Phagocytosis along with the production of ROS mediates neutrophil apoptosis. Neutrophils are exposed to both pro and anti-apoptotic factors and the net result on neutrophil survival depends on the balance between these two factors (Geering and Simon, 2011 and Luo and Loison, 2008).

1.4. Th17 Cells

The concept of Th1/Th2 paradigm has played a vital role in our understanding of the molecular and cellular mechanisms in asthma. Both Th1 and Th2 cells are derived from CD $^{+}$4 T cells. Th1 cells are considered to have a regulatory role in asthma. Their principal function is in the phagocyte mediated defense against infections like intracellular microbes, but they also secrete IFN-γ, which inhibits proliferation of Th2 cells. In asthma, there is Th1/Th2 imbalance, involving an exaggerated T helper 2 (Th2) cells mediated inflammatory response to allergens. Th2 cells promote IgE and eosinophil/mast cell mediated immune reactions resulting in airway eosinophilia, smooth muscle cell hyperplasia, mucus secretion and AHR (Abbas *et al*. 2007: 305 and Park and Lee 2010).

A third type of differentiated $CD^{+}4$ T cells has generated a lot of interest with regards to its role in immune and inflammatory responses in asthma. These are Th17 cells which produce a number of inflammatory mediators especially interleukin-17 (IL-17) (Al-Ramli *et al*.2009).

1.4.1 Differentiation of Th17 cells from naïve T cells

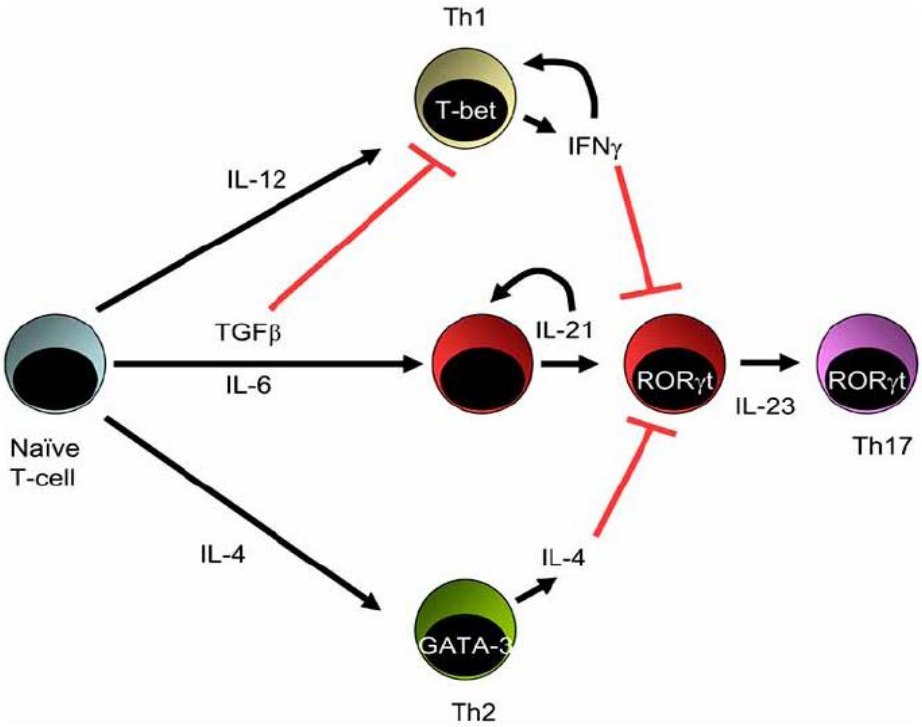

Figure 4. Differentiation of naïve T cells into Th1, Th2 and Th17 cells. Exposure of naïve T cells to IL-12 drives Th1 cell expression as there is up regulation of transcription factor, T-bet. Th2 cell differentiation depends on exposure of naïve T cells to IL-4 with up-regulation of transcription factor GATA-3.Diffrentiation into Th17 cells is driven by TGFβ and IL-6, which also stimulates the release of IL-21. IL-21 acts in an autocrine manner up-regulating Th17 specific transcription factor RORγt and also up-regulation of IL-23 receptors. This helps in the propagation of these cells. IFNγ from Th1 cells and IL-4 from Th2 cells inhibit Th17 cell differentiation. Similarly Th1 cell differentiation is inhibited by TGFβ favouring Th17 cell differentiation (Reproduced from Traves and Donnelly, 2008)

Th17 cells are derived from naïve T cells in the presence of TGFβ and IL-6 (Figure 4). It is possible that TGFβ and IL-6 are expressed by macrophages and dendritic cells stimulated by the presence of bacteria in the airways. Macrophages may also be the source of IL-23 propagating Th17 cells. Both TGFβ and IL-6 are elevated in the airways of asthma patients. This suggests that inflammatory milieu of the lung in asthma could promote differentiation of Th17 cells. (Traves and Donnelly, 2008)

1.4.2. IL-17 family of cytokines

IL-17 family of cytokines includes six members. These are IL-17A (synonymous with IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and IL-17F. Out of this family of IL-17 cytokines, IL-17A, IL-17F and IL-17 E have been best studied. IL-17B, IL-17C and IL-17D have been least characterised.

IL-17A is a homodimeric glycoprotein. It consists of 155 amino acids with molecular weight of 35 kDa. IL-17F is most homologous to IL-17A (50% sequence similarity). Therefore both the cytokines share many biological properties. Both the cytokines induce the expression of various inflammatory mediators, involved in neutrophil expansion from the bone marrow, neutrophil survival and recruitment to the airways, from airway epithelial cells. Therefore these cytokines play a role in bacterial and fungal infections (Park and Lee 2010, Pappu *et al*. 2011 and Nembrini C *et al*. 2009)

IL-17E has a role in allergic inflammation. In mice, intranasal instillation with IL-17E caused asthma-like symptoms. These included eosinophil infiltration, up-regulation of IL-4/5/13, mucus production in the lungs along with airway hyperresponsiveness (Pappu *et al*. 2011)

1.4.3. IL-17 sources and receptors

IL-17 is predominantly produced by Th17 cells. Other cellular sources include $CD^{+}8$ T cells, γδ T cells and natural killer T cells. IL-17 might also be produced by granulocytes and macrophages. Similar to IL-17 cytokine family, IL-17 family of receptors includes IL-17 receptor R A-E. IL-17 RA and RC form a receptor complex that is stimulated by IL-17. Both of these receptors are transmembrane proteins. The functional characteristics of receptors IL-17 RB, RD and RE remain to be determined (Ivanov and Linden 2009).

1.4.4. IL-17 and asthma

IL-17 has been reported to be increased in the BAL fluid, sputum, lung tissues and peripheral blood of patients with asthma. Studies have also demonstrated a positive correlation between asthma severity and the levels of IL-17 along with Th17 cells in plasma and airway of these patients.

IL-17 signalling can induce the accumulation of neutrophils into the airways. This biological role of IL-17 is demonstrated in a study which showed increased levels of IL-17 mRNA expression correlated with number of neutrophils in the sputum of asthmatic patients (Park and Lee 2010).

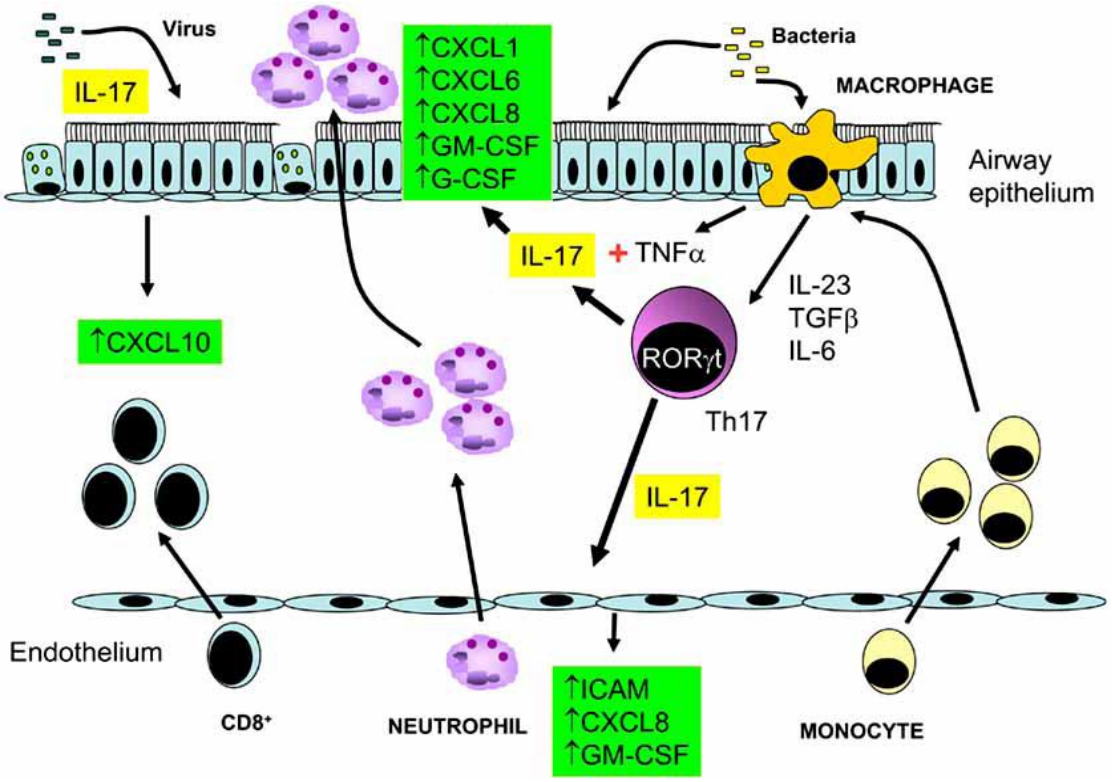

Figure 5. IL-17 as a mediator in airway inflammation. IL-17 can induce the expression and secretion of CXCR2 cytokines from airway epithelium and endothelium. CXCR2 cytokines include CXCL1, CXCL6 and CXCL8. It can up-regulate ICAM on endothelial surface. These actions promote accumulation of neutrophils in the airways and are enhanced in the presence of viral and bacterial infections. IL-17 also increases neutrophil survival by inducing expression of GM-CSF and G-CSF, which are neutrophil survival factors (Reproduced from Traves and Donnelly 2008)

IL-17 can induce the production of neutrophil chemokines from airway epithelium and endothelium, promoting accumulation of neutrophils in the airways (Figure 5). However, chemotactic and chemkinetic activity of IL-17 on neutrophils has not been examined.

Increased expression of CXCR2 cytokines can also mediate migration of monocytes into the airways. In the lungs monocytes differentiate into macrophages. Activated macrophages can produce TGFβ, IL-6 and IL-23 which are required for Th17 cell differentiation. Also, TNF-α produced by activated macrophages can synergise with IL-17 resulting in enhanced inflammatory mediator release from the airway epithelium. IL-17 may also up-regulate CXCL10 from airway epithelium mediating $CD8^+$ cell recruitment (Traves and Donnelly 2008).

Interestingly, IL-17 mRNA levels have been shown to correlate with IL-5 mRNA levels in the sputum of asthma patients. IL-5, a cytokine produced by Th 2 cells, is involved in eosinophilic airway inflammation. Also, mouse models have demonstrated up regulation of IL-17 in the airways after allergen exposure. These data suggest an association of IL-17 with neutrophilic and Th2 mediated eosinophilic airway inflammation (Park and Lee 2010).

1.5. Glucocorticoids – Anti-inflammatory Therapy in Asthma

Glucocorticoids are used as the main anti-inflammatory therapy to control eosinophilic airway inflammation in asthma. These drugs reduce the number of eosinophils by suppressing the synthesis and effects of eosinophil survival factors, inducing eosinophil apoptosis and stimulating uptake of apoptotic eosinophils by macrophages (Druilhe *et al.*2003)

Glucocorticoids exert their anti-inflammatory effects by switching off multiple inflammatory genes that are activated during the process of inflammation. These genes encode for multiple cytokines, chemokines, inflammatory enzymes, receptors, proteins and adhesion molecules.

The anti-inflammatory properties of glucocorticoids are mediated through glucocorticoid receptors. Glucocorticoids can diffuse across the cell membranes to bind to GRs. There are of two isoforms of glucocorticoid receptors – hGRα and hGRβ. GRα is present in the cytoplasm, binds to its ligand and is trans located to the nucleus. Inside the nucleus, it affects gene transcription, resulting in the execution of glucocorticoid actions. On the other hand, hGRβ is present in the nucleus and does not bind to glucocorticoids. hGRβ does not affect gene expression by itself but may repress the transcriptional activity of GRα. hGRβ might be involved in glucocorticoid resistance in asthma (Barnes PJ, 2006 and Lewis-Tuffin and Cidlowski, 2006)

1.5.1. Glucocorticoids – effects on neutrophils (numbers, survival, activation)

Glucocorticoids increase the number of circulating neutrophils in blood by increasing their release from the bone marrow and reducing the adherence of neutrophils to the vasculature. Decreased adherence also decreases the capacity of neutrophils to migrate from the vasculature to the site of inflammation (Parillo and Fauci, 1979 and Crockard *et al.* 1998). As previously mentioned, glucocorticoids cause airway neutrophilia by increasing the expression of IL-8 and IP-10 in airway epithelium. IL-8 and IP-10 are neutrophil chemokines (Fukakusa *et al*.2005)

Glucocorticoids also prolong neutrophil survival by delaying apoptosis in vitro. The increased survival of neutrophils may also contribute to glucocorticoid induced neutrophilia (Cox G, 1994). Glucocorticoids induce neutrophil survival by up regulation

of anti-apoptotic Bcl-2 family proteins like Mcl-1 (intrinsic pathway of apoptosis). These also cause stabilization and induction of IAPs (inhibitors of apoptosis) and suppression of components of extrinsic pathway of apoptosis (Saffar et al, 2011)

Glucocorticoids do not affect neutrophil activation in terms of production of superoxide (Cox G, 1994), but do have inhibitory effects on neutrophil degranulation in vitro, as demonstrated by decreased release of granule-associated enzymes in response to glucocorticoids (Liu.L *et al.* 2005 and Smith and Iden 1980)

1.6. Neutrophil Influx after Allergen Stimulation

Increased number of eosinophils seen in asthmatic airways is usually a response to inhaled allergens. Some studies have also reported increased number of neutrophils in BAL and bronchial biopsies after endobronchial allergen instillation in allergic asthmatic patients. In contrast, a study has demonstrated that neutrophilia observed after allergen challenge might be due to contamination of allergen extract with an endotoxin and not a response to allergen challenge. Initially in this study segmental bronchoprovocation with an allergen was used and neutrophil recruitment was observed in 24 hour BALF. The allergen extract used tested positive for endotoxin. Subsequently, subjects challenged with endotoxin free allergen extract demonstrated preferential recruitment of eosinophils rather than neutrophils. Neutrophil numbers in BALF were significantly less when endotoxin free allergen extract was used. It has also been suggested that the neutrophil influx observed after allergen challenge might also be a non-specific consequence of bronchoscopy procedure (Imaoka H *et al.* 2011 and Hunt LW *et al.* 1994)

1.7. Neutrophils and Severe Asthma

As neutrophils along with their inflammatory products have been found to be increased in the airways of patients with severe asthma, these inflammatory cells might have a physiological role in causing asthma severity by causing damage to the airways through their products.

A study demonstrated significantly increased number of neutrophils and eosinophils along with IL-8, free neutrophil elastase activity, TNF-α, IL-6 and ECP (eosinophil cationic protein) in the intubation tracheal aspirates of patients with acute severe asthma as compared to control subjects. IL-8, neutrophil elastase and TNF-α are produced by neutrophils. IL-6 is produced by airway epithelial cells, monocytes/macrophages, lymphocytes and endothelial cells. These patients were treated with high doses of intravenous corticosteroids.

Interestingly the number of neutrophils was significantly higher and number of eosinophils was significantly lower in extubation tracheal aspirates as compared to intubation samples of these severe asthma patients. In addition, levels of IL-8, TNF-α and free neutrophil elastase activity were also significantly higher in extubation tracheal aspirates as compared to intubation samples. Levels of ECP and IL-6 showed little change. Increased number of neutrophils along with its inflammatory mediators in extubation aspirates might be due to high dose glucocorticoid therapy and intubation. Glucocorticoids promote neutrophil survival which might explain increase in neutrophils in extubation samples. In turn, prolonged survival of neutrophils might lead to increased inflammatory mediators secreted by these cells.

There was clinical improvement in these patients at the time of extubation, although inflammatory mediators were increased. This might be due to the effects of glucocorticoids as they promote resolution of airway obstruction. These drugs render

airways structures like goblet cells, submucosal gland cells and blood vessels less responsive to inflammatory mediators (Ordonez *et al*. 2000)

Other studies done in severe asthma patients have demonstrated similar results. A study showed increased numbers of neutrophils and eosinophils along with elevated levels of IL-8 and MPO in the sputum samples of patients with severe persistent asthma (Jatakanon *et al.* 1999). Another study showed increased numbers of neutrophils in the airways in fatal attacks of asthma (Carroll *et al*. 1996)

Although these studies suggest that neutrophils may have a pathophysiological role in severe asthma, they have failed to establish a causal role of neutrophils in this disease. These studies only demonstrate an association of increased neutrophil numbers in the airways along with its mediators with severe asthma. It is possible that neutrophilic airway inflammation observed in patients with severe asthma might be due to an airway infection or glucocorticoid therapy. Assessing neutrophil numbers, activation and migration under controlled airway inflammation, can determine if neutrophils are intrinsically activated, migrating more towards lungs in patients with severe asthma resulting in lung tissue injury.

1.8. Study Hypotheses

1) Neutrophils are increased in severe asthma in blood and sputum as compared to less severe asthma (severity is defined based on the dose of corticosteroids required to maintain asthma control)

2) Neutrophils are no more activated in patients with severe asthma as compared to less severe asthma.

3) IL-17 is raised in sputum of patients with severe asthma and is a direct chemoattractant for neutrophils; this migratory response would be more in patients with severe asthma as compared to normal controls.

1.9. Specific Objectives

The study is divided into two parts. The first part (neutrophil activation study) examined blood and sputum neutrophil numbers, viability and activation in patients with varying asthma severity in a cross sectional observational study.

In the second part, sputum IL-17 was measured and migration of blood neutrophils towards IL-17 was examined in two groups of subjects, normal controls and patients with severe asthma.

The specific objectives are:

1) To compare neutrophil numbers in blood and sputum of patients with varying asthma severity

2) To compare neutrophil viability and activation in blood and sputum of patients with varying asthma severity.

3) To measure IL-17 in the sputum and to examine migration of blood neutrophils towards IL-17 in normal control subjects and patients with severe asthma.

CHAPTER 2

METHODS

2.1. General Design of Experiments

For the neutrophil activation study, three groups of asthma subjects with varying severity of asthma were enrolled into the study. The study design was cross sectional observational. These subjects were divided into three groups, 10 in each group for a total of 30 subjects. These groups were as follows,

- Severe asthma - Prednisone dependent (daily oral steroid)
- Moderate to severe asthma - On high dose of inhaled corticosteroids (>500 mcg daily of fluticasone or equivalent)
- Mild asthma - On low dose of inhaled corticosteroids (≤ 500 mcg daily of fluticasone or equivalent)

Peripheral blood drawn from these subjects was used to isolate neutrophils through Percoll discontinuous gradient method (Cox G, 1995). Sputum induction with hypertonic saline was used to collect sputum samples. Isolated blood neutrophils and sputum neutrophils were tested for activation and viability. Blood neutrophil viability (survival) was also tested at 24 hours. A blood sample was sent to the core lab for complete blood count (CBC) to obtain neutrophil numbers in blood. Sputum was analyzed to get total and differential cell counts.

Neutrophil activation was tested by Dihydrorhodamine assay (van Pelt L.J *et al* .1996). In this assay, Dihydrorhodamine was used to measure the production of

hydrogen peroxide during the oxidative burst in the neutrophils. Dihydrorhodamine is a non-fluorescent compound which is converted to rhodamine in the presence of hydrogen peroxide. The resulting fluorescence can be measured by flow cytometry.

Neutrophil activation was also measured by measuring the expression of primary and secondary granule proteins on the surface of neutrophils as described previously (Bjornsson et al. 2008). Primary granule protein, CD63, and secondary granule protein, CD66b, were measured by using specific antibodies conjugated with fluorescent probes. The resulting fluorescence was measured by flow-cytometry.

Neutrophil viability was measured by annexin V method (BioVision, Mountain View, CA - Catalog#: K101-400). This method could identify viable, apoptotic and necrotic cells. Annexin V conjugated with a fluorescent probe and propidium iodide, a fluorescent dye were used to identify apoptotic and necrotic cells. Samples were analyzed by flow-cytometry. Blood neutrophil viability was also measured at 24 hours by fluorescent light microscopy. Acridine orange, a fluorescent stain was used to identify apoptotic cells as described previously (Cox G, 1995)

For neutrophil migration study, an in vitro experimental model was used. The study subjects were divided into two groups, 10 in each for a total of 20. The groups were as follows,

- Severe asthma - Prednisone dependent asthma patients
- Normal controls – non asthmatic and non-atopic.

Normal controls were screened to exclude asthma by methacholine challenge testing. (Juniper E.F et al 1994). Allergies were excluded in these subjects by allergy prick testing. The method used has been described previously (St. Joseph's healthcare,

Hamilton, Ontario). Peripheral blood was drawn from study subjects to isolate neutrophils by Percoll discontinuous gradient method. Migration of neutrophils towards IL-17 was tested using disposable 96-well chemotaxis chamber (ChemoTx, Neuro Probe, Gaithersburg, MD, USA) as described previously (Frevert CW et al, 1998). Calcein a fluorescent dye, was used to label neutrophils to assess their migration. Migration was measured by a fluorescent plate reader (Molecular devices, Sunnyvale, CA – Spectra Max, Gemini EM). This fluorescence-based assay is rapid, less labor intensive and more sensitive. Neutrophils isolated from blood were exposed to various concentrations of IL-17 (0.05 ng/ml to100 ng/ml). Validation experiments to measure IL-17 in the sputum supernatants were performed using three methods. These were Enzyme Linked Immunosorbent Assay (R&D systems Inc. Minneapolis, MN, USA), Bio-Plex Cytokine assay (Bio-Rad, Mississauga, Ontario, Canada) and Mesoscale Discovery Electrochemiluminescence Detection System ((Meso Scale Discovery, Gaithersburg, Maryland, USA).

2.2. Subjects

Asthma subjects were recruited from patients of the Firestone Institute for Respiratory Health. Normal controls were recruited from the staff at St. Joseph's hospital. The study was approved by Research Ethics Board of St. Joseph's Healthcare. All the study subjects signed consent form before participating in the study.

Criteria used to enroll study subjects are as follows,

Inclusion criteria

1) Subjects were 18 to ≤ 70 years of age, of either sex or any race and asthma subjects had a documented diagnosis of asthma

2) Asthma subjects had no exacerbation of asthma within the past 4 weeks (Asthma control questionnaire score <2)

3) Nonsmokers or smokers who had <20 pack year history and not smoked in the past 6 months.

Exclusion criteria

1) Subjects who were diagnosed with any other relevant lung disease (eg. Cystic fibrosis, pulmonary fibrosis, bronchiectasis).

2) Subjects suffering from upper and or lower respiratory tract infection within the past 4 weeks.

3) Subjects who were on medications, for example cytotoxic agents, investigational drugs, antibiotics and antiviral drugs.

2.3. Sample Size

Based on our estimate of the variance of sputum neutrophil %, group sample sizes of 9 and 9 achieve 81% power to detect a difference of 20% in neutrophil% between the two groups. We have included a 3rd group. As a result we would require less than 9 subjects per group, but we have kept 9-10 subjects per group to be conservative in our estimate.

2.4. Isolation of Blood Neutrophils

Neutrophils were isolated from blood as described previously (Cox G, 1995). 20 ml blood was drawn into vacutainers (BD, Franklin Lakes, NJ) containing 3.2% buffered sodium citrate. Blood was centrifuged at 300g for 20 minutes (Beckman T J-6**,** Beckman Coulter Canada, Inc. Mississauga, Ontario). Platelet rich plasma was removed and stored at -80˚C in sterile eppendorf tubes. The remaining RBC/WBC pellet was then resuspended in 2.8 ml of 6% dextran (ICN Biomedicals, Inc, Costa Mesa, CA), in the ratio of 0.14ml dextran/ml of blood. Endotoxin-free saline (Baxter Corp., Mississauga, ON) was then added to bring the volume to 20 ml. The sample was left to stand for 50 minutes.

Supernatant was transferred into a new tube and spun for 6 minutes at 300g. The supernatant was discarded and the WBC pellet was resuspended in 2 ml saline. The cells were underlayed with 2 ml of freshly prepared 60% Percoll (1.8 ml 100% Percoll mixed with 1.2 ml saline) and then with 2 ml of freshly prepared 70% Percoll (2.1 ml 100% Percoll mixed with 0.9ml saline) using a sterile Pasteur pipette. Percoll was obtained from GE Healthcare, Biosciences, Baie d'Urfe Quebec. The sample was centrifuged for 10 minutes at 300g without brake. The cells at the interface of the 60% and 70% Percoll layers were carefully transferred to a new tube and washed with saline.

The cells were centrifuged at 300g for 10 minutes. The pellet was resuspended in 9 ml of cold, sterile endotoxin free water. (Baxter, Deerfield, IL). After 20 seconds, 1 ml of 10 x HBSS without calcium and magnesium (Sigma-Aldrich, St. Louis, MO) was added. 10 micro liters of the cell solution was added to 10 micro liters of trypan blue dye (Mediatech, Inc, Herndon,VA) and neutrophils were counted using a haemocytometer (Bright Line, Fisher Scientific Company, Ottawa, Ontario).The calculation for neutrophil count is PMN/ml= (# of neutrophils counted x dilution x 10^4) / # of large squares counted.

The suspension was centrifuged for 10 minutes at 300g. The neutrophil pellet was resuspended in the desired solution at the required density.

2.5. *Hydrogen Peroxide Measurement by Dihydrorhodamine (DHR) Assay*

Dihydrorhodamine assay was performed to assess the production of hydrogen peroxide (H_2O_2) in the neutrophils. Dihydrorhodamine 123 (DHR) is a non-fluorescent compound. DHR reacts with hydrogen peroxide, a reactive oxygen intermediate produced in the respiratory burst, and is converted to highly fluorescent compound rhodamine 123. The cell associated fluorescence produced as a result can be detected with a flow cytometer. The assay performed was similar to a previously described method (van Pelt L.J et al 1996).

Dihydrorhodamine (DHR) assay for blood neutrophils

Neutrophils isolated from blood (Cox G, 1995) were suspended at the cell density of 4 x 10^6 cells /ml in the buffer solution (140mM NaCl, 4mM KCl, 10mM Glucose, 10mM HEPES, 1mM $MgCl_2$ and1mM $CaCl_2$). The buffer solution was kept incubated at 37°C. Five Eppendorf and flow tubes each were marked as 1) Untreated DHR 2) DHR 3) DHR + FMLP 4) DHR + PMA 5) DHR + H_2O_2 .

Dilutions were made at the start of experiment. 250 µl of neutrophil suspension was added to the Eppendorf tubes. All the tubes were incubated for 10 minutes at 37°C and 5% CO_2.

Cytochalasin (Sigma-Aldrich, St. Louis, MO) 5µg/ml was added to the fMLP tube and all tubes were incubated at 37°C. At t = 5 minutes, DHR (Sigma-Aldrich, St. Louis, MO) 100nM was added to all the tubes except untreated DHR tube and all tubes were incubated. At *t* =10 minute incubation, fMLP (Sigma-Aldrich, St. Louis, MO) 10^{-6}M,

PMA (Sigma-Aldrich, St. Louis, MO) 100 ng/ml and H2O2 (Fisher Scientific Company, Ottawa, ON) 0.3% were added to the respective tubes. All the tubes were incubated for 15 minutes at 37°C. After that, tubes were put on ice to stop the reaction.

0.75 ml of 2% Para formaldehyde (EM Science, Darmstadt, Germany) was added to all Eppendorf tubes. The solution was transferred to the flow tubes (Becton Dickinson Company, Franklin Lakes, NJ). The tubes were kept covered at 4˚C until analysis was performed through flow Cytometry (FACSCalibur flow cytometer , BD Bio Sciences, San Jose, CA).

Neutrophils were identified using their density and size in forward- angle light scatter (FSC) and side-angle light scatter (SSC). 10^4 events were collected in the neutrophil gate. 488 nm was the fluorescence excitation wavelength used. Results are expressed as mean fluorescence intensity (MFI). DHR was analyzed using a FL1 histogram of gated events. The emission wavelength for FL1 was 530nm and for FL2 was 585nm. FACSCalibur from Becton Dickinson with CellQuest software analysis program was used.

Dilutions were made as follows 1) FMLP (10^{-6}M) – 1µl of ¼ dilution of 10^{-3}M stock solution 2) PMA (100ng/ml) – 2.5 µl of 1/100 dilution of 1mg/ml stock solution 3) DHR (100nM) – 1 µl of 1/40 dilution of 1mM stock solution 4) H_2O_2 (0.3%) - 3µl of 1/100 dilution of a 30% stock solution 5) Cytochalasin (5µg/ml) - 1µl of ½ dilution of 125mg/ml stock solution. Aliquots were kept on ice, covered. All these agents were dissolved in dimethyl sulfoxide (DMSO) except for H_2O_2, to make stock solutions and stored at -20˚C. These were diluted at final concentrations in PBS when needed.

Dihydrorhodamine (DHR) assay for sputum neutrophils

The assay for sputum is similar to the one described previously for blood neutrophils. The sputum sample was pelleted, supernatant was aspirated and discarded. The pellet was resuspended in 3.25 ml of buffer solution (mentioned previously). 250 µl of this suspension was then added to the five eppendorf tubes. The rest of the experiment is the same as DHR assay for blood neutrophils. For flow cytometry, gating used for blood neutrophils was used as a guide to gate sputum neutrophils.

2.6. Granule Protein Expression Measurement – CD 63 and CD 66b

CD63 and CD66b are markers of neutrophil activation. CD63 is present in the membrane of primary granules and CD66b in the membrane of secondary granules. Their expression on the surface membrane of neutrophils is increased upon stimulation of these cells (Cham BP et al. 1994 and Faurschou and Borregaard 2003). These markers can be measured by flow cytometry. Method used has been modified (Bjornsson et al. 2008). A marker for neutrophils, CD16, is used this experiment. Measurements were made both in blood and sputum.

Measurement of granule protein expression on blood neutrophils

Neutrophils isolated from blood (Cox G, 1995) were resuspended at the cell density of 4×10^6 cells/ml in the buffer solution (140mM NaCl, 4mM KCl, 10mM Glucose, 10mM HEPES, 1mM $MgCl_2$ and1mM $CaCl_2$). The buffer solution was kept in the incubator at 37°C. 250 ul of this suspension was added to the pre marked Eppendorf

tubes 1) CD 63 2) CD 66b 3) CD63 + CD66b + CD16 4) FMLP + CD63 + CD66b + CD16 5) PMA + CD63 + CD66b + CD16 6) Untreated 7) CD16 + Isotype). Tubes were incubated for 10 minutes at 37°C and 5% CO_2.

Cytochalasin (Sigma-Aldrich, St. Louis, MO) 5µg/ml was added to the fMLP tube. After 10 minutes of incubation, fMLP (Sigma-Aldrich, St. Louis, MO) 10^{-6}M and PMA (Sigma-Aldrich, St. Louis, MO) 100 ng/ml were added to the respective tubes. The tubes were incubated for 15 minutes. These were then put on ice to stop the reaction. The tubes were pelleted, top aspirated and resuspended in 50 µl of the second buffer solution (buffer solution mentioned above + 0.5% BSA). This buffer solution was kept on ice. 10 µl of antibodies were added to the respective tubes (CD63, CD66b and Isotypes – Beckman Coulter, Inc., Brea, CA and CD16 – BioLegend, San Diego, CA, Catalog # 302012). These antibodies were conjugated with fluorescent compounds. CD63 was phycoerythrin (PE) conjugated, CD66b was fluorescein isothiocyanate (FITC) conjugated and CD16 was conjugated with allophycocyanin (APC). The tubes were covered in foil and rocked for thirty minutes in the cold room. The cells were washed twice in 500 µl of second buffer solution. Finally cells were resuspended in 500 µl of 1% Para formaldehyde (EM Science, Darmstadt, Germany). The suspensions were transferred to the corresponding flow tubes (Beckton, Dickinson and Company, Franklin lakes, NJ). The tubes were kept covered and stored at 4˚C.

The samples were analyzed by flow cytometry (FACSCalibur flow cytometer , BD Bio Sciences, San Jose, CA). Neutrophils were gated using their density and size in side angle light scatter and forward angle light scatter respectively. Compensation was

achieved using FITC and PE labeled antibodies individually.

The identity of neutrophils was further confirmed by their CD16 signal. CD63 was detected using FL2 detector and CD66b was detected using FL1 detector. For CD63, excitation wavelength was 488 nm and emission wavelength was 585 nm. For CD66b, excitation wavelength was 488 nm and emission wavelength was 530 nm. Ten thousand events were collected in the neutrophil gate. Results are expressed as mean fluorescence intensity (MFI). FACSCalibur from Becton Dickinson with CellQuest software analysis program was used

Measurement of granule protein expression on sputum neutrophils

The sputum sample was pelleted, supernatant was aspirated and discarded. The pellet was resuspended in 3.25 ml of buffer solution (140mM NaCl, 4mM KCl, 10mM Glucose, 10mM HEPES, 1mM $MgCl_2$ and1mM $CaCl_2$). 250 µl of this suspension was added to each of the pre marked Eppendorf tubes (1) CD16+ CD63+CD66b 2) fMLP+CD16+CD63+CD66b 3) PMA+ CD16 + CD63+CD66b 11) Untreated 12) Isotype+CD16).

The tubes were incubated for 10 minutes at 37°C and 5% CO_2. The rest of the experiment is the same as the one described for blood. For flow cytometry, gating used for blood neutrophils was used as a guide to gate sputum neutrophils.

Dilutions were made as follows 1) fMLP (10^{-6}M) – 1µl of ¼ dilution of 10^{-3}M stock solution 2) PMA (100ng/ml) – 2.5 µl of 1/100 dilution of 1mg/ml stock solution 3) Cytochalasin (5µg/ml) - 1µl of ½ dilution of 125mg/ml stock solution. Aliquots were kept

on ice covered. All these agents were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions and stored at -20°C. These were diluted at final concentrations in PBS when needed.

2.7. Neutrophil Viability – Annexin V Method

Neutrophil viability was examined using the annexin method. In cells during apoptosis, membrane phosphatidyleserine (PS) is trans located from the inner surface of the plasma membrane to the outer cell surface. This translocation occurs during early apoptosis during which there is no loss of plasma membrane integrity. PS on the cell surface can be detected by staining with fluorescent conjugate of annexin V, which is a protein that has a high affinity for PS. Since PS also trans locates during necrosis, Annexin V is not an absolute marker of apoptosis. Therefore it is often used with propidium iodide (PI), a fluorescent dye, which binds to nucleic acids which can only occur when there is loss of plasma membrane integrity which occurs in the later stages of apoptosis or in case of necrosis. Annexin V and PI can be used to assess cell viability. Viable cells do not stain for Annexin V and PI, early stage apoptotic cells stain for annexin V only and late stage apoptotic or necrotic cells stain for both for annexin V and PI. Neutrophil viability can be analyzed by flow cytometry. Method and kit used was from by BioVision, Mountain View, CA (Catalog#: K101-400).

Blood neutrophil viability

Four Eppendorf and four flow tubes were marked as 1) Annexin 2) PI 3) Annexin + PI 4) Untreated/Control. Neutrophils isolated from blood (Cox G, 1995) were resuspended in four eppendorf tubes in 500 µl of Binding Buffer at the cell density of 4×10^6 cells/ml. 5 µl of annexin V-FITC was added to the respective tubes and 5 µl of propidium iodide (PI)

was added to the respective tubes. The suspension was transferred to the corresponding flow tubes. The tubes were covered and kept at 4°C until analysis was done. Analysis was performed using FACSCalibur flow cytometer (BD Bio Sciences, San Jose, CA). 10,000 granulocytes were counted. FITC-annexin excites at 495nm and emits at 520nm and was detected using the FL1 detector. PI excites at 495nm and emits at 639nm and was detected using FL2 detector. FACSCalibur from Becton Dickinson with CellQuest software analysis program

Sputum neutrophil viability

Two Eppendorf and flow tubes were marked as 1) Untreated /Control 2) Annexin + PI. An aliquot of sputum cells was resuspended in 1 ml of Binding Buffer. 500 ml was added to the Eppendorf tubes. Annexin – V-FITC 5 µl and PI 5 µl were added to the respective tubes. The suspension was transferred to the flow tubes. The tubes were covered and kept at 4°C until analyzed. Analysis was performed by flow cytometry. Due to a low number of sputum cells to be used in all the analyses, blood neutrophil compensation was used for the sputum neutrophils. FITC-annexin excites at 495nm and emits at 520nm and was detected using the FL1 detector. PI excites at 495nm and emits at 639nm and was detected using FL2 detector.

2.8. Assessment of Cell survival by Fluorescent Microscopy

Isolated blood neutrophils were resuspended at a concentration of 4 x 10^6 in 2% FCS+RPMI (Lonza Cologne AG, Cologne Germany and PAA Laboratories Inc., Etobicoke, ON). This was kept overnight in the incubator at 37°C and 5% CO_2. At 24 hours, cultured cells were resuspended by gentle pipetting. An aliquot of cultured cells was mixed with acridine orange (Sigma-Aldrich, St. Louis, MO) at a final concentration of 5 µg/ml. The cells, both normal and apoptotic, were then counted using fluorescent light microscope. Neutrophil survival was expressed as the percentage of non-apoptotic cells. Apoptotic cells were identified by nuclear morphology, specifically the appearance of rounded lobes of the nucleus.

2.9. Cytospins

Isolated blood neutrophils were resuspended at the concentration of 1 x 10^6 cells/ml in1 x PBS (Sigma-Aldrich, St. Louis, MO). Two slides for cytospins were made using 65µl of this suspension for each slide. The suspension was spun for 6 minutes at 450 rpm (Shandon III cytocentrifuge; Shandon Southern Instruments, Sewickley, PA). The slides were then stained with Geimsa/ Wright's stain and covered with mounting medium and a cover slip. These were then examined using a light microscope to count neutrophils, eosinophils and monocytes. A total of 100 cells were counted. Results were expressed as the percentage of cells present. Neutrophil purity was 94% in mild asthma subjects, 94.4% in moderate asthma subjects, 95.8% in severe asthma subjects and 97% in normal control subjects.

2.10. Asthma Control Questionnaire

This was used to assess asthma control in subjects participating in the study (Juniper EF et al,1999). It comprised of six questions regarding asthma symptoms. These symptoms include night time and morning symptoms, limitation of activities because of asthma, shortness of breath, wheezing and use of short acting brochodilator like ventolin. Symptoms are scored from 0 to 6 in terms of severity in an ascending order. Study subjects were asked to do the scoring. Question no.7 is regarding FEV_1, which is also scored from 0 to 6 in terms of decline in lung function. FEV_1 of study subjects was measured by spirometry. All the scores were added and divided by 7. A cut off point of 2 was used. Subjects having a score of less than 2 were considered to have good asthma control and were included into the study.On the next page is the sample of the questionnaire used for the study

ASTHMA CONTROL QUESTIONNAIRE ©

PATIENT ID _____

DATE _____

Please answer questions 1 - 6.

Circle the number of the response that best describes how you have been during the past week.

1. On average, during the past week, how often were you **woken by your asthma** during the night?
   - 0 Never
   - 1 Hardly ever
   - 2 A few times
   - 3 Several times
   - 4 Many times
   - 5 A great many times
   - 6 Unable to sleep because of asthma

2. On average, during the past week, how **bad were your asthma symptoms when you woke up** in the morning?
   - 0 No symptoms
   - 1 Very mild symptoms
   - 2 Mild symptoms
   - 3 Moderate symptoms
   - 4 Quite severe symptoms
   - 5 Severe symptoms
   - 6 Very severe symptoms

3. In general, during the past week, how **limited were you in your activities** because of your asthma?
   - 0 Not limited at all
   - 1 Very slightly limited
   - 2 Slightly limited
   - 3 Moderately limited
   - 4 Very limited
   - 5 Extremely limited
   - 6 Totally limited

4. In general, during the past week, how much **shortness of breath** did you experience because of your asthma?
   - 0 None
   - 1 A very little
   - 2 A little
   - 3 A moderate amount
   - 4 Quite a lot
   - 5 A great deal
   - 6 A very great deal

ASTHMA CONTROL QUESTIONNAIRE ©

PATIENT ID _____

DATE _____

5.	In general, during the past week, how much of the time did you **wheeze**?	0 Not at all 1 Hardly any of the time 2 A little of the time 3 A moderate amount of the time 4 A lot of the time 5 Most of the time 6 All the time
6.	On average, during the past week, how many **puffs/inhalations of short-acting bronchodilator** (eg. Ventolin/ Bricanyl) have you used each day? *(If you are not sure how to answer this question, please ask for help)*	0 None 1 1 - 2 puffs/inhalations most days 2 3 - 4 puffs/inhalations most days 3 5 - 8 puffs/inhalations most days 4 9 - 12 puffs/inhalations most days 5 13 - 16 puffs/inhalations most days 6 More than 16 puffs/inhalations most days

To be completed by a member of the clinic staff

7.	FEV_1 pre-bronchodilator: . . . . . . FEV_1 predicted: . . . . . . FEV_1 % predicted: . . . . . . (Record actual values on the dotted lines and score the FEV_1 % predicted in the next column)	0 > 95 % predicted 1 95 - 90 % 2 89 - 80% 3 79 - 70 % 4 69 - 60% 5 59 - 50% 6 < 50% predicted

2.11. Cold Questionnaire

This questionnaire was used to assess the cold symptoms of potential subjects. It was adapted from the one described previously (Meschievtz *et a*l, 1984). Symptoms were recorded in four categories: Nasal, throat, systemic and cough. A four-point category scale was used to score the symptoms as none=0, mild=1, moderate=2 or severe=3. Subjects were classified as "not having cold" if there were no symptoms, as having "definite cold" if they had symptoms in two or more categories with at least one symptom scoring 2 or more, "doubtful cold" if there were symptoms in only one category with symptoms scoring no more than 1.Subjects having no symptoms or a doubtful cold were included into the study.

COLD HISTORY AND SYMPTOMS SEVERITY

Symptoms	Date:dd/mm/year	Absent (0)	Mild (1)	Moderate (2)	Severe (3)
Nose category					
Runny nose					
Stuffy nose					
Sneezing					
Increased tissues					
Throat category					
Sore throat					
Hoarseness					
Systemic category					
Malaise					
Muscles aches					
Fever					
Chills					
Red/watery eyes					
Face/ear ache					
Painful neck glands					
Cough category					
Cough					

Symptoms in 2 or more categories (nose, throat, cough or systemic)? Yes G No G
At least one symptom scoring 2 or more? Yes G No G
DEFINITE COLD Yes G No G

Symptoms in only 1 category (nose, throat, cough or systemic)? Yes G No G
Symptoms scoring no more than 1? Yes G No G
DOUBTFUL COLD Yes G No G

2.12. Migration Study

Disposable 96-well chemotaxis chamber (ChemoTx, Neuro Probe, Gaithersburg, MD, USA) was used to measure neutrophil migration towards IL-17. This method has been described previously (Frevert CW et al, 1998). Calcein AM (Cayman Chemical, Ann Arbor, MI, catalogue no. 400146) was used to label neutrophils to assess their migration. Calcein AM is a non-fluorescent dye which is hydrolysed by intracellular esterases into fluorescent Calcein.

Neutrophils were isolated from blood as described previously (Cox, 1995) at a concentration of 3 x 10^6 cells/ml of 1 x HBSS+FCS (without phenol red) (Sigma-Aldrich, St. Louis, MO and PAA Laboratories Inc, Etobicoke, ON respectively). Calcein AM was added at a concentration of 5ul/ml of neutrophil suspension. This suspension with and without Calcein was incubated at 37°C and 5% CO_2 for 30 minutes. Cells were pelleted and washed with 1 x PBS (Lonza, Walkersville, MD) twice. Finally, cells were resuspended in 1 ml of 1 x HBSS+FCS (without phenol red). Neutrophils were exposed to various concentrations of IL-17 (0.05, 0.1, 1, 10 1and 100 ng/ml) (R&D systems Inc. Minneapolis, MN) in addition fMLP (4.5 ng/ml) (Sigma-Aldrich, St. Louis, MO) as a positive control. Chemotactic agents were diluted in PBS-BSA (Lonza, Walkersville, MD and Sigma-Aldrich, St. Louis, MO respectively). Migration of neutrophils towards PBS-BSA was also measured as a baseline measurement.

Chemotactic agents (29 µl) were added to the wells in the bottom chamber of the microplate. Measurements were made in duplicates. Also to measure total fluorescence, 25 ul of neutrophil suspension was also placed in two wells of the bottom chamber. Filters were positioned onto the microplate. 25 µl of neutrophil suspension were placed directly onto the filter sites and the chamber was incubated for 1 hour at 37°C and 5% CO_2.

The non-migrating cells on top of the filter were removed by gentle wiping with a kimwipe (Kimberly-Clark Professional, Roswell, GA). The chamber was placed in the fluorescence plate reader (Molecular devices, Sunnyvale, CA – Spectra Max, Gemini EM). Calcein fluorescence signal was used to measure the cells that had migrated into the bottom chamber. The probe in the fluorescent plate reader was in the bottom read position, which allowed measurement of fluorescence in the bottom chambers (Excitation ,485 nm and emission, 530 nm). Results were expressed as the percentage of migrating neutrophils using the formula (mean total fluorescence in wells of interest / mean total fluorescence of neutrophils) x 100.

2.13. Methacholine Challenge Testing

Airway responsiveness was measured as described previously (Juniper E.F et al 1994). This test is used to determine asthma severity and also to exclude asthma in non-atopic subjects. The test was performed by breathing in a mist of provocholine, which has a bronchoconstrictive effect in asthmatics. This effect was measured through spirometry. Tidal breathing method was used. Asthma was excluded in subjects whose FEV_1 did not reduce by 20% at 8 mg/ml of provocholine (They did not have a bronchoconstrictive effect in response to provocholine)

2.14. Allergy Skin Prick Testing

The method used has been described previously (St. Joseph's healthcare, Hamilton, Ontario). Allergy skin prick testing was done to exclude allergies in non-asthmatic subjects. Allergies were excluded to common allergens like molds, pollens, animal dander, dust mites and some foods. Subjects should not have taken Hismanal for a month or anti histamines in the last 4 days. Drops of allergens were placed on the skin and tiny pricks were made under the skin. After 15 minutes the subjects arm was assessed If the subject had a reaction, it was compared to the reaction to histamine. If the size of the swelling (wheal) was 1-2mm, it was scored as +1. If it was 3-5 mm, it was scored as +2 and if 6-9 mm, it was scored as +3 and so on. The reaction to the allergens was positive if the swelling was equal to or greater than the histamine reaction. Those non asthmatics that tested positive for allergies were not included into the study.

2.15. Sputum Induction, Processing and Examination

2.15.1 Sputum induction

Sputum was induced as described previously (Firestone Institute for Respiratory Health: sputum induction procedure, revised version December 2009). Sputum induction is a relatively non-invasive method. It is used to obtain sputum for cell or fluid phase inflammatory indices, culture or cytology.

The first step in sputum induction is spirometry to assess the lung function of study subjects. This was done according to American Thoracic Society criteria (ATS Standardization of Spirometry). Next step is administration of a beta agonist followed by sputum induction by inhalation of an aerosol of normal saline followed by hypertonic saline (1.5, 3, 4 and 5%), generated by an ultrasonic nebulizer (Pin et al. 1992) with an output of 0.87 ml/minute and particle size of 5.58μm aerodynamic mass-median diameter.

2.15.2. Sputum Processing and Examination

Sputum was selected from the entire expectorate under an inverted microscope, and processed as previously described (Pizzichini et al, 1996). Briefly, the selected portion of sputum was, treated with 4 volumes of ditheothreitol (DTT) to disperse the mucus, rocked for 15 minutes followed by the addition of 4 volumes of D-PBS and filtered through an ACCUFILTER® (Cellometrics, Hamilton, Ontario). A manual total cell count (expressed as cells/gram of sputum $x10^6$) was performed using a haemocytometer by the trypan blue exclusion criteria, to determine cell viability.

Cytospins were prepared and stained with Wright's (Dacie JV and Lewis SM, 1991) and a differential cell count was performed on 400 non squamous cells and reported as percentage. The remaining sputum filtrate was centrifuged at 3000rpm for 4 minutes and the supernatant was collected in micro tubes and stored at -70^0C for subsequent analysis. The cell pellet was used for measuring sputum neutrophil activation and viability.

2.16. Validation of IL-17 Measurement in Sputum Supernatant.

Validation experiments were performed as it was our intention to measure IL-17 in the sputum supernatants of participating severe asthma subjects and normal control subjects. Spike and recovery experiments were performed using three different methods. Methods used were Enzyme-linked immunosorbent assay (ELISA), Bio-Plex Cytokine assay and Mesoscale Discovery Electrochemiluminescence Detection System. The methods used are as follows,

2.16.1. ***ELISA***

Enzyme-linked immunosorbent assay was used to measure IL-17A. Quantitative sandwich enzyme immunoassay technique was used (R&D systems Inc. Minneapolis, MN, USA). Six replicates of sputum supernatant samples were examined. Standard solutions were tested with and without DTT.

Briefly, monoclonal antibody for IL-17A was pre-coated onto a microplate. Standard and sputum supernatant samples were pipetted into the wells and any IL-17A present in the samples was bound by the immobilised antibody. Unbound substances were washed away and an enzyme linked polyclonal antibody specific for IL-17A was added to the wells. A substrate solution was added to develop colour which was proportional to the bound IL-17A. Color development was stopped by the addition of acid. Optical density was measured in each well within thirty minutes using a microplate reader (Bio-Rad, Model 550) set at 450nm. Standard curve was obtained by plotting optical density on the y axis against the IL-17A concentration on the x axis.

Sputum supernatant samples were spiked as part of spike and recovery experiment. Two types of spikes were used. Supernatants were spiked at two concentrations to mimic a sputum supernatant concentration of 10pg/ml and1000pg/ml IL-17A.

2.16.2. Bio-Plex Cytokine Assay

Bio-Plex assay from Bio-Rad (Mississauga, Ontario, Canada) was used to measure IL-17A. Six replicates of sputum supernatant samples were examined.

Bio-Plex assay is designed in a capture sandwich immunoassay format. This assay utilizes color coded polystyrene beads which are fluorescently dyed microspheres. A flow cytometer with two lasers along with optics is used to measure biochemical reactions associated with microspheres. Antibody directed against IL-17A was coupled to the colour coded beads. The antibody and bead complex was reacted with sputum supernatant solution and the standard solution. After removing unbound protein through series of washes, a biotinylated detection antibody specific for a different epitope on the IL-17A was added to the beads. This lead to the formation of a sandwich of antibodies around the cytokine. Streptavidin-phycoerythrin was added which binds to biotinylated detection antibody. The samples were analysed using a BioPlex 200 System based on Luminex xMAP technology which identifies and quantifies the specific reaction based on bead color and fluorescence.

Two experiments were carried out with sputum supernatants. Initially, sputum supernatants prepared with DTT and later those prepared without DTT were used.

In the first experiment with sputum supernatants treated with DTT, supernatants were spiked at two concentrations to mimic a sputum supernatant concentration of 10pg/ml and1000pg/ml IL-17A. Linearity-of-dilution experiments were also carried out with high spiked sputum samples.

In the second experiment, sputum supernatant samples were prepared as follows,

1) Samples with DTT as in the first experiment (not spiked).

2) Samples without DTT (treated with PBS only). These samples were spiked to final concentration of 1000pg/ml.

Both types of samples mentioned above were examined with and without antiproteases. (Antiproteases added to sputum supernatants before they were frozen. These were Aprotonin+ Leuprotonin+ PMSF+ sodium vanadate). Standard solutions were also tested with and without DTT.

2.16.3. Mesoscale Discovery Electrochemiluminescence Detection System (MSD)

Mesoscale Discovery electrochemiluminescence detection system was used (Meso Scale Discovery, Gaithersburg, Maryland, USA). Six replicates of sputum supernatant samples were examined

This method uses plates with wells that contain up to 10 carbon electrodes per well. Each electrode is coated with a different capture antibody. This method is designed in a sandwich ELISA format. Analytes of interest are captured on the electrodes. The analytes are detected with an analyte specific ruthenium-conjugated secondary antibody. The ruthenium label emits light at the surface of electrodes upon electrochemical stimulation. This allows the concentration of the analyte to be determined (Chowdhury F. et al, 2009). DTT treated sputum supernatant samples were spiked to a final concentration of 1000pg/ml.

2.17. Ethics

Ethics approval was obtained from the Research Ethics Board of St. Joseph's Healthcare to draw blood, do sputum induction, methacholine challenge testing and allergy skin prick testing in addition to use of questionnaires. The research labs at St. Joseph's Healthcare are well equipped and staffed to conduct the experiments. The procedures used are well established and were safe as no inadvertent events occurred.

2.18. Statistical Analysis

Statistical analysis was performed by both parametric test (one-way ANOVA with Bonferonni correction for multiple comparisons) and non-parametric test (Kruksal Wallis ANOVA). SPSS software package was used. Data was assumed statistically significant when the p-value was less than 0.05.

CHAPTER 3

RESULTS OF NEUTROPHIL ACTIVATION STUDY

The aim of this study was to assess if neutrophil numbers and their activity are increased in subjects with severe asthma as compared to less severe asthma in order to explore the causal relationship between neutrophils and severe asthma. Therefore, neutrophil numbers, activation and viability were measured in blood and sputum samples of clinically stable subjects with varying asthma severity.

3.1. Clinical Features of Study Subjects

The clinical characteristics of study subjects are summarized in Table1. Atopy was more prevalent in subjects with mild (80%) and moderate asthma (77.7%) as compared to severe asthma (40%). The pulmonary function parameters (FEV1 % predicted and FEV_1/FVC; Table 1) were significantly lower in severe asthma group as compared to mild asthma group. Mild asthma subjects were either taking inhaled corticosteroids at a daily dose of $\leq$ 500 µg or were taking these when needed. All the subjects with moderate and severe asthma were on daily dose of > 500µg of inhaled steroids.

Blood and sputum samples were collected from ten mild, nine moderate and ten severe asthma subjects. Neutrophils were isolated from all the blood samples. Measurements of neutrophil numbers, viability and activation were made in all the collected blood and sputum samples.

Table 1 Clinical features of study subjects

Variables	Mild asthma	Moderate asthma	Severe asthma
N	10	09	10
Age years*	47(16)	51(17)	53(13)
Gender M/F	7/3	4/5	5/5
Smoking Hx*	1.1(3.3)	1.7(4.9)	7.7(6.9)
ACQ score*	0.46(0.3)	1.00 (0.6)	1.17 (0.6)
BMI*	29 (3.3)	29.5 (4.3)	30.4 (7.0)
Atopy, n (%)	8 (80)	7 (77.7)	4 (40)
FEV_1% predicted*	91(10)	78(10)	71(17) #
FEV_1/FVC%*	77(6)	69(8)	68(10)#
ICS dose ǂ Ψ	0(0-500)	1000(750-3000)	1000(500-2000)
Oral steroid dose¶ Ψ	0	0	9.75 (3.5-20)

N: number of study subjects
*: Data shown as mean (SD)
M: male; F: female.
Hx:history
ACQ: Asthma Control Questionnaire
BMI: Body Mass Index (kg/m^2)
n: Number of study subjects with positive skin prick test(%)
ǂ: Inhaled corticoid dose (Fluticasone equivalent µg/day,median and range)
¶: Prednisone equivalent mg/day (Median and range)
Ψ: median and range of daily dose of steroids
#: $p < .05$ versus mild asthma group

3.2. Neutrophil counts in Blood and Sputum

Neutrophil counts were obtained in blood and sputum samples of asthma patients as shown in Figure 6. Results of blood neutrophil counts are expressed as percentage (%) of total leucocyte count and numbers ($x10^9$/liter of blood). Blood neutrophil numbers were significantly increased in severe asthma subjects as compared to mild and moderate asthma subjects when results are expressed as absolute numbers ($x10^9$/L) only ($p<0.05$).

Results of sputum neutrophil counts are also expressed as percentage (%) of the total leucocyte count and numbers ($x10^6$/ml of sputum). Significant differences were not observed amongst the three groups of subjects, although increased sputum neutrophil counts are observed in severe asthma subjects when compared to the other two groups.

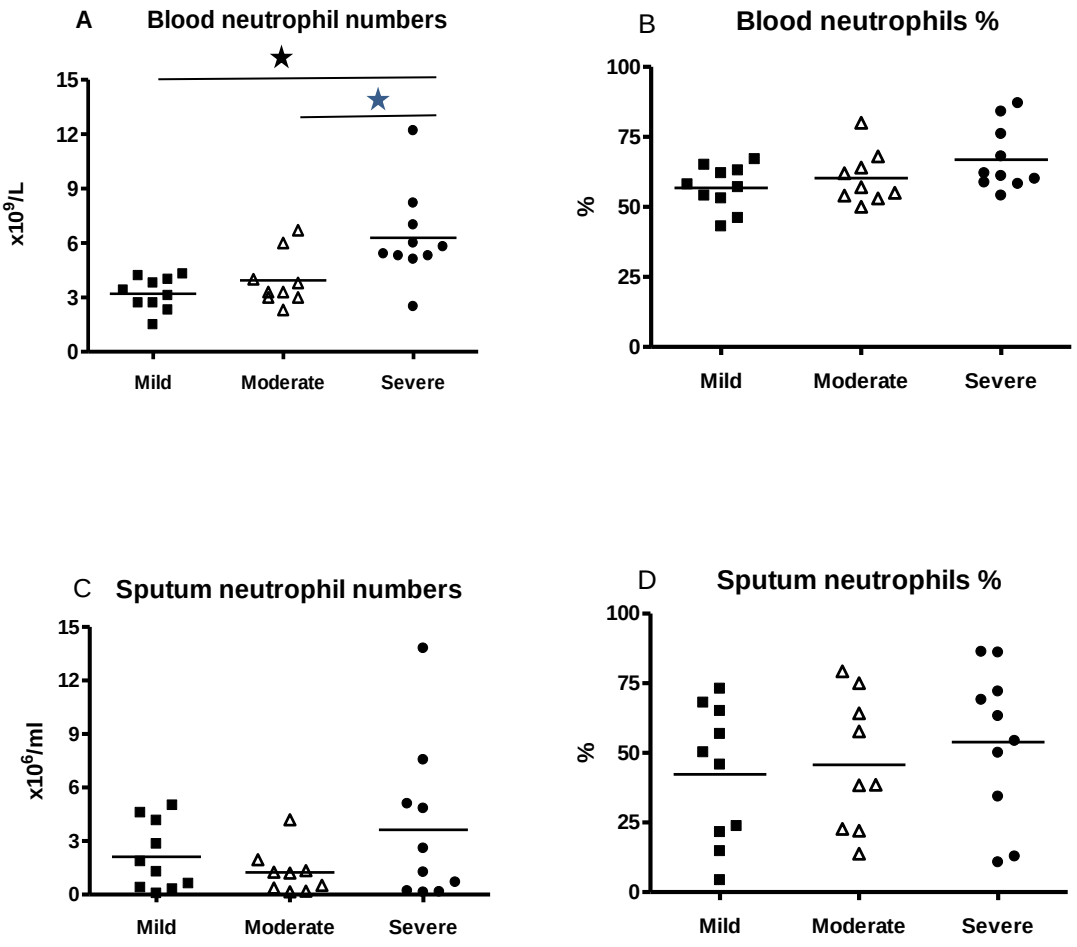

Figure 6. Neutrophil counts in blood (A & B) and sputum (C& D) of asthma subjects. Neutrophil counts in blood were significantly increased in severe asthmatics as compared to mild and moderate asthmatics, when results are expressed as absolute numbers. There is increase in sputum neutrophils in severe asthma subjects, although not statistically significant. The dot plots show the mean values as black horizontal lines.

3.3. Eosinophil counts in Sputum

Eosinophil counts were obtained in sputum samples of asthma patients (Figure 7). Results are expressed as percentage (%) of total leucocyte count and numbers ($x10^6$/ml of sputum). Significant differences were not observed amongst the three groups of subjects, although increased sputum eosinophil counts are observed in severe and moderate asthma subjects when compared to mild asthma group, especially when results are expressed as a percentage.

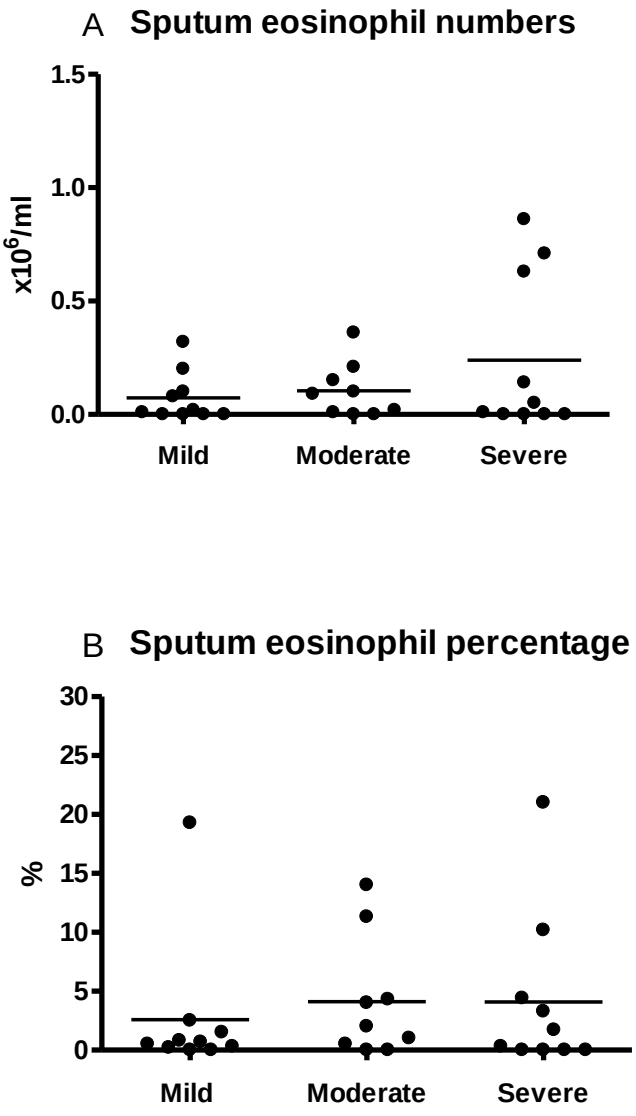

Figure 7. Eosinophil counts in sputum (A & B) of asthma subjects.

No significant differences were observed amongst the three groups of asthma subjects.

The dot plots show the mean values as black horizontal lines.

3.4. Macrophage counts in Sputum

Macrophage counts were obtained in sputum samples of asthma patients (Figure 8). Results are expressed as percentage (%) of total leucocyte count and numbers ($x10^6$/ml of sputum). Macrophage counts in sputum were increased in mild asthma subjects as compared to moderate and severe asthma subjects, although not statistically significant.

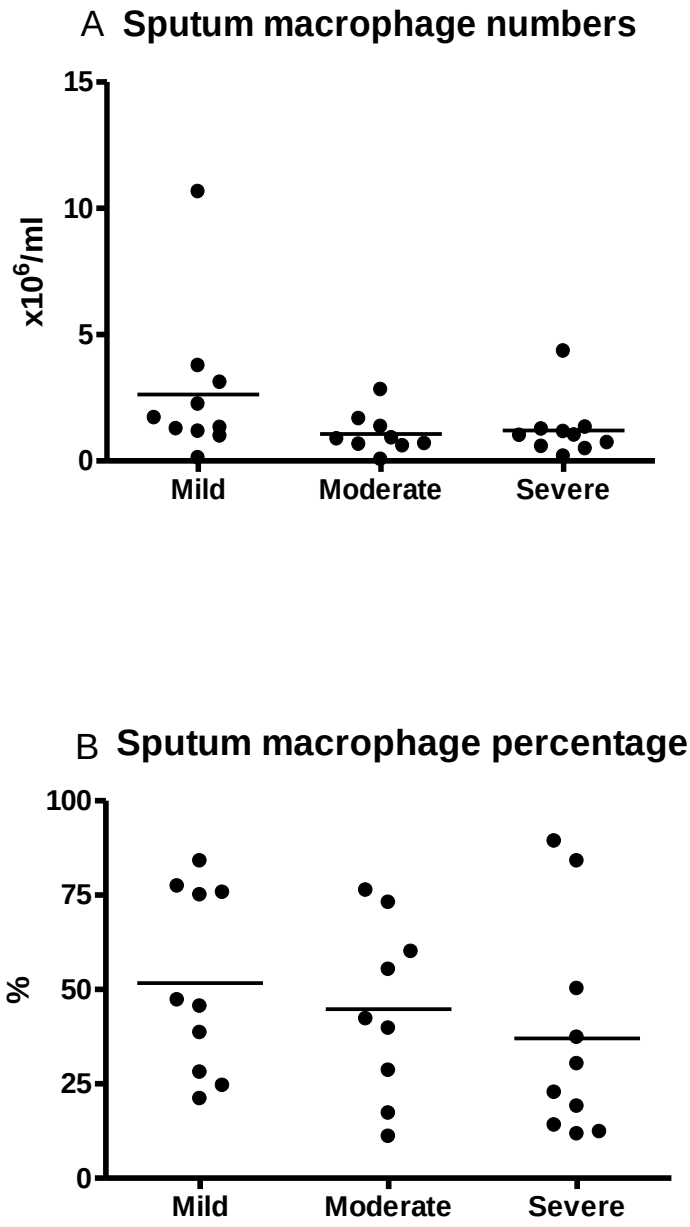

Figure 8. Macrophage counts in sputum (A & B) asthma subjects. Macrophage counts are increased in mild asthma subjects as compared to moderate and severe asthma subjects. The difference is not statistically significant. The dot plots show the mean values as black horizontal lines.

3.5. Neutrophil viability in Blood and Sputum

Viability of neutrophils was examined by annexin V method (BioVision, Mountain View, CA (Catalog#: K101-400) in blood (Figure 9A) and sputum (Figure 9B). Viable neutrophils did not stain with annexin and propidium iodide. Results are expressed as percentage of total neutrophils. Percentage of viable neutrophils was more in blood than in sputum. No significant differences in viability were observed in blood and sputum neutrophils amongst the three groups of asthma patients. However, blood neutrophil viability in severe asthma group was more than the mild asthma group ($p=0.06$). Results are also shown as dot plots (Figure 10).

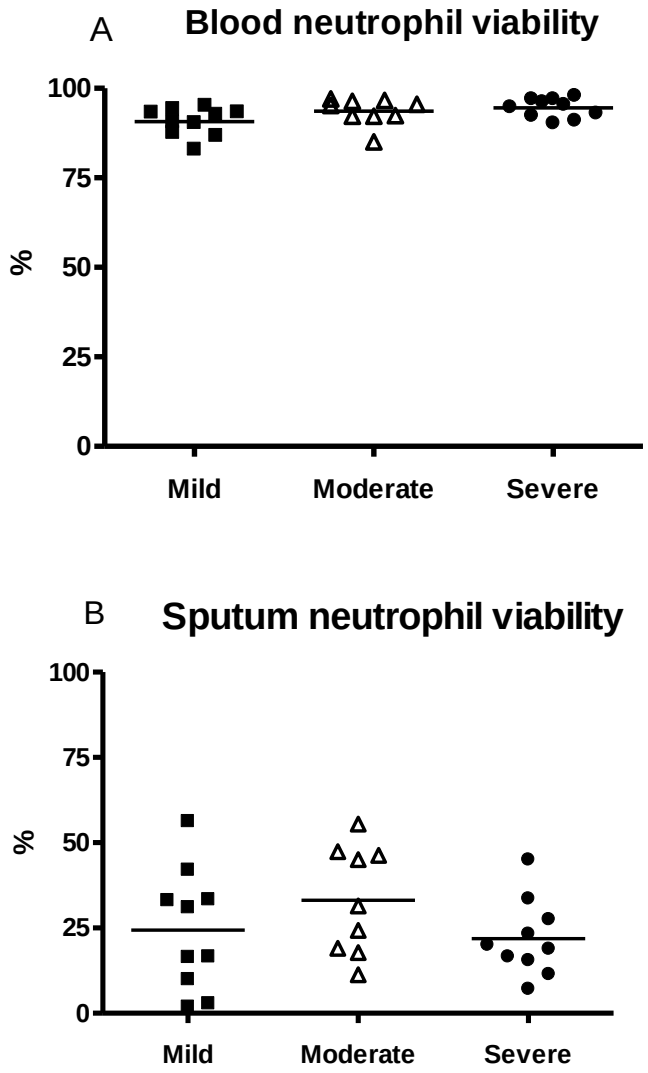

Figure 9. . Neutrophil viability in blood (A) and sputum (B) of asthma subjects. Results are expressed as the percentage of total neutrophils. No significant differences in viability were observed in blood and sputum neutrophils amongst the three groups of asthma patients. Increased percentage of viable neutrophils were observed in blood than in sputum amongst all the three groups of asthma subjects. Blood neutrophil viability in severe asthma group was more than the mild asthma group (p=0.06). The dot plots show the mean values as black horizontal lines.

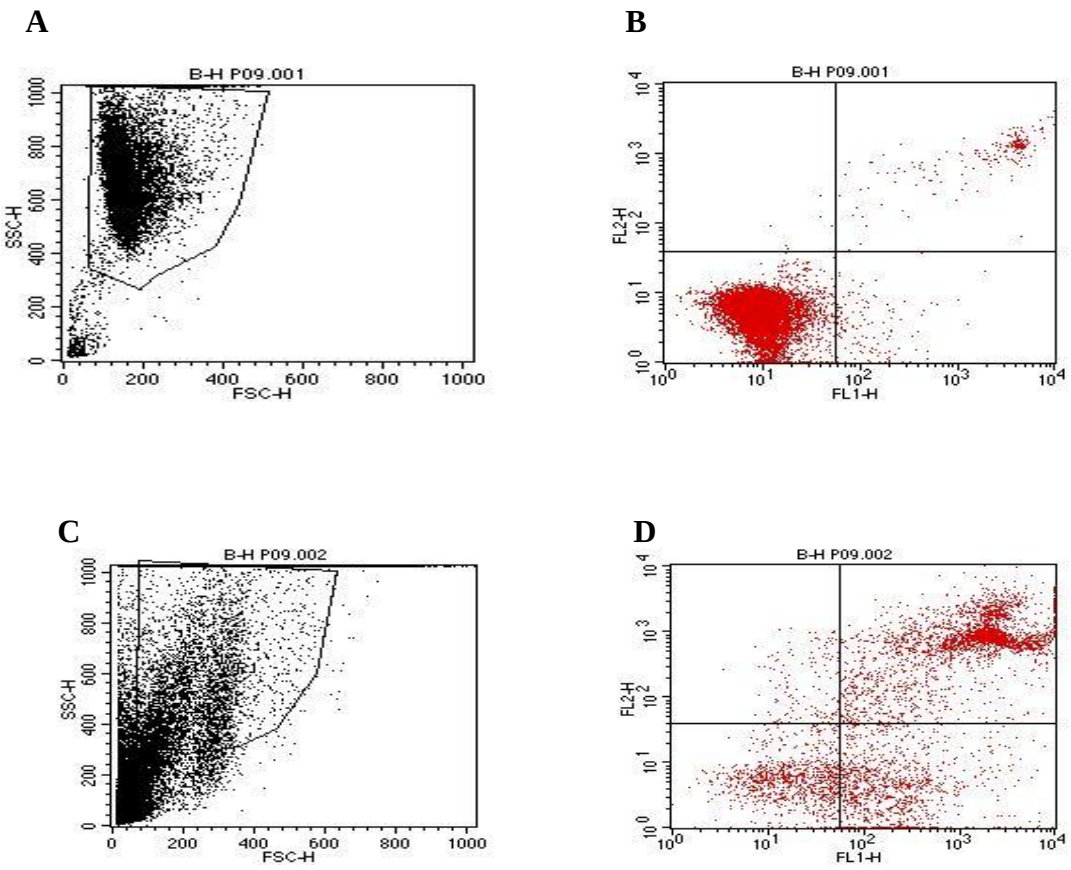

Figure 10. Detection of apoptosis of blood (A &B) and sputum neutrophils (C& D) by annexin V-FITC and propidium iodide (PI) labeling followed by flow cytometry. Neutrophils in blood (A) and sputum (C) were gated using their density and size in forward-angle light scatter (FSC) and side-angle light scatter (SSC). Fluorescence from annexin V-FITC and PI labeled cells was detected at FL1 detector and FL2 detector respectively. Dots in the bottom left quadrants represent viable neutrophils that do not bind annexin V and PI. Dots in the bottom right quadrants represent neutrophils in the early stages of apoptosis and bind only annexin V. Dots in the top right quadrants represent neutrophils that are in late stages of apoptosis or are necrotic, binding with both annexin V and PI. The number of viable neutrophils is more in blood than in sputum.

3.6. Neutrophil Survival at 24 hours

Blood neutrophil survival was examined at 24 hours in three groups of asthma subjects. Acridine orange stain was used to detect morphological changes of apoptosis as described previously (Cox G, 1995). The cells were examined by fluorescent light microscopy. Both normal and apoptotic cells were counted. Neutrophil survival was expressed as the percentage of non-apoptotic viable cells. Apoptotic cells were identified by nuclear morphology, specifically the appearance of rounded lobes of the nucleus.

Results are expressed as percentage of viable cells. Blood neutrophils show increased survival in severe asthma subjects as compared to moderate asthma subjects, the difference being statistically significant(Figure 11)

Sputum neutrophil survival was also examined in the initial phase of the study. Since there were no surviving cells present in the sputum samples at 24 hours, this test was discontinued.

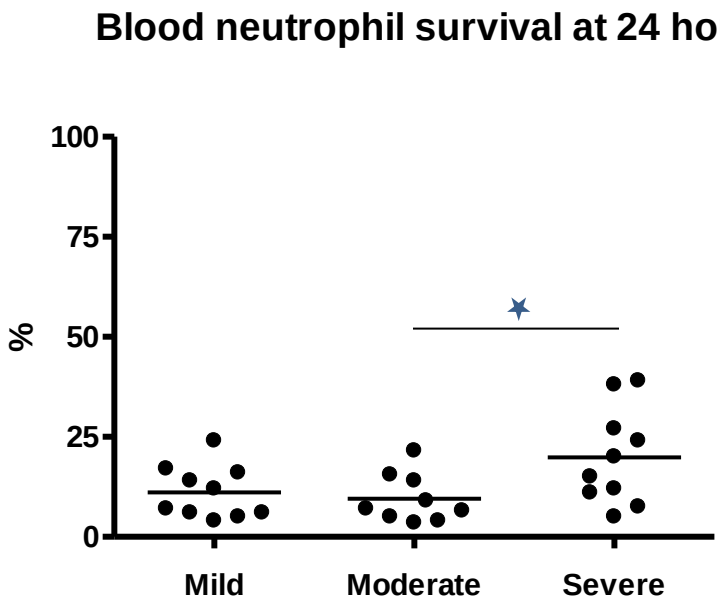

Figure 11. Blood neutrophil survival at 24 hours in asthma subjects. There was significantly increased survival in severe asthma group as compared to moderate asthma group. Results are expressed the percentage of surviving (viable) cells. Horizontal bars represent the mean values.

3.7. Hydrogen Peroxide measurement by Dihydrorhodamine (DHR) Assay

Activation of neutrophils in blood (Figure 12) and sputum (Figure13) was examined by measuring the production of hydrogen peroxide during the oxidative burst. The method used was the Dihydrorhodamine (DHR) assay (van Pelt L.J et al 1996). DHR, a non-fluorescent compound converts to fluorescent compound rhodamine 123 in the presence of hydrogen peroxide. This fluorescence is measured by flow cytometry. Neutrophils were examined under controlled (without adding DHR), unstimulated (DHR added) and stimulated (DHR added along with stimulation with fMLP) states. Results are expressed as mean fluorescence intensity (MFI).

In case of blood neutrophils, there is no difference in activation of neutrophils amongst the three groups of asthma subjects under controlled, unstimulated and stimulated states. Similarly, sputum neutrophils show no difference in the level of activity amongst the three groups under controlled, unstimulated and stimulated states. Blood neutrophils show an enhanced response to stimulation with fMLP as they are at a low level of activation otherwise.

In contrast, sputum neutrophils cannot be further activated as they are already in an activated state even before stimulation with fMLP. The results are also shown as dot plots and histograms (Figure 14)

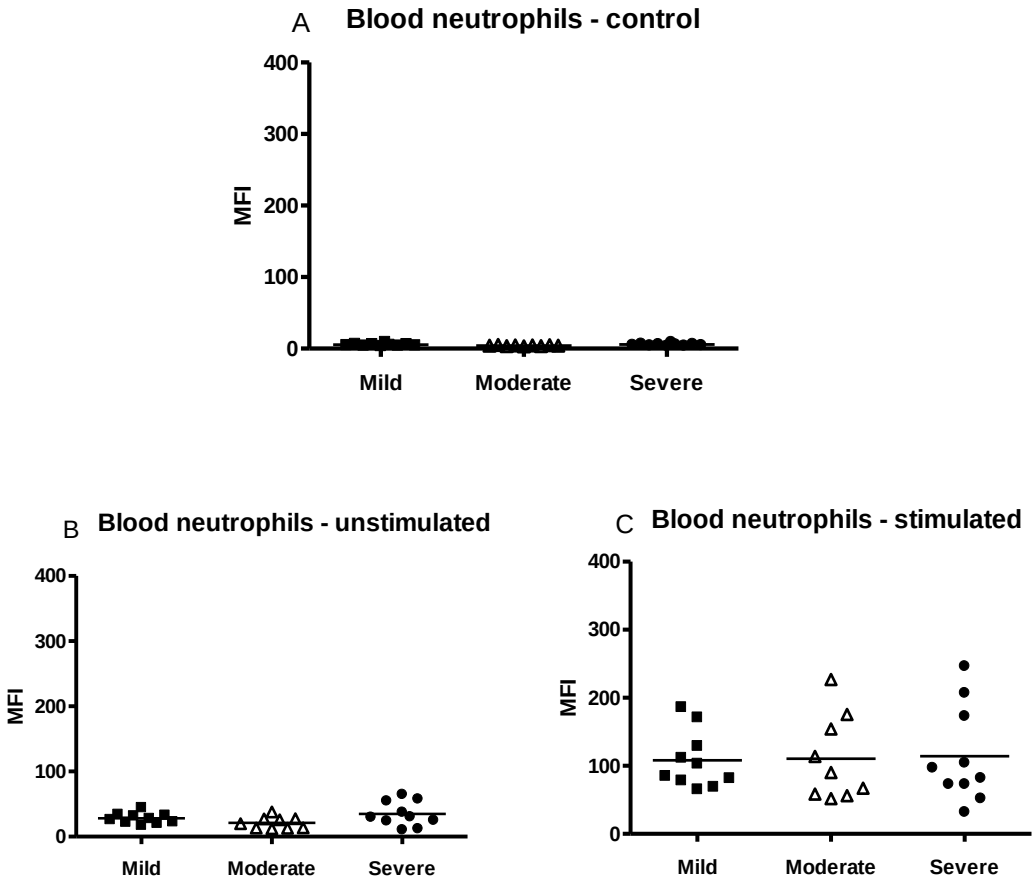

Figure 12. Blood neutrophil activation – Dihydrorhodamine assay. Neutrophils were examined for the production of hydrogen peroxide during the oxidative burst. Cells were tested under controlled (A), unstimulated (B) and stimulated states (C). There is no difference in the level of activation of blood neutrophil activation amongst the three groups. Blood neutrophils show an enhanced response to stimulation with fMLP, which suggests that circulating blood neutrophils are not in an activated state. Results are expressed as mean fluorescence intensity (MFI). Horizontal bars represent the mean values.

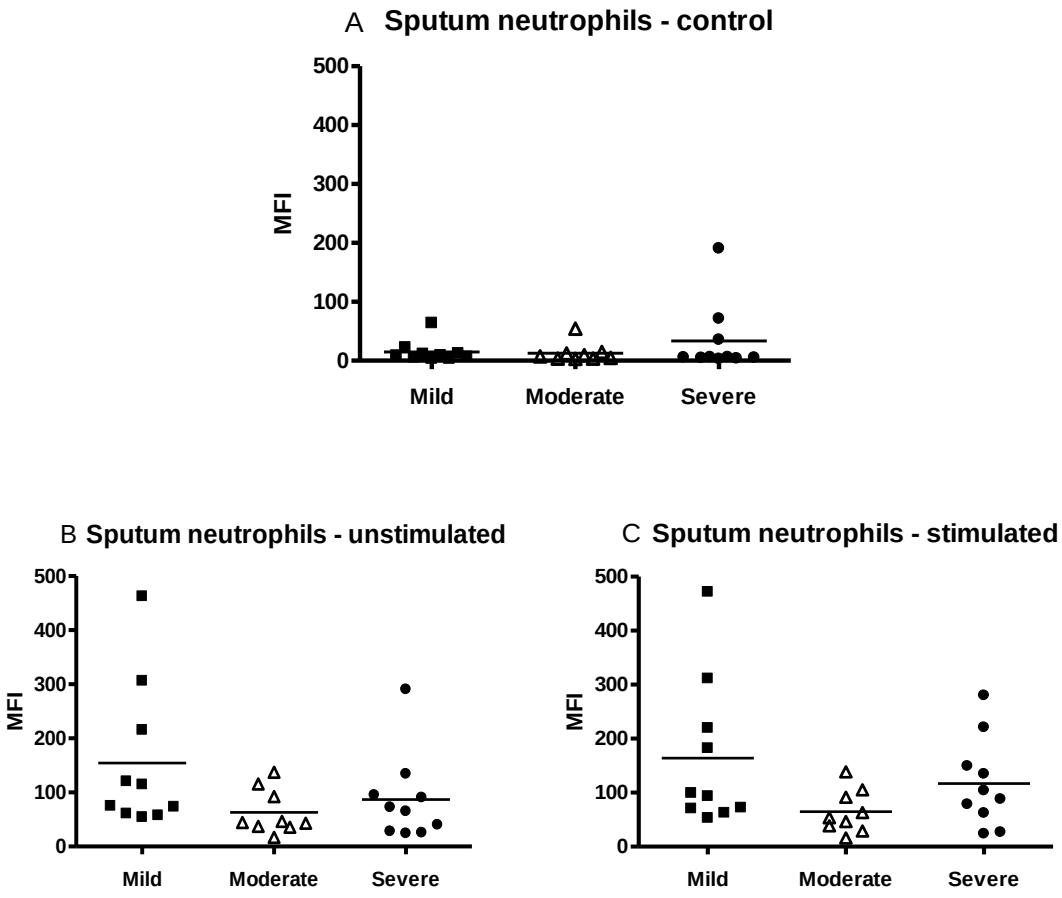

Figure 13. Sputum neutrophil activation by Dihydrorhodamine assay. Neutrophils were examined for the production of hydrogen peroxide during the oxidative burst under controlled (A), unstimulated (B) and stimulated states (C). There is no difference in the level of activation of sputum neutrophils amongst the three groups. Sputum neutrophils show an increased level of stimulation under unstimulated in addition to stimulated states. This suggests that sputum neutrophils are already activated and cannot be further activated with fMLP. Results are expressed as mean fluorescence intensity (MFI). Horizontal bars represent the mean values.

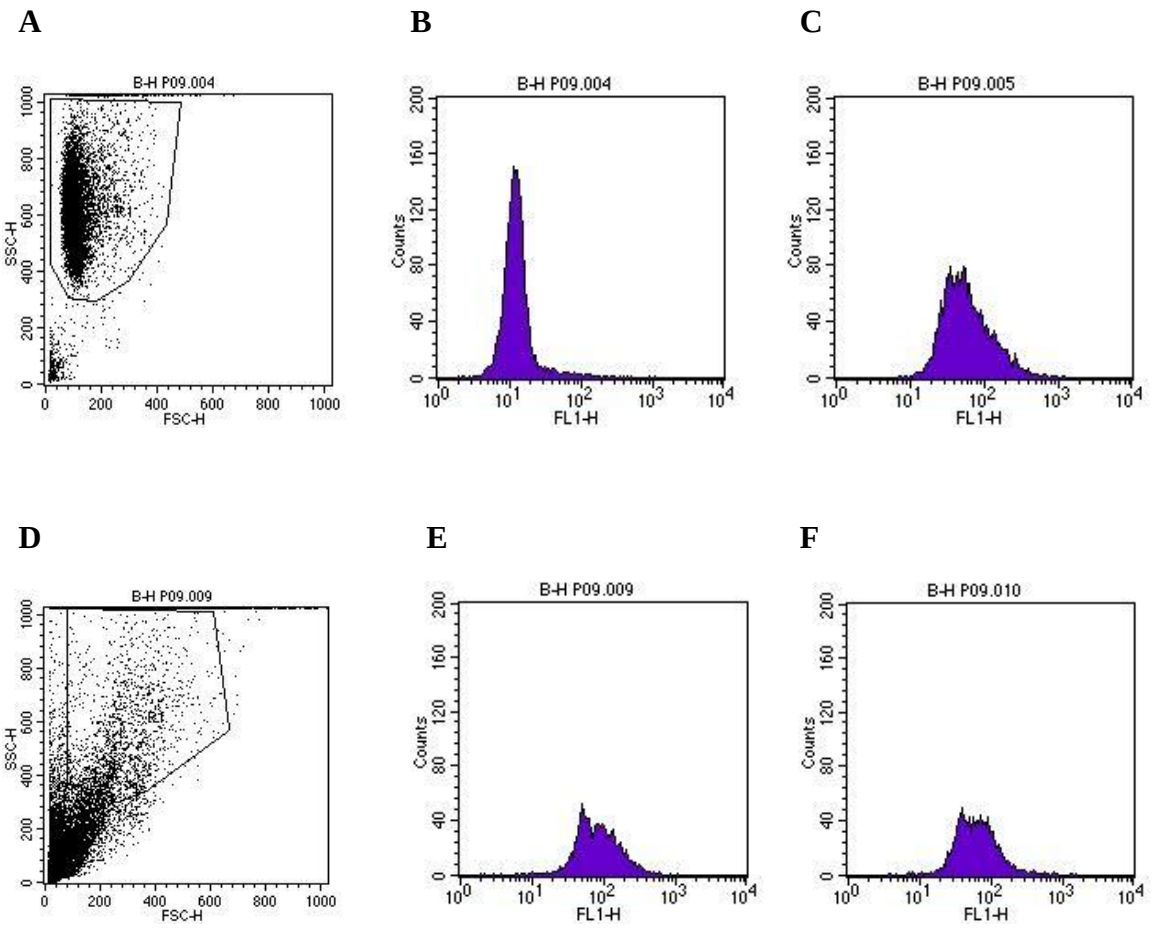

Figure14. Measurement of hydrogen peroxide in neutrophils in blood (A, B and C) and sputum (D, E and F) by Dihydrorhodamine assay. Production of hydrogen peroxide was measured at the baseline (B & E) and in the presence of fMLP (C & F). Neutrophils were analysed by flow cytometry. Cells were gated (A & D) using their density and size in forward-angle light scatter (FSC) and side-angle light scatter (SSC). The y axis on histograms represents neutrophil counts and x axis represents fluorescence from rhodamine 123 representing hydrogen peroxide production. Blood neutrophils show an increase in hydrogen peroxide production upon stimulation (B & C) in contrast to sputum neutrophils which cannot be further activated (E & F)

3.8. Granule Protein Expression measurement – CD 63 and CD 66b

Expression of primary and secondary granule proteins on the surface of blood and sputum neutrophils was also examined under controlled (isotype), unstimulated and stimulated states. These granule proteins are CD63 (primary granules) and CD66b (secondary granules).

In case of blood neutrophils, the expression of primary (figure 15) and secondary (figure 18) granule proteins is similar amongst the three groups. A similar picture is seen in case of sputum neutrophils (figure 16 &19) as they show no difference in the level of activity amongst the three groups. Blood neutrophils show an enhanced expression of granule proteins when stimulated with fMLP as circulating neutrophils are not in an activated state. On the other hand, sputum neutrophils cannot be activated any further as they are already in an activated state even before stimulation with fMLP. Results are also shown as dot plots and histograms (Figures 17 & 20)

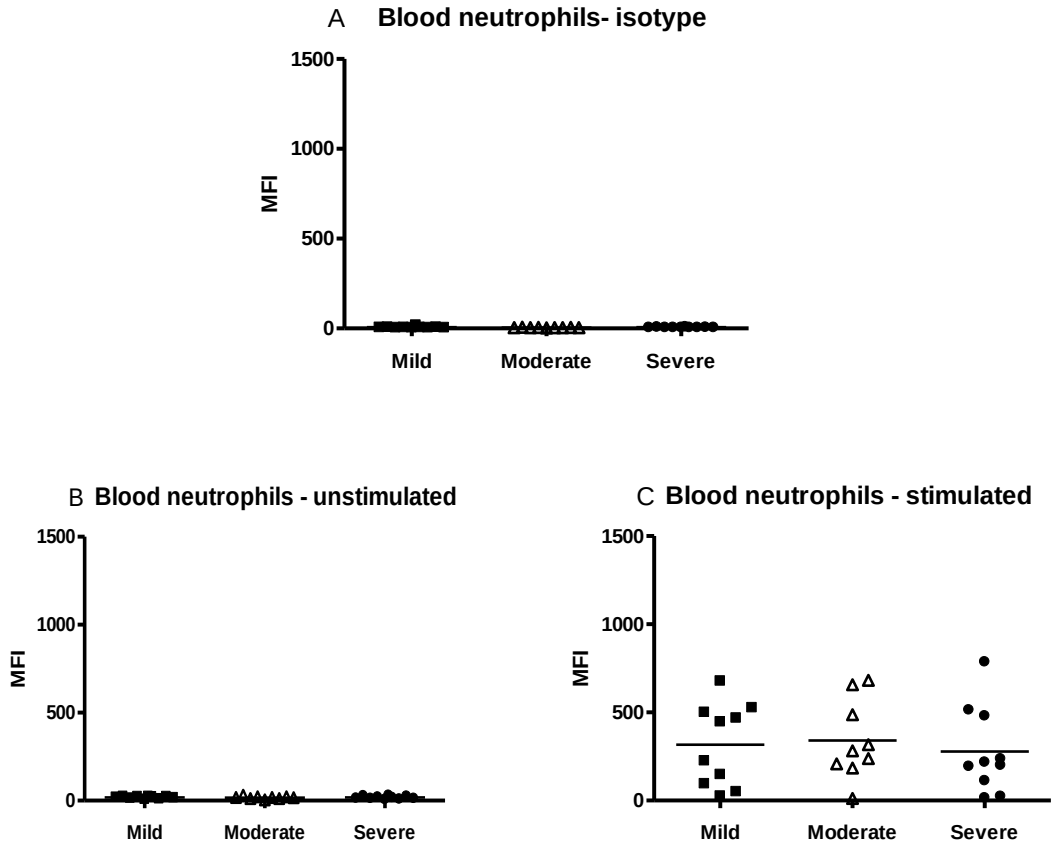

Figure 15. Expression of primary granule protein CD63 on the surface of blood neutrophils. Cells were tested under controlled- isotype (A), unstimulated (B) and stimulated states (C). There is no difference in the expression of CD63 on the surface of blood neutrophils amongst the three groups. Blood neutrophils show an enhanced response to stimulation with fMLP, which suggests that circulating blood neutrophils are not in an activated state. Results are expressed as mean fluorescence intensity (MFI). Horizontal bars represent the mean values.

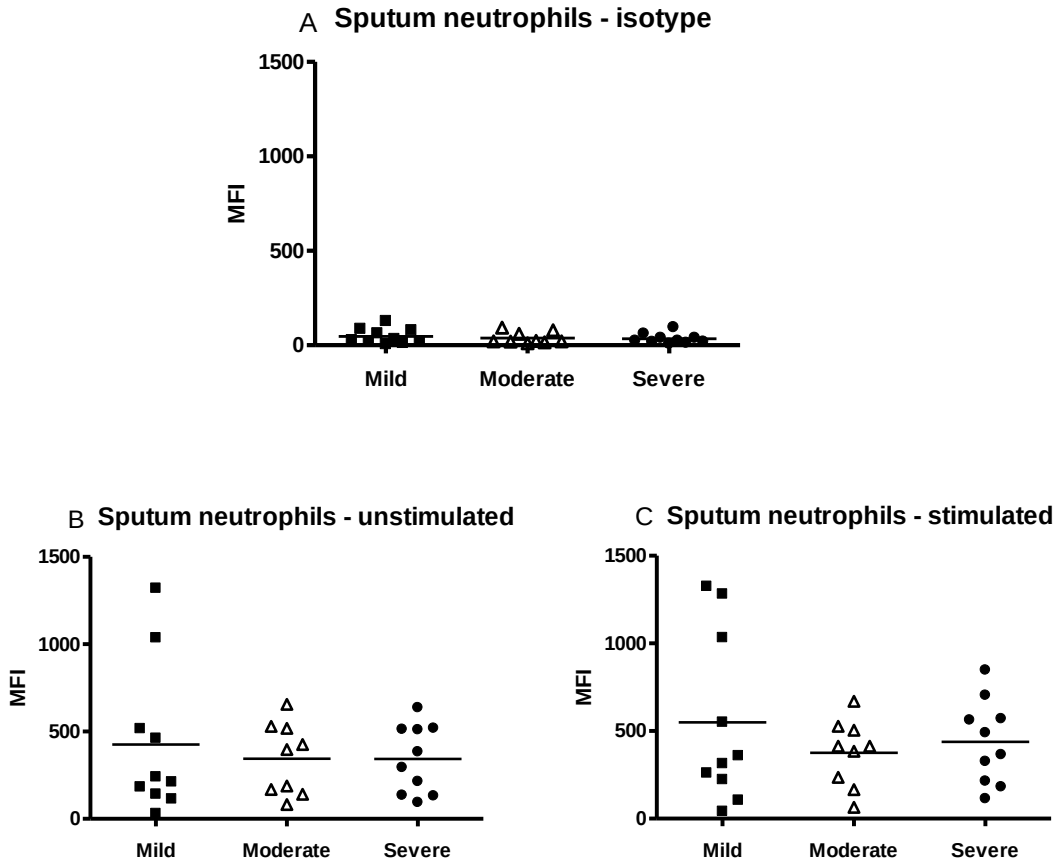

Figure 16. Expression of primary granule protein CD63 on the surface of sputum neutrophils. Cells were tested under controlled - isotype (A), unstimulated (B) and stimulated states (C). There is no difference in the expression of CD63 on sputum neutrophils amongst the three groups. Sputum neutrophils show an increased expression of primary granule protein under unstimulated in addition to stimulated states. This suggests that sputum neutrophils are already activated and cannot be further activated with fMLP. Results are expressed as mean fluorescence intensity (MFI). Horizontal bars represent the mean values.

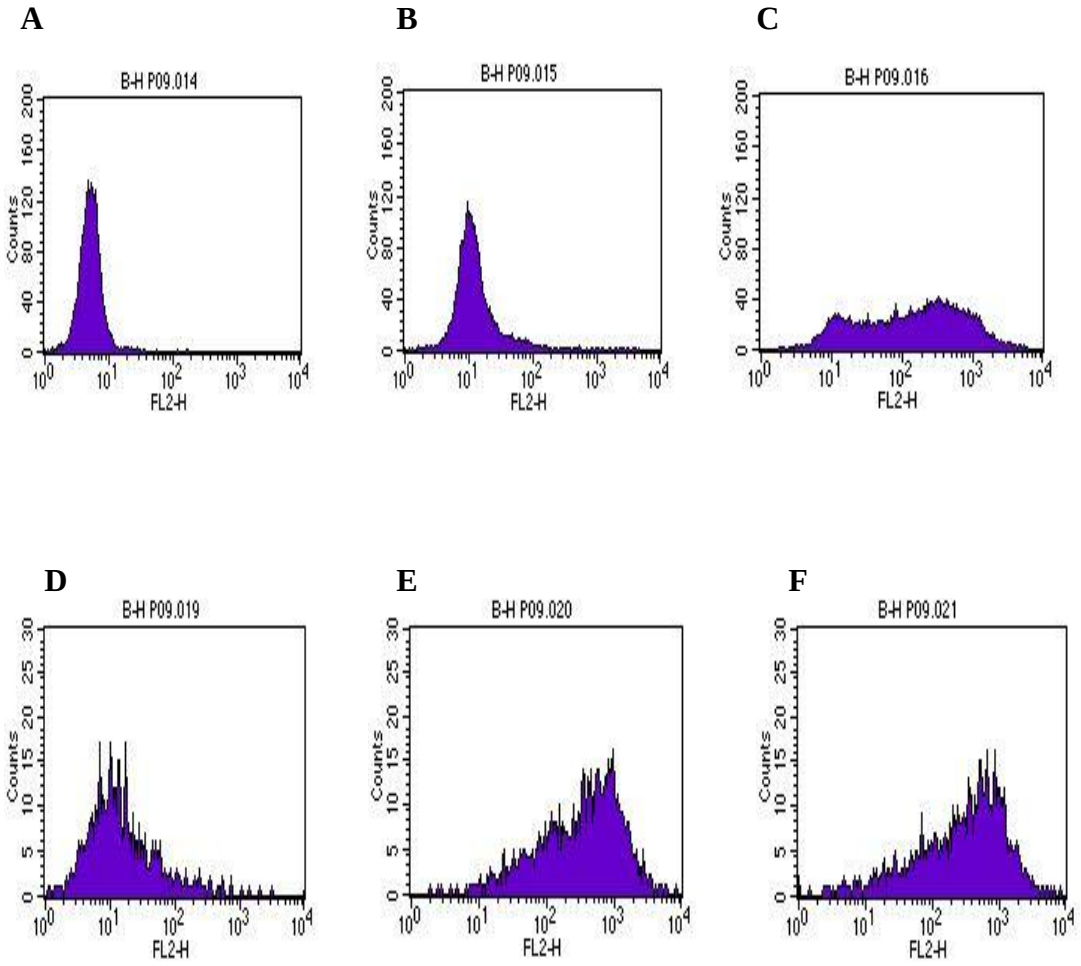

Figure 17. Expression of primary granule protein CD63 on blood (A, B & C) and sputum (D, E & F) neutrophils was examined by flow cytometry. Expression of CD63 was examined with isotype control antibody (A & D), with specific CD63 antibody in unstimulated (B & E) and stimulated states with fMLP (C & F). On the histograms, the y axis represents neutrophil counts and x axis represents fluorescence from labeled cells using FL2 detector. Blood neutrophils show a response to activation with fMLP (B & C) which is not observed in sputum neutrophils as they are already activated (E & F).

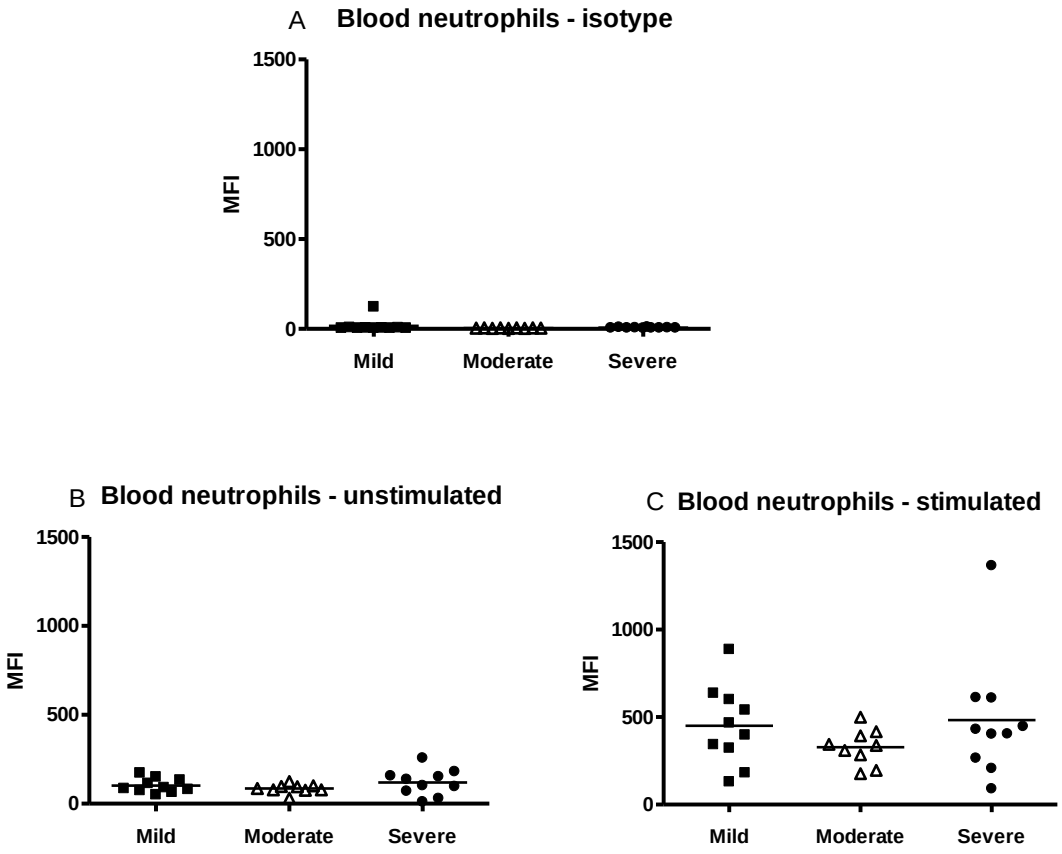

Figure 18. Expression of secondary granule protein CD66b on the surface of blood neutrophils. Cells were tested under controlled- isotype (A), unstimulated (B) and stimulated states (C). There is no difference in the expression of CD66b on the surface of blood neutrophils amongst the three groups. Blood neutrophils show an enhanced response to stimulation with fMLP, which suggests that circulating blood neutrophils are not in an activated state. Results are expressed as mean fluorescence intensity (MFI). Horizontal bars represent the mean values.

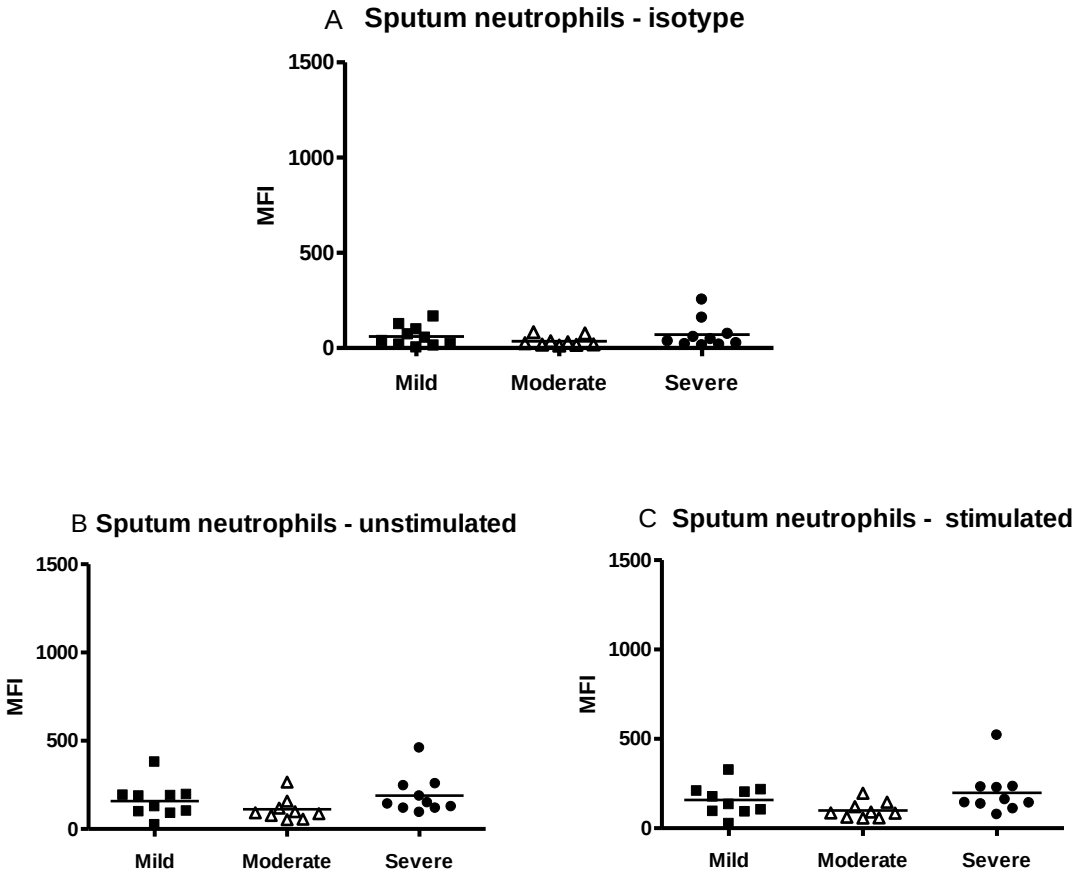

Figure 19. Expression of secondary granule protein CD66b on the surface of sputum neutrophils. Cells were tested under controlled - isotype (A), unstimulated (B) and stimulated states (C). There is no difference in the expression of CD66b on sputum neutrophils amongst the three groups. Sputum neutrophils show an increased expression of secondary granule protein under unstimulated in addition to stimulated states. This suggests that sputum neutrophils are already activated and cannot be further activated with fMLP. Results are expressed as mean fluorescence intensity (MFI). Horizontal bars represent the mean values.

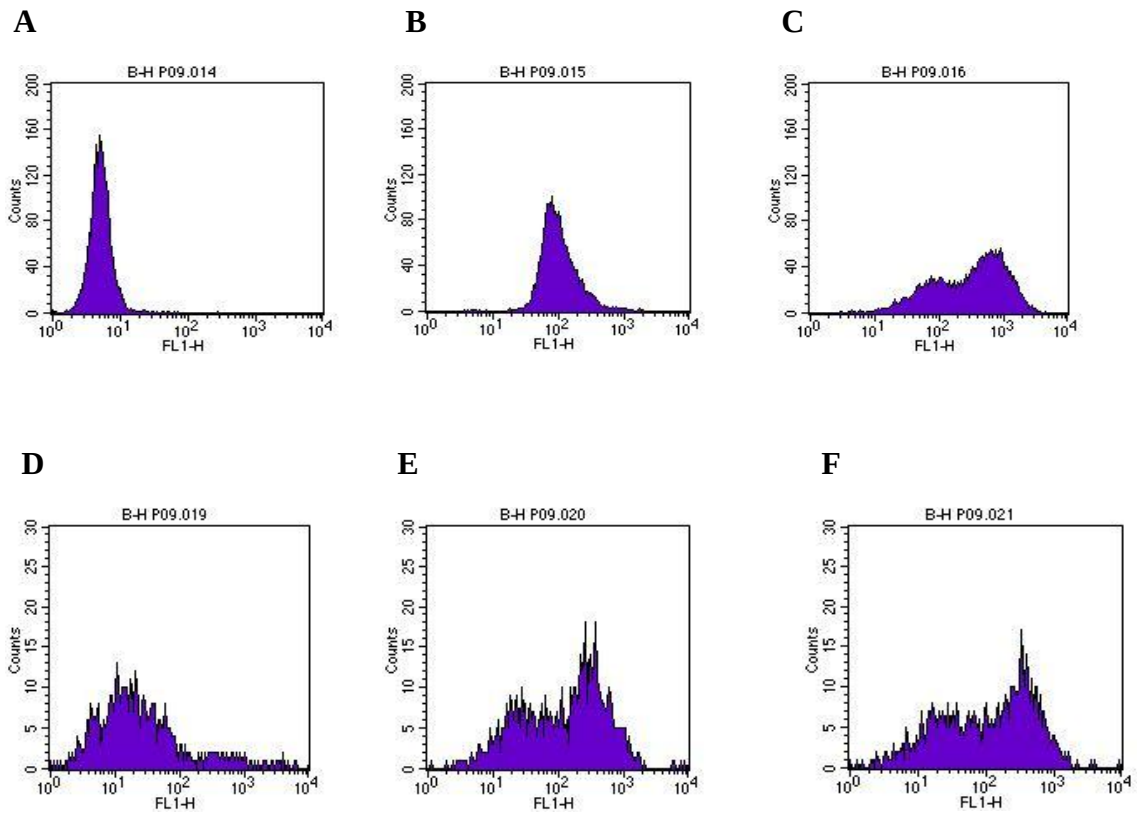

Figure 20. Expression of secondary granule protein CD66b on blood (A, B & C) and sputum (D, E & F) neutrophils was examined by flow cytometry. Expression of CD66b was examined with isotype control antibody (A & D), with specific CD66b antibody in unstimulated (B & E) and stimulated states with fMLP (C & F). On the histograms, the y axis represents neutrophil counts and x axis represents fluorescence from labeled cells using FL1 detector. Blood neutrophils show a response to activation with fMLP (B &C) which is not observed in sputum neutrophils as they are already activated. (E &F)

RESULTS OF IL-17 VALIDATION EXPERIMENTS IN SPUTUM SUPERNATANTS

Validation of IL-17 measurement in the sputum supernatants was performed before measuring IL-17 in sputum samples of subjects with severe asthma and normal control subjects. Spike and recovery experiments were performed using three different methods. Methods used were Enzyme-Linked Immunosorbent Assay (ELISA), Bio-Plex Cytokine Assay and Mesoscale Discovery Electrochemiluminescence Detection System.

3.9. ELISA

DTT treated supernatants were spiked at two concentrations to mimic a sputum supernatant concentration of 10pg/ml and1000pg/ml IL-17A. Levels of IL-17 were too low to be detected from sputum supernatant solutions (Table II).

Standard curves from standard solutions were obtained as given in the protocol (Figure 21). The observed concentration in standard solutions treated with DTT is 32.5% less than standard solution prepared without DTT (Table III).

Table II – Cytokine assays of DTT treated sputum samples

Immunoassay	Spike Level	Expected value (pg/ml)	Observed value (pg/ml)
ELISA	Low	10	ND
	High	1000	ND
Bio-Plex	Low	1185	1187
	High	2175	1268
MSD	High	1005	6.92

Low spike at 10 pg/ml
High spike at 1000 pg/ml
ND: not detected (IL-17 levels were too low to be measured accurately)

Table III – ELISA (Effect of DTT on Immunoassay)

Standard without DTT	Standard with DTT
31.3	20.3
62.5	42.8
125	84.9
250	182.4
500	340.6
1000	683.9
2000	1239.9

All values are in picograms per milliliter
DTT: Dithiothreitol

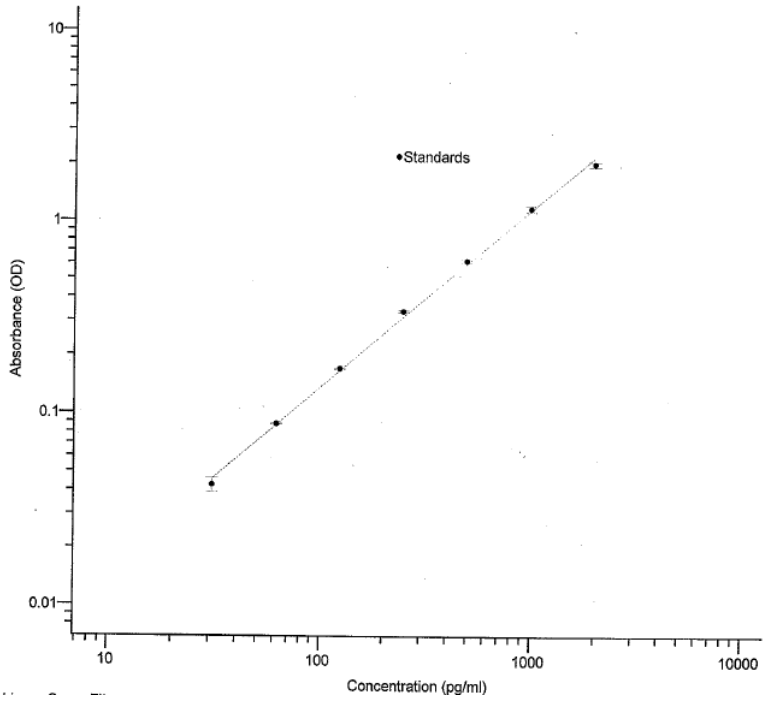

Figure 21. IL-17 ELISA standard curve. Concentrations of IL-17 in pg/ml have been plotted on the x axis against optical densities (OD) on the y axis.

3.10. Bio-Plex Cytokine Assay

In the first experiment, DTT treated sputum supernatants were spiked at two concentrations to mimic a sputum supernatant concentration of 10pg/ml and1000pg/ml IL-17A. Standard curve from standard solution from the kit were obtained as according to the protocol. Although, recovery from low spiked samples was as good as the expected concentration (expected value was 1185 pg/ml and observed value was 1187.6 pg/ml), recovery was low from high spiked samples (expected value was 2175 pg/ml and observed value was 1268 pg/ml) (Table II)

Also, linearity-of-dilution experiments were performed in high spiked sputum samples. Dilutions used were 1:2 and 1:5. The observed concentration increased as dilution increased. The expected concentration after spiking was 2175 pg/ml. The recovery in case of 1:2 dilutions was 102.5% as the observed concentration was 2231.2 pg/ml. Recovery with 1:5 dilutions was 267.7% as the observed concentration was 5823.9 pg/ml , exceeding the expected concentration (Table IV)

In the second experiment, two types of samples were analysed, DTT and non-DTT treated. In the DTT treated samples, IL-17 levels were too low to be detected with or without anti-proteases. IL-17 was not measured in this experiment in DTT treated samples without anti-proteases, although IL-17 was measured in similar samples in the first experiment mentioned above. Also, in non-DTT treated samples, IL-17 levels were too low to be detected with or without anti-proteases, spiked or unspiked (Table V).

In this second experiment, standard solutions were examined with and without DTT. Standard curves were obtained according to the protocol (Figure 22). In DTT treated solutions, the observed concentration is 54% less than non DTT treated standard solutions (Table VI)

Table IV- Linearity-of-dilution experiments (Bio-Plex cytokine assay)

Samples	Observed conc. (pg/ml)	Expected conc. (pg/ml)	Recovery (%)
Neat	1175		
High spike	1268.2	2175	58
Dilution 1:2	2231.2		102.5
Dilution 1:5	5823.9		267.7

Neat: Observed concentration of IL-17 before spiking

Table V – Bio-Plex cytokine assay

Samples	% Recovery (without anti-proteases)	% Recovery (with anti-proteases)
With DTT	ND	ND
Without DTT	ND	ND
Without DTT (High spiked)	ND	ND

DTT: Dithiothreitol
ND: not detected (IL-17 levels were too low to be measured accurately)

Table VI - Bio-Plex Cytokine assay (Effect of DTT on Immunoassay)

Standard without DTT	Standard with DTT
22388.5	15371.8
6073	2057
1400	598
366.3	150.5
92.8	37.3
21.9	11.8
5.73	4.4
1.42	0

All values are in picograms per milliliter
DTT: Dithiothreitol

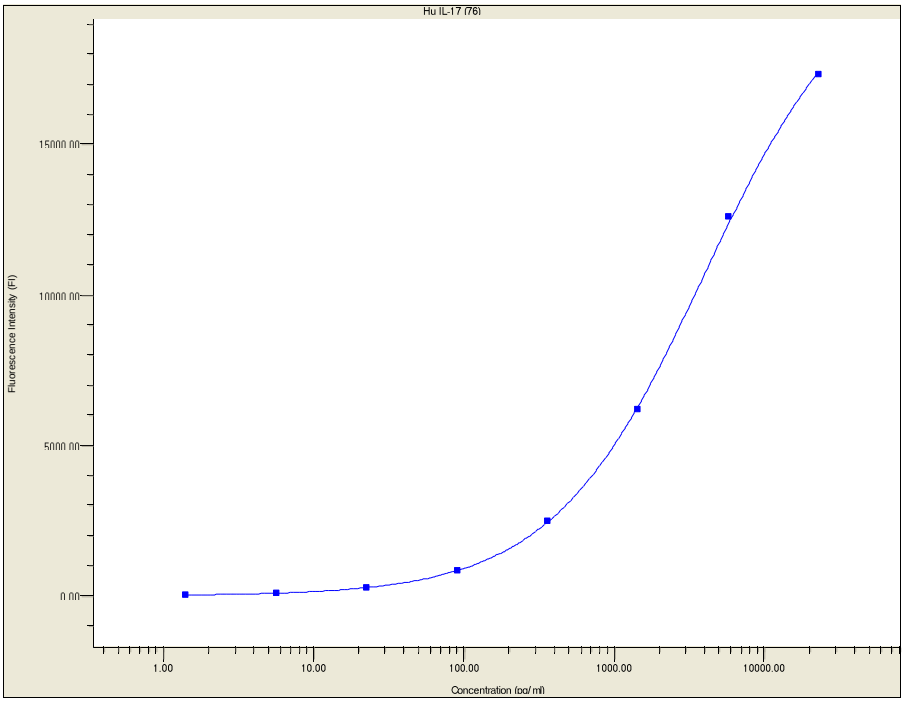

Figure 22. IL-17 Bio-Plex standard curve. Concentrations of IL-17 in pg/ml have been plotted on the x axis against fluorescence intensities on the y axis.

3.11. Mesoscale Discovery Electrochemiluminescence Detection System (MSD)

Sputum supernatant samples were spiked to a final concentration of 1000pg/ml. Standard curve was obtained from the standard solution (Figure 23). Recovery from spiked samples was only 0.7% (Table II)

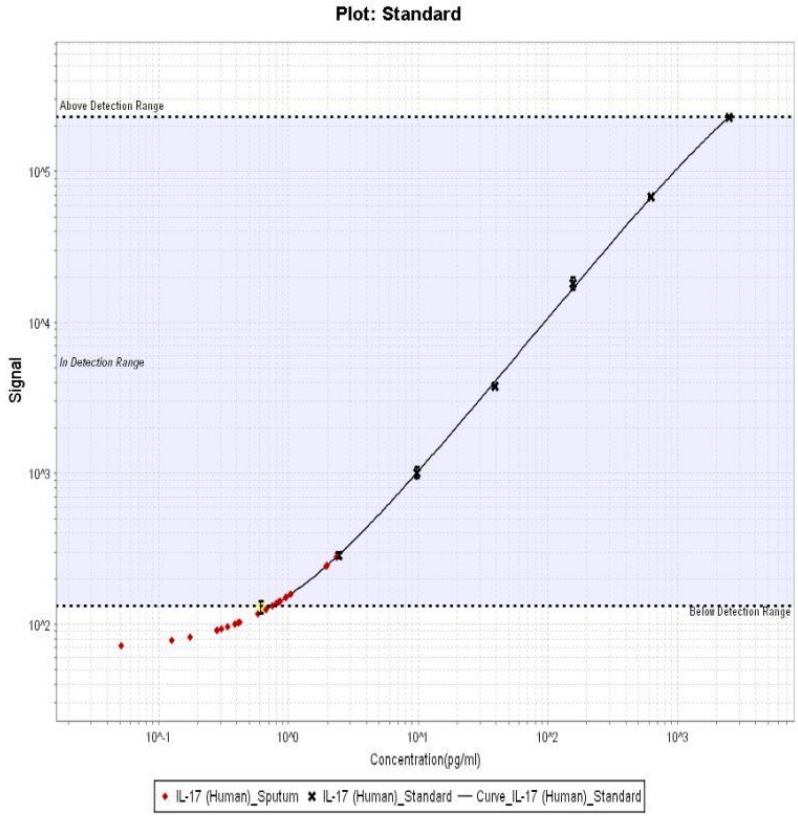

Figure 23. IL-17 MSD standard curve. Concentrations of IL-17 in pg/ml have been plotted against the electrochemiluminescence signal on the y axis.

RESULTS OF NEUTROPHILS MIGRATION STUDY

3.12. Neutrophil Migration towards IL-17

The chemotactic activity of IL-17 on neutrophils has not been studied, therefore migration of neutrophils towards IL-17 was examined in this study. Neutrophil migration was examined in two groups of study subjects – normal control group (non-asthmatic and non-atopic) and prednisone dependent asthma group. Ten subjects were in each group. Blood was drawn from these subjects and neutrophils were isolated by Percoll discontinuous gradient method. Isolated neutrophils were exposed to various concentrations of IL-17. These were 0.05, 0.1, 1, 10 and 100 ng/ml. Neutrophil migration or chemotaxis was also examined at the baseline (no chemotactic agent added) and with a positive control (fMLP) in a disposable 96-well chemotaxis chamber. Calcein AM was used to label neutrophils. Calcein AM is a non-fluorescent compound which is hydrolysed by intracellular esterases into fluorescent Calcein. This fluorescence is measured by a fluorescent plate reader. Results are expressed as the percentage of migrating neutrophils (Figure 20).

There is no difference in chemotaxis or migration of neutrophils towards various concentrations of IL-17 and baseline migration, in both the study groups. This suggests that IL-17 does not cause direct chemotaxis of neutrophils.

In order to confirm that the IL-17 that we used was biologically active, we performed an activity assay and demonstrated that IL-17 could stimulate the synthesis of IL-8 by cultured human airway smooth muscle cells at concentrations of 10ng/ml and 100 ng/ml and is therefore biologically active (Figure 25). The method used has been described previously (Wuyts *et.al*.2005).

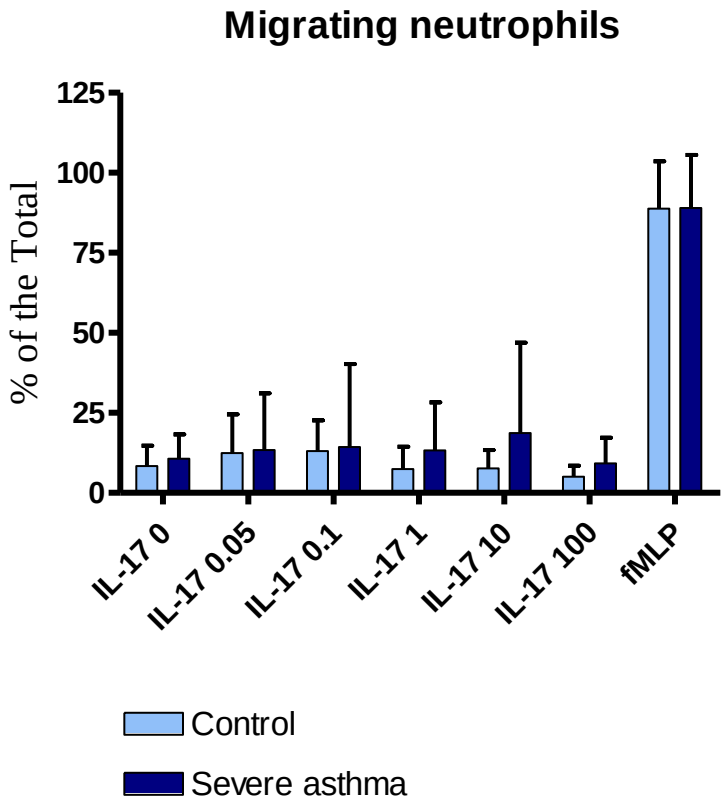

Figure 24. Neutrophil chemotaxis towards IL-17. Neutrophils were exposed to various concentrations of IL-17 – 0.05, 0.1, 1, 10 and 100 ng/ml. Migration was also examined at the baseline and in the presence of fMLP as a positive control. There is no difference in chemotaxis or migration of neutrophils towards various concentrations of IL-17 and baseline migration, in both the study groups. The results show that IL-17 does not cause direct chemotaxis of neutrophils. Results are expressed as the percentage of migrating neutrophils. Lighter bars represent the normal control group and darker bars represent the severe asthma group.

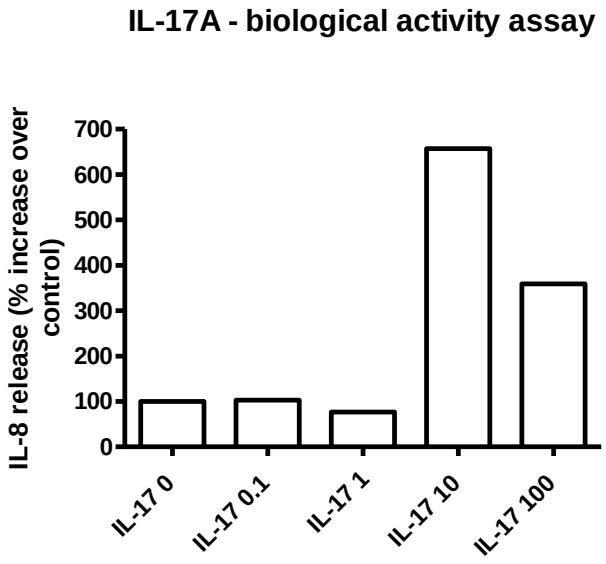

Figure 25. Biological activity of IL-17A. Human airway smooth muscle cells (HASMC) were stimulated with various concentrations of IL-17A to induce the production of IL-8. IL-8 was measured by ELISA in HASMC culture supernatants. Concentrations of IL-17A are expressed as ng/ml on x-axis. The y-axis represents IL-8 released as a percentage increase over control. Increased release of IL-8 is observed at 10 ng/ml and 100ng/ml of IL-17.

CHAPTER 4

DISCUSSION

4.1. Summary and main results

In this study the role of neutrophils in severe asthma was assessed in stable asthma subjects with varying asthma severity. We have examined the numbers, viability and activation of neutrophils in blood and sputum samples of asthma subjects after controlling their airway inflammation. We have also tested migration of neutrophils towards IL-17 in two groups of subjects. These were non-asthmatics and severe asthma subjects. Calcein AM, a fluorescent dye, was used to label neutrophils. A 96 well disposable chemotaxis chamber was used for this method. We also attempted to validate measurement of IL-17 in sputum supernatants.

In neutrophil activation study, asthma subjects were divided into three groups according to asthma severity. These were mild asthma subjects taking ≤ 500 µg/day of fluticasone or equivalent, moderate asthma subjects taking > 500 µg/day of fluticasone or equivalent and severe asthma subjects taking daily oral steroids. Ten mild, nine moderate and ten severe asthma subjects participated in the study. All the subjects were free from an asthma exacerbation and had no cold or chest infections in the last four weeks. Their asthma control questionnaire score was < 2. Blood samples were drawn and sputum was induced in all the subjects.

Neutrophil numbers were estimated in blood through complete blood count report with differential (CBC and differential). Neutrophil numbers were obtained in the sputum through sputum analysis. Neutrophil viability was examined through annexin stain method. Neutrophils were analysed by flow cytometry. The results were expressed as percentage of viable cells in blood and sputum. These cells were non-apoptotic and non-

necrotic. Neutrophil viability was also examined at 24 hours by fluorescent light microscopy. Neutrophil activation was examined by two methods. First the production of hydrogen peroxide during the oxidative burst within the neutrophils. This was measured by Dihydrorhodamine assay. The second method used was to measure the expression of primary (CD63) and secondary (CD66b) granule proteins on the surface of neutrophils. Neutrophils were tested with and without stimulation. Neutrophils were analysed by flow cytometry in both the methods.

The results of neutrophil activation study show that neutrophils are increased in blood in severe asthma subjects as compared to mild and moderate asthma subjects when expressed as numbers. Although sputum neutrophil counts were increased in severe asthma subjects, significant differences were not observed amongst the three groups of asthma subjects.

Neutrophil viability in blood and sputum also showed no difference amongst the three groups of study subjects. However percentage of viable neutrophils was more in blood than in sputum. On the other hand, blood neutrophil survival at 24 hours was significantly more in severe asthma subjects as compared to moderate asthma subjects.

With regards to neutrophil activation, no significant differences were observed amongst the three groups of asthma subjects, whether it was the production of hydrogen peroxide or expression of primary and secondary granule expression on neutrophils. In both the experiments, blood neutrophils were found to be not activated when tested in unstimulated state and showed a substantial response to activation. This was in contrast to sputum neutrophils which could not be further activated upon stimulation. This suggests that sputum neutrophils are already in an activated state in the airways.

In neutrophil migration study, migration of blood neutrophils was similar at baseline and when exposed to various concentration of IL-17. This suggests that IL-17 is

not directly chemotactic for neutrophils. Also, measurement of IL-17 in sputum supernatants could not be validated as IL-17 levels in the sputum were below the detectable limits of three independent assay systems (ELISA, Bio-Plex and Mesoscale)

4.2. Neutrophil counts in Blood and Sputum

Blood neutrophil counts are expressed as percentage (%) and numbers ($x10^9$/L). Neutrophil counts, when expressed as absolute numbers ($x10^9$/ml) were significantly increased is severe asthma group than mild and moderate asthma groups. The severe asthma subjects were on daily dose of oral steroids in addition to taking daily inhaled corticosteroids.

Corticosteroids have been implicated in causing blood neutrophilia. These drugs increase neutrophil viability, increase egress of neutrophils from the bone marrow and decrease adhesion of neutrophils to the endothelium (Cox G, 1994, Parillo and Fauci, 1979, Crockard *et al.* 1998). Blood neutrophilia observed in severe asthma patients could be due to the effect of steroids on blood neutrophils.

In contrast to our observation in blood, neutrophils in the sputum were not significantly increased in severe asthma subjects as compared to the other two groups, although increased counts were observed in severe asthma group. Results are expressed as percentage (%) and numbers ($x10^6$/ml)

These results are in contrast to previous studies (Ordonez *et al*, 2000, Uddin *et al*, 2010) which have shown significantly increased numbers of neutrophils in the airways of patients with severe asthma. One possible explanation for this difference in the results is that airway neutrophilia observed in these studies might have been due to high dose intravenous or oral steroid therapy given to these patients as these were suffering from symptoms of an asthma exacerbation or having severe persistent symptoms of

asthma. The other explanation would be perhaps subjects in previous studies were suffering from an airway infection which resulted in increased recruitment of neutrophils into the airways. Airway infection was not excluded in these studies. In previous studies airway inflammation was not controlled in contrast to the present study. In our study, only clinically stable asthma subjects on optimum doses of steroids were included. These subjects were not suffering from an asthma exacerbation or cold symptoms. Our study shows that in clinically stable severe asthma patients, sputum neutrophil counts are similar to those of less severe asthma.

4.3. Eosinophil counts in Sputum

Sputum eosinophil counts are expressed as percentage (%) and numbers ($x10^6$/ml). No significant differences were observed amongst the three groups of asthma subjects. In our study, asthma subjects on optimum doses of inhaled or oral steroids were included. This was done to include only those asthma subjects whose airway eosinophilic inflammation was under control. Our target was <3% eosinophils in the sputum. Sputum eosinophil percentages achieved were 2.6% in mild asthma subjects and 4.1% in both moderate and severe asthma subjects. These percentages are very close to our target percentage. Testing neutrophils in asthma subjects on optimum doses of steroids provides a better picture with regards to numbers, viability and activity of neutrophils as the results are not masked by increased eosinophils in blood and sputum samples.

4.4. Macrophage counts in Sputum

Results are expressed as percentage (%) and numbers ($x10^6$/ml). No significant differences were observed amongst the three groups of asthma subjects. However, macrophage counts were increased in mild asthmatic subjects as compared to moderate and severe asthmatic subjects. Although steroids have been shown to decrease the airway macrophages in addition to eosinophils (John *et al*. 1998), this effect was not observed in our study.

4.5. Neutrophil Viability in Blood and Sputum

Neutrophil viability was measured in blood and sputum samples of asthma subjects. The method used was annexin V method. Neutrophils were isolated from blood only. Viable neutrophils did not stain for either annexin or propidium iodide (PI). Early apoptotic cells stained for annexin only and late apoptotic and necrotic cells stained for annexin and PI. The viability of neutrophils was analysed by flow cytometry. The results show that neutrophil viability is less in sputum than in blood. The results also show that there is no difference in neutrophil viability amongst the three groups of asthma subjects in blood and sputum. High doses of steroids or an airway infection could account for increased neutrophil viability or decreased apoptosis observed in previous studies.

4.6. Neutrophil Survival at 24 hours

Neutrophils were isolated from blood samples of asthma subjects. These were kept in the incubator for 24 hours in 2% FCS+RPMI. Neutrophils were tested for their 24 hour survival by examining morphological changes of apoptosis. Cells were stained with acridine orange stain and were examined by fluorescent light microscopy. Both normal and apoptotic neutrophils were counted. Neutrophil survival was expressed as the percentage of non-apoptotic viable cells. Apoptotic cells were identified by nuclear morphology, specifically the appearance of rounded lobes of the nucleus.

Results were expressed as percentage of viable cells. The 24 hour blood neutrophil survival was significantly more in severe asthma subjects as compared to moderate asthma subjects. Similar results have been reported previously (Cox G, 1994). The study demonstrated that steroids have a significant increased neutrophil viability at 24 hours. In the presence of significant difference with moderate asthma subjects, similar results were expected when compared to mild asthma subjects but were not observed in this study.

In addition to testing blood neutrophils for their survival at 24 hours, sputum neutrophils were also tested in the initial phase of the study for their survival. Since there were no surviving cells at 24 hours, the testing of sputum neutrophils was discontinued.

4.7. Hydrogen Peroxide measurement by Dihydrorhodamine (DHR) Assay

Neutrophils in blood and sputum samples from asthma subjects were tested for the production of hydrogen peroxide by Dihydrorhodamine assay. Hydrogen peroxide is one of the reactive oxygen species produced during an oxidative burst in neutrophils. Production of hydrogen peroxide represents neutrophil activation. Dihydrorhodamine is a non-fluorescent compound which converts to rhodamine 123 in the presence of hydrogen peroxide. Rhodamine 123 is fluorescent and resulting fluorescence can be analysed by flow cytometry. The study demonstrates no difference in the production of hydrogen peroxide in blood and sputum neutrophils amongst the three groups of asthma subjects. No differences was observed when neutrophils were tested with and without stimulation with fMLP.

The results also show that blood neutrophils are not activated and show a greater response to activation by fMLP as compared to sputum neutrophils which are already activated even in the absence of stimulation by fMLP.

The results are in agreement with a previous study that showed that glucocorticoids do not affect the production of superoxide by neutrophils (Cox G, 1994). Superoxide is one of the reactive oxygen species produced during an oxidative burst.

4.8. Granule Protein Expression measurement in Blood and Sputum – CD 63 and CD 66b

Expression of primary and secondary granules proteins on the surface of blood and sputum neutrophils was also tested. CD 63 is the primary granule protein and CD66b is the secondary granule protein expressed on the surface of neutrophils when these cells are activated. Spontaneous and fMLP stimulated expression of these granule proteins was measured in all the three groups of asthma subjects. Granule protein specific antibodies were conjugated with fluorescent probes. The resulting fluorescence was measured by flow cytometry. The results of the study show that there is no difference in the expression of primary and secondary granule proteins on the surface of blood and sputum neutrophils, either spontaneously or when stimulated with fMLP.

In a previous study, blood neutrophils were found to be activated in severe asthma patients as compared to less severe asthma and non-asthmatic controls (Mann BS and Chung KF, 2005) .The activation markers measured were CD35 and CD11b. CD 35 mediates neutrophil binding and phagocytosis of C3b-coated particles and CD 11b is involved in transendothelial migration of neutrophils. Current study measured different granule proteins and shows no difference in their expression amongst the asthma subjects, both in blood and sputum.

4.9. IL-17 Validation Experiments in Sputum Supernatant.

Three types of assays were used to validate IL-17 measurement in sputum supernatant. We could not validate measurement of IL-17 in these assays. Standard curves were obtained in all the assays used.

In ELISA, IL-17 levels could not be measured in DTT treated spiked sputum supernatants. This is contrast to a previous study in which IL-17 validation was demonstrated in DTT treated sputum supernatant samples using ELISA (Barczyk *et al*. 2003).

In Bio-Plex assays, in the first experiment, IL-17 was measured in unspiked DTT treated samples. These samples were spiked to mimic sputum concentrations of 10 pg/ml (low spike) and 1000 pg/ml (high spike). The recovery from low and high spiked samples was 100% and 58% respectively. Linearity-of-dilution experiments were also performed to assess the effects of dilution on IL-17 measurement. Although the linearity-of-dilution experiments showed that measurement of IL-17 increased as dilution of samples increased, the observed concentrations after 1:5 dilution exceeded the expected concentration. It was 267.7% of the expected concentration. This result cannot be explained and it puts the validity of the assay into question.

In the second set of experiments performed with Bio-Plex assay, DTT and non-DTT treated samples were analysed. In contrast to the first experiment with Bio-Plex, IL-17 could not be measured in unspiked DTT treated samples. This shows that we could not reproduce the results of the first experiment. If the first experiment with Bio-Plex is considered valid, then the validity of the second experiment with Bio-Plex is questionable

or vice versa. Also, IL-17 could not be measured in non-DTT treated (spiked and unspiked).

In MSD assay, the recovery was only 0.7% of the DTT treated samples.

The results from standard solutions from ELISA and Bio-Plex cytokine assays show that DTT is interfering with measurement of IL-17. In case of ELISA standard solutions, the observed concentration in DTT treated solutions is 32.5% less than non-DTT treated solutions. Similarly, in Bio-Plex, observed concentration in standard solutions treated with DTT is 54% less than non-DTT treated standard solutions.

In conclusion, we could not validate IL-17 measurement in sputum supernatants; whether DTT treated or non-DTT treated samples. In case of DTT treated samples, IL-17 was either too low to be measured as observed in ELISA or the recovery was very small as observed in MSD assay. Bio-Plex assay was also used to examine DTT treated samples. Although IL-17 was measured in spiked samples, the linearity-of-dilution experiments measured 267.7% more IL-17 than the expected concentration. This makes the validity of the assay questionable.

Non-DTT treated samples, analysed by Bio-Plex assay, IL-17 was too low to be detected. Also, unspiked DTT treated samples were also examined as part of this experiment but measurement of IL-17 could not be reproduced as in the previous experiment, making the validity of the assay questionable. However, the results do show that DTT is interfering with measurement of IL-17 in standard solutions as demonstrated in ELISA and Bio-Plex assays.

4.10. Neutrophil Migration towards IL-17

Neutrophils were isolated from blood samples of severe asthma subjects and non-asthmatic, non-atopic controls. Migration of neutrophils towards IL-17 was assessed using a 96-well chemotaxis chamber. Calcein, a fluorescent dye, was used to label the neutrophils. It is the hydrolysis product of Calcein AM. Neutrophils were exposed to various concentrations of IL-17A. The concentrations used were 0.05, 0.1, 1, 10 and 100 ng/ml. fMLP was used as a positive control in addition to baseline measurements. The chemotaxis chamber was then placed in a fluorescent plate reader and Calcein fluorescence was used to measure the cells that had migrated to the bottom chamber.

According to the results of the study, there was no difference in migration of neutrophils at the baseline and towards various concentrations of IL-17A in both groups of study subjects. This shows that IL-17 A is not directly chemotactic for neutrophils. These results are supported by a recent study (Pelletier M et al. 2010) that showed that neutrophils are not responsive to either IL-17A and IL-17F as they do not express the receptors for the action of these cytokines. The biologic activities of these two cytokines are dependent on their binding to a receptor complex composed of at least 2 IL-17RA subunits and 1 IL-17RC subunit. The study demonstrated that neutrophils do not express IL-17RC and therefore do not respond to either IL-17A or IL-17F. This was in contrast to human monocytes which were shown to express both IL17RA and IL-1RC.

4.11. Limitations of the Study and Technical Difficulties

4.11.1. Study subject selection

In the neutrophil activation study, we examined three groups of asthma subjects divided on the basis of asthma severity. These were mild, moderate and severe asthma groups divided on the basis of daily dose of inhaled and oral steroids. The limitation of this study is that normal non-asthmatic controls were not included into the study. This group of subjects has been included in previous studies (Mann BS and Chung KF 2006, Uddin *et al*. 2010). Perhaps, if this group was added, we could have shown differences in neutrophil numbers, viability and activation between asthma subjects and non-asthmatic control subjects.

4.11.2. Sputum neutrophils

In neutrophil activation study, neutrophils were examined in both blood and sputum samples. Neutrophils or granulocytes were isolated from blood. Isolated granulocytes included both neutrophils and eosinophils. Neutrophils were not isolated from sputum samples as this is technically difficult. Blood and sputum samples were analysed by flow cytometry. Granulocytes, which included both neutrophils and eosinophils, were gated. To increase the identification of neutrophils, a specific antibody for neutrphils,CD16, was used. This antibody was used when expression of granule proteins was examined and not during Dihydrorhodamine assay in which production of hydrogen peroxide was measured. Since eosinophils counts were within normal range in blood samples and eosinophil percentage was $\leq 4\%$ in sputum samples, we assumed that most of the fluorescent signal is coming from neutrophils.

4.11.3. Difficulties in recruiting study subjects

As strict criteria were followed to include only clinically stable asthma subjects, it made recruitment difficult. This was especially true in case of moderate and severe asthma subjects as they appeared to suffer from more frequent exacerbations or infections as compared to mild asthma subjects. Also, many stable asthma subjects were not able to produce a sputum sample.

4.12. Clinical Relevance

Neutrophils are an important part of innate immune response. Their primary function is phagocytosis of invading microbes which involves identifying, ingesting and killing of microbes (Abbas *et al*. 2007:29). Neutrophils produce reactive oxygen species and granule products to kill the microbes (Nordenfelt and Tapper 2011). These cytotoxic granule contents and ROS can cause host tissue damage if released into the surrounding tissues.

A number of studies have demonstrated an association between neutrophilic inflammatory response and severe asthma (Jatakanon *et al.* 1999, Fahy *et al.* 1995, Ordonez *et al*. 2000). Neutrophils along with their inflammatory mediators have been found to be increased in the airways of asthma patients. These studies suggest that neutrophils might play a role in the pathophysiology of severe asthma and are causing damage to the airways. We cannot conclude from the results of these studies that neutrophils are intrinsically activated in severe asthma subjects and causing airway damage. Increased neutrophilic response could have been due to an airway infection, high steroid dose and just an epiphenomenon of severe asthma.

In this study, we have carefully included those patients who were clinically stable and were on optimum doses of steroids. This would give a better idea of the activity of neutrophils.

The results of the study show that neutrophils are not intrinsically activated in severe asthma subjects when compared to mild and moderate asthma subjects. There is no difference in numbers, viability and activity of sputum neutrophils amongst the three asthma groups when the subjects are clinically stable.

In fact, increase in neutrophils and neutrophil elastase have been shown to facilitate asthma recovery following an asthma exacerbation.(Innes *et al*. 2009). The study showed increased neutrophils and neutrophil elastase in airway secretions of patients suffering from an asthma exacerbation. Neutrophil elastase helped in breaking down mucus plugs, restoring airway patency. Both the neutrophils and elastase were increased further in airway secretion during recovery phase.

Our study also shows that IL-17 is not directly chemotactic for neutrophils. It is possible that increased IL-17 in the airways in the presence of an infection is causing neutrophil migration towards lungs by inducing the release of neutrophil chemotactic factors from airway epithelium and endothelium.

It is possible that recurrent airway infections in asthma patients (Gern JE and Busse WW, 1999), lead to tissue damage and airway remodelling. The tissue damage depends on the balance between the beneficial and harmful effects of mediators released by inflammatory cells including neutrophils. It is also possible that high doses of steroids given to patients suffering from an asthma exacerbation results in increased number of neutrophils in the airways by increasing their viability. The increased number of neutrophils in the airways might result in increased levels of inflammatory mediators released these cells.

Although neutrophils play a beneficial role as part of the innate immune response, during this process of fighting microbial infections, they may cause damage to the surrounding tissues. As our study has demonstrated that airway neutrophils are neither increased in numbers nor intrinsically activated in stable asthma patients, contrasting results seen in previous studies might be due an airway infection or high doses of steroids.

CHAPTER 5

CONCLUSIONS

We have shown that in severe asthma subjects, blood neutrophils are increased as compared to mild and moderate asthma subjects. Additionally, blood neutrophils showed increased survival at 24 hours when compared to moderate asthma subjects. Both the increased number of blood neutrophils and their increased survival in severe asthma subjects could be due to an effect of steroids, as these subjects take oral steroids to control their eosinophilic airway inflammation.

The study results did not show significant differences in sputum neutrophil counts and viability amongst the three groups of asthma subjects. Also, no significant differences were observed in neutrophil activation, both in blood and sputum. These results are in contrast to previous studies (Ordonez *et al*. 2000, Mann BS and Chung KF, 2006, Uddin *et al. 2010*). The reason for this difference in observation might be the use of strict criteria to include only clinically stable patients in our study who were on optimal doses of steroids and were not suffering from an airway infection. As previously mentioned, high steroid doses and airway infections can both increase neutrophil numbers and viability. Presence of an airway infection can increase neutrophil activity. Previously, steroids have been shown to decrease neutrophil degranulation (Liu.L *et al.* 2005 and Smith and Iden 1980) but do not affect oxidative burst (Cox G, 1994). It is possible that oral steroids taken by severe asthma subjects only increased the blood neutrophil numbers and survival at 24 hours. The doses were not high enough to affect the sputum neutrophil numbers, viability and activity.

The results of the study show that airway neutrophils are neither increased in numbers, nor more viable or more activated amongst subjects with varying asthma severity when they are clinically stable.

The study also demonstrates that IL-17 is not directly chemotactic for neutrophils. This is in agreement with another study that has shown that neutrophils are not responsive to IL-17 as they lack one of the two receptors required for IL-17 activity (Pelletier M *et al*.2010). Neutrophils migrate towards airways in response to chemokines including IL-8. IL-17 can induce release of IL-8 from airway epithelium and endothelium and is therefore indirectly involved in neutrophil migration towards lungs (Traves and Donnelly 2008). As IL-17 has also been found to be increased in the sputum samples of asthma patients as compared to normal controls and COPD patients (Barczyk et al. 2003), we attempted to measure it in sputum supernatants in our study subjects. It was our expectation that IL-17 would be increased in sputum of severe asthma subjects. Validation experiments were performed as a first step. However, despite carefulmeasurements by 3 methods, we were not able to measure IL-17 reliably in DTT treated and non-DTT treated sputum.

Our observations have important clinical implications, particularly in relation to new drug discovery. Since the neutrophil number in patients with severe asthma were not significantly different from that of patients with milder asthma, this questions the rationale for targeting pathways of neutrophil recruitment and activity such as the IL-8/CXCR2 pathway, IL-17 and its receptors etc. Since most of the association between airway function in asthma and airway neutrophils have been when asthma was exacerbated, it is likely that there was an unrecognized infection that may have contributed to the recruitment of airway neutrophilia and as such we suggest that further efforts in understanding the relationship between airway neutrophilia and airway function may be directed to better understanding of the airway microbiome associated with asthma exacerbations.

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