NEUTROPHIL PHENOTYPE AND FUNCTION IN OCULAR INFLAMMATORY DISORDERS.

BY

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Abstract

Ocular inflammatory diseases are the result of the breakdown of protective ocular barriers. Behçet's disease (BD) and Ocular mucous membrane pemphigoid (OcMMP) are chronic inflammatory eye disorders, in which neutrophils have been implicated in their pathogenesis. In this thesis the phenotype and function of neutrophils from patients with these diseases was assessed.

Peripheral blood neutrophils were obtained from patients with BD, OcMMP and healthy controls. Percoll isolation was used to isolate all neutrophils, while Ficoll-Hypaque was used to obtain subpopulations. Phagocytic capacity and production of reactive oxygen species (ROS), neutrophil extracellular traps (NETs) and heterogeneity were assessed in all populations.

Neutrophils from both patient cohorts showed a reduced phagocytic capacity and ROS production, but greater NET production by total neutrophils compared to cells from healthy controls. Also, patients had elevated numbers of low density and lower number of normal density neutrophils, but showed a similar phenotype as total neutrophils. Granulocytic myeloid-suppressor cells were evident within the Low density neutrophil (LDN) and Normal density neutrophil (NDN) population of both patient cohorts when compared healthy controls.

The results suggest that a chronic inflammatory state reduces phagocytosis and ROS production but increases NET production in circulatory neutrophils. Therefore, Further analysis of neutrophil heterogeneity may explain these phenotypic and functional differences.

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List of publications ready to submit:

- Low density neutrophils are increased in patients with Behcet's Disease but do not explain differences in neutrophil function. Mariam Murad, Liying Low, Matthew Davidson, Philip I Murray, Saaeha Rauz and Graham R Wallace. *Immunology*
- Control of neutrophil extracellular trap formation by commercial ocular eye drops Matthew Davidson, Mariam Murad, Mohith Shamdas and Saaeha Rauz. *Ocular Surface*
- Peripheral neutrophil phenotype and function in Ocular Mucous Membrane Pemphigoid. Mariam Murad, Liying Low, Matthew Davidson, Philip I Murray, Graham R Wallace and Saaeha Rauz. *Ocular Surface*

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Abbreviations

AECA	Anti-endothelial cell antibodies
ALDH	Aldehyde dehydrogenase
ANCA	Anti-Neutrophilic Cytoplasmic Autoantibodies
APC	Antigen presenting cells
APC/CY7	Allophycocyanin: Cy-7 Tandem Conjugate
Arg1	Arginase 1
B cells	B lymphocyte
BAFF	B cell-activating factor
BD	Behçet's Disease
BDCAF	BEHÇET'S DISEASE CURRENT ACTIVITY FORM
BMEC	Birmingham Eye Centre
BMZ	Basement membrane zone
BP180	Bullous pemphigoid antigen 1
BV605	BD Horizon Brilliant™ Violet 605
BV711	BD Horizon™ Brilliant Violet™ 711
C3	Complement receptor 3
C5a	Complement component 5a
CC	Cicatrising conjunctivitis
CCL20	CC-chemokine ligand 20
cfDNA	Cell free deoxyribonucleic acid

CLTA-4	Cytotoxic T-lymphocyte antigen-4
CRP	C-reactive proteins
CR	Complement receptor
CXCR2	Chemokine receptor2
CXCR4	Chemokine receptor4
DC	Dendritic cells
DED	Dry eye disease
DHA	Docasahexonoic acid
DIF	Direct immunofluorescence
DNA	Deoxyribonucleic acid
DNase 1	Deoxyribonuclease 1
E. coli	Escherichia coli
EC	Endothelial cell
ECM	Extracellular matrix
ERAP1	Endoplasmic reticulum aminopeptidase
ESR	Serum neopterin and erythrocyte sedimentation rate
F	Female
FACS	Fluorescence-activated cell sorting
FCS	Heat-inactivated foetal calf serum
FcR	Fc receptor
FITC	Fluorescein Isothiocyanate Conjugate

FMLP	N-formylmethionyl-leucyl-phenylalanine
G-CSF	Granulocyte-colony stimulating factor
GITR	Glucocorticoid induced tumour receptor
GMCSF	Granulocyte-macrophage colony-stimulating factor
GMDSC	Granulocytic myeloid-derived suppressor cells
G-MDSCs	Granulocytic myeloid-derived suppressor cells
GMPs	Granulocyte–monocyte progenitors
GPI	Glycophosphatidylinositol
GPS	L-glutamine, penicillin, streptomycin solution
GWAS	Genome-wide association analysis
HIV	Human immunodeficiency viruses
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
HNA	Human neutrophil antigens
HOCL	Hypochlorous acid
HSC	Hematopoietic stem cells
HSV	Herpes simplex virus type 1
ICAM 1,2	Intercellular adhesion molecule 1,2
ICBD	International Criteria for Behçet's disease
IF	Immunofluorescence
IFNAR1	Interferon Alpha And Beta Receptor Subunit 1
IFNs	Interferon type I

IFN-α	Interferon-alpha
IFN-γ,	Interferon gamma
IgA	Immunoglobin A
IgD	Immunoglobin D
IgE	Immunoglobin E
IgG	Immunoglobin G
IgM	Immunoglobin M
IL13	Interleukin-13
IL17	Iinterleukin-17
IL-1RA	IL-1 receptor antagonist
IL21	Interleukin-21
IL22	Interleukin-22
IL23	Interleukin 23
IL4	Interleukin-4
IL5	Interleukin-5
IL8	Interleukin-8
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-5	Interleukin 5
ISG	International Study Group
JAM	Junctional adhesion molecule
KIR	killer-inhibitory receptors

LDG	Low density granulocytes
LDN	Low Density neutrophils
LFA-1	Lymphocyte function-associated antigen-1
L-GPS	L-Glutamine–Penicillin–Streptomycin
LMPP	Lymphoid-primed multipotent progenitors
LPS	Lipopolysaccharide
LXA4	Lipoxin A4
M	Male
MAC-1	Macrophage antigen-1
MADCAM-1	Mucosal vascular cell-adhesion molecule-1
MET	Mesenchymal–epithelial transition
MHC	Major histocompatibility complex
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide–related sequence A
Ml	Millilitre
MM	Multiple myeloma
MM9	Metalloproteinase-9
MP3	Membrane proteinase 3
MPO	Myeloperoxidase
MPP	Multipotent progenitor cells
MPV	Mean platelet volume

mRNA	Messenger ribonucleic acid
MTX	Methotrexate
Myd88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NBH	B-cell-helper neutrophils
NDN	Normal Density neutrophils
NET	Neutrophil extracellular traps
NK	Natural Killer
NLR	Neutrophil-to-lymphocyte
NOS	Nitrogen oxygen synthase
OcMMP	Ocular mucous membrane pemphigoid
OLFM4	Olfactomedin-4
PAD4	Protein arginine deiminase 4
PAN	Proangiogenic neutrophil
PAPA	Pyogenic arthritis, gangrenosum and acne
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reactions
PD	Pemphigus diseases
pDCs	Plasmacytoid dendritic cells
PDL1	Programme death ligand 1
PE	Phycoerythrin Conjugate

PE/CY7	Phycoerythrin: Cy-7 Tandem Conjugate
PECAM-1	Platelet endothelial-cell adhesion molecule-1
PI3K	Phosphoinositide 3-kinase
PLR	Platelet-to-lymphocyte ratio
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocytes
PMN-N	normal neutrophil population
PPAR	Peroxisome proliferator-activated receptor
PR3	Granule proteinase 3
PTPN22	Protein tyrosine phosphatase non receptor type 22
RA	Retinoic acid
RA	Rheumatoid Arthritis
RAF	Rapidly Accelerated Fibrosarcoma.
ROS	Reactive oxygen species
RXR	retinoid x receptor
S. aureus	Staphylococcus aureus
SD	Standard deviation
SDF-1	Stromal-derived factor-1
SJS	Stevens-Johnson Syndrome
SJS-TEN	Stevens–Johnson syndrome and toxic epidermal necrolysis
SLE	Systemic lupus erythematosus

STAT1	Signal Transducer and Activator of Transcription 1
STAT4	Signal Transducer and Activator of Transcription 4
T cells	T lymphocyte
T reg	Regulatory T cell
TAN	Tumour-associated neutrophils
TGF-β	TGF-beta
TGF-β	Transforming growth factor beta
Th	T helper cells
Th1	T helper 1
Th2	T helper 2
TICAM1	Toll Like Receptor Adaptor Molecule 1
TIRAP	TIR domain-containing adaptor protein
TLR4	Toll-like receptor 4
TLR4	Toll-like receptor 4
TNF	Tumour necrosis factor
TNF-a	Tumour necrosis factor alpha
TNFR	Total homocysteine and soluble necrosis factor receptor
TRAIL	TNF-associated apoptosis inducing ligand
VCAM1	Vascular cell adhesion molecule 1
VCAM-1	Vascular cell-adhesion molecule-1
VEGF-A	Vascular Endothelial Growth Factor A)

VLA-4	Very late antigen 4
WT	Wild type
α4β1	Integrin α4β1
α-MSH	Alpha-melanocyte-stimulating hormone
γδ+ T	Gamma delta T cells

CHAPTER 1

GENERAL INTRODUCTION

1.0. Introduction

1.1. A general overview of the Immune response

The immune system is recognised to defend and protect the host from different and constantly evolving pathogenic microbes. In order to do this, the immune system helps to eradicate pathogens and toxic elements that enter via mucosal surfaces (Chaplin, 2010). For efficient and successful eradication of different pathogens, the immune system is separated into two distinct phases known as innate and adaptive immune responses which are determined by the speed and specificity of the reaction (Akira, 2011). The innate immune response, also termed as innate immunity, includes physical, chemical and microbiological barriers (Fig.1). Innate immunity involves cells such as neutrophils, monocytes, macrophages, and humoral complement, cytokines and many different acute phase protein elements, which together, deliver an immediate host defence important for survival (Parkin *et al.,* 2001).

The adaptive immune response describes antigen-specific reactions through T and B lymphocytes. The difference between the phases of the immune response is that the innate immune response is rapid but non-specific, whereas the adaptive immune response is highly precise, but can take several days to develop (Fig.1). Moreover, the adaptive immune response generates memory such that following exposures of the same pathogen encounters a more vigorous and rapid response (Parkin *et al.,* 2001).

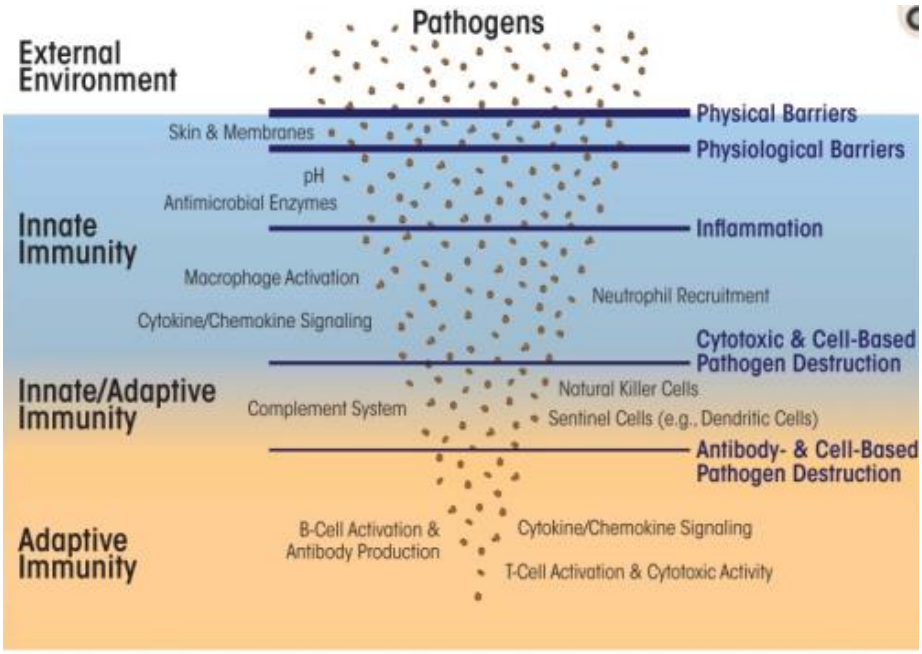


Figure 1- The overview of the immune response. Innate immunity involves many non-specific protective mechanisms against infection that trigger pathogen recognition (via cytokines and chemokines or short peptides). The adaptive immune response is formed of specialised cells and proteins (B and T cells and antibodies) that eradicate specific pathogens, and chemical signals to recruit other immune cells. The complement system connects the innate and adaptive immunity during an immune response (Spiering 2015).

1.1.1. The innate immune response

The main role of innate immunity is to deal with challenges in tissues, either invading pathogens or sterile inflammation. The innate immune response is facilitated by mast cells, macrophages, dendritic cells, neutrophils, natural killer cells, basophils, and monocytes (cellular barriers), that react to many different infections that have breached physical barriers (Agarwal, 2010).

1.1.1.1. The anatomical and physiological barriers of the innate immune response.

The anatomical barriers of the innate immune response are comprised of intact skin and mucosal tissues that provide substantial protection against infectious agents. The skin prevents entry of microbes and provides an acidic environment to inhibit growth of microorganisms. The mucous membranes of all mucosal tissues secrete mucus to trap foreign ***microorganisms*** and can express cilia help to expel microorganisms out the body (Agarawal 2010; Turvey *et al.,* 2010).

1.1.1.2. The chemical barriers of the innate immune response

The chemical barriers include the immune mechanisms such as the complement system and antimicrobial peptides. The complement system, more than 30-blood borne acute-phase proteins created in the liver, aid the killing of pathogens either directly or by recognition of antibodies bound to bacteria. A biochemical cascade is triggered, and bacteria are coated with complement proteins which lead to perforating cell walls and death. Moreover, the complement system facilitates clearance of foreign cells through opsonisation, lysis and pathogen recognition (Schifferli *et al.,* 1986). Complement is also involved in the clearance of immune complexes and apoptotic cells the circulation and damaged tissues (Davies *et al.,* 1994; Dunkelberger *et al., 2009*).

Antimicrobial peptides are produced at epithelial cell surfaces, including defensins, lysosymes, cathelicidin and histatins (Kobayashi *et al.,* 2005). Lysosymes produced by neutrophils disturbs the cell wall component of bacteria. Cathelicidin is found in neutrophils, epithelial cells, monocytes, NK cells, T and B cells and displays an antimicrobial activity against both Gram-positive and Gram-negative bacteria (Tjabringa *et al*, 2006). Cationic peptides such as histatins are present in human saliva and play an important role in providing efficient

antimicrobial and antifungal actions to maintain oral health (De Smet *et al.,* 2005; Agrawal 2010).

1.1.1.3. The cellular barriers of the innate immune response

In barrier tissues, such as mucosa, macrophages and mast cells recognise pathogens and their components via Toll-like receptors and dectin-1 on the cell surface, and opsonisation by complement or antibody. Subsequent degranulation of mast cells releases histamine, leukotrienes and prostaglandins which induce vasodilation and permeability in local blood ***vessels.*** Stimulated macrophages release cytokines including tumour necrosis factor (TNF) and interleukin-8 (IL-8) which activates blood vessels and attract neutrophils, basophils and monocytes to the site (Tosi, 2005; Kobayashi *et al.,* 2005).

Macrophages play a major role in resolution of inflammation by phagocytosing dead and dying neutrophils and clearing cell debris which is known as efferocytosis (Agrawal 2010; Minakami *et al.,* 2006).

Neutrophils are the most abundant white blood cells with the concentrations up to $5x10^6$/ml in the blood, and upon injury or infection move rapidly to the site of infection or injury, where they detect and phagocytose pathogens (Spiering 2015; Selders *et al.,* 2017).

Monocytes, the largest blood borne cells, mature in the bone marrow and on entry into tissues differentiate into macrophages and dendritic cells (Geissmann *et al.,* 2010). Macrophages as described will contribute to pathogen clearance and resolution while dendritic cells ingest pathogens, migrate to the draining lymph node and initiate the adaptive response (Krzyszczyk *et al.,* 2018).

Basophils initiate from bone marrow and circulate in blood, migrate to the injured or infected site, and release anticoagulant components (heparin and vasoactive compounds such as histamine and serotonin) to reduce blood clotting and contribute towards the inflammatory response (Stone *et al.,* 2010).

Eosinophils are involved in the innate immune response and functional roles include antigen presentation, cytokine mediators, lipid-derived peptides are released in response to acute and chronic inflammation (Kovalszki *et al.,* 2018).

Natural killer cells (NK cells) are also part of the innate immune response and respond rapidly to virus infected and tumour cells with proteolytic enzymes and cytotoxic proteins and initiate apoptosis (Spiering 2015; Agrawal 2010).

1.2. The adaptive immune response

The main functions of adaptive immunity are ***to*** initiate pathogen specific immunologic effector pathways and to develop immunologic***al*** memory. Immunologic***al*** memory allows a quick elimination of specific pathogens if subsequent infections occur (Bonilla *et al.,* 2010; Marshall et al., 2018). The cells of the adaptive immune response are: antigen-specific T lymphocytes (T cells) and B lymphocytes (B cells). T cells are stimulated to differentiate and proliferate via the expression of antigen-derived peptides in the context of major histocompatibility complex (MHC) molecules on antigen presenting cells (APC). Effector and memory T cells have several different subsets (Marshall *et al.,* 2018).

MHC class I encodes proteins human leukocyte antigen [HLA], on all nucleated cells as three isotypes A, B and C, and present intracellular antigen derived peptides to cytotoxic $CD8^+$ T cells. MHC class II isotypes, HLA DP, DQ and DR that are found on only specific APC dendritic

cells, macrophages and B cells, and present peptides derived from extracellular pathogens to $CD4^+$helper (Th) cells (Bonilla *et al.,* 2010; Murphy *et al.,* 2007) (Fig. 2).

Stimulation of T cells takes place in draining lymph nodes when they come in contact with DC that have internalised an antigen and presented peptides bound to its MHC molecules. The function of $CD8^+$ cytotoxic T cells is to destroy cells infected by viruses or tumour cells displaying the suitable antigens (Murphy *et al.,* 2007; Bonilla et al., 2010). $CD4^+$ Th cells have different functions. Th1 cells are classified by the production of interferon-gamma (IFN-γ) help in the killing of bacteria by macrophages, improves anti-viral immunity and the immune response to intracellular pathogens. Th2 cells classified by IL-4, IL-5 and IL-13 contribute to ***B cell*** differentiation and antibody production that increases the efficacy of opsonisation and phagocytosis of bacteria and neutralisation of viruses (Marshall *et al.,* 2018).

Th17 cells are characterised by the production of IL-17, IL-21 and IL-22, are involved in host protection against microbes that are not eradicated by Th1 and Th2 immune cells this includes extracellular bacteria and some fungi. IL-17 facilities powerful effects on stromal cells leading to the production of inflammatory cytokines and recruiting leukocytes especially neutrophils and as a result a link is created between the innate and adaptive immune response (Tesmer *et al.,* 2008; Yang *et al.,* 2016).

A FoxP3 expressing regulatory T cell (T reg) suppresses and limits the immune response and control abnormal reactions to self-antigens and the growth of autoimmune disease (Marshall *et al.,* 2018). $CD4^+CD25^-$ naïve T cells which are transfected with Foxp3 (gene) convert to $CD4^+CD25^+$ Treg-like cells that in response produce inhibitory cytokines, IL-10 and express cytotoxic T-lymphocyte antigen-4 (CLTA-4) and glucocorticoid induced tumour necrosis factor

(TNF) and receptor-related protein (GITR), that combine to inhibit the immune response (Ohue and Nishikawa 2019).

1.2.1. The role of B lymphocytes in adaptive immune response

B lymphocytes originate from hematopoietic stem cells in the bone marrow. All B cells express a unique antigen binding receptor (antibody) on their membrane after being fully matured and leaving the bone marrow. B cells can directly recognise antigens via their receptor and do not require APC. The main function of B cells is to produce specific antibodies (which can involve further differentiation) to bind to and destroy foreign species or antigens (Bonilla *et al.,* 2010; Murphy *et al.,* 2007). When stimulated B cells proliferate and differentiate into antibody-releasing plasma cells or memory B cells (Fig. 2). Antibodies released into the circulation and tissues by plasma cells contribute to the clearance of pathogens. Memory B cells respond rapidly by producing antibodies and eradicating the antigen upon re-exposure (Marshall *et al.,* 2018). Different forms of antibody have different functions that aid different responses to pathogens (Table 1).

Table 1- The locations and functions of the different antibody subtypes.

Ig antibody	*Location*	*Function*	*Reference*
IgM	Blood and Lymph fluid	First Ig expressed during B cell development, complement fixation and coating antigen for destruction	Schroeder and Cavacini (2010)
IgG	Blood and Lymph fluid	Main Ig during secondary immune response, the only antibody capable of crossing the placental barrier, coating antigen for destruction and complement fixation	Schroeder and Cavacini (2010)
IgD	On the surface of B lymphocytes	Involved in homeostasis	Vladutiu, 2000
IgA	mucosal surfaces	protects mucosal surfaces from toxins and neutralization or prevention of binding to mucosal surface and	Woof and Kerr 2006
IgE	mucosal surfaces	Associated with hypersensitivity and allergic reactions and encounters a role in in immune response to parasites	Schroeder and Cavacini (2010)

Immunoglobin M (IgM), Immunoglobin G (IgG), Immunoglobin D (IgD), Immunoglobin A (IgA), Immunoglobin E (IgE)

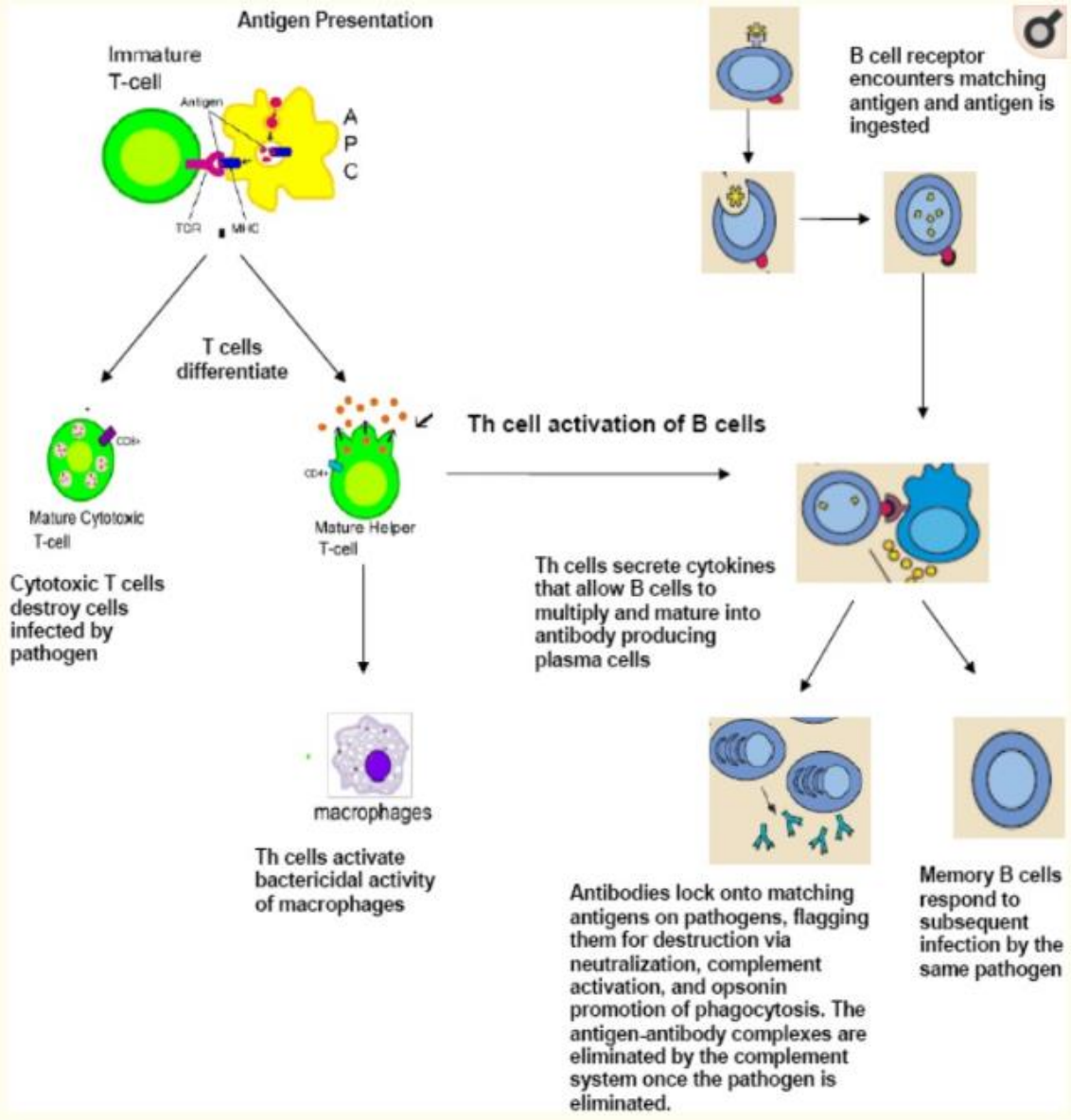


Figure 2-The illustration of adaptive immunity in specifically T and B cell activation and function. The process of antigen presentation stimulates T cells to differentiate into cytotoxic T cells (CD8+ cells) or T-helper (Th) cells known as CD4+ cells. B cells endure proliferation and differentiate into antibody-secreting plasma cells or memory B cells, when activated by foreign antigens to which they have appropriate antigen specific receptor. The secreted antibodies bind to antigens presented on the surface of the pathogens instructing them for destruction via complement activation, opsonisation, phagocytosis, and removal of pathogen by immune effector cells. The antigen-antibody complexes are cleared by the complement cascade upon the elimination of the pathogen (Marshall et al., 2018).

1.3 Overview of neutrophils in an inflammatory response.

1.3.1. The neutrophil life cycles.

This thesis investigates neutrophil phenotype and function in chronic inflammatory eye disease. Neutrophils, also known as polymorphonuclear (PMN) leukocytes, are 70% of all leukocytes in the blood and are constantly produced (more than $10^{11)}$ every day in the bone marrow (Dancey *et al.,* 1976). Neutrophils leave the bone marrow into the blood where they circulate until they migrate into the tissues. When the neutrophils reach the end of their lifespan within the tissues, they traffic back to the bone marrow and destroyed (Bratton *et al.,* 2011). The numbers of neutrophils in the circulation continue to remain constant via these homeostatic mechanisms.

1.3.2. The process of granulopoiesis in neutrophils

Neutrophils produced in the bone marrow originate from hematopoietic stem cells (HSC) that differentiate into multipotent progenitor (MPP) cells. MPP further transform into lymphoid-primed multipotent progenitors (LMPPs) which in response to transcription factors differentiate into granulocyte–monocyte progenitors (GMPs), then to myeloblast, ***a*** process controlled by granulocyte colony-stimulating factor (G-CSF). The myoblasts further go through maturation process that entails the stages of promyelocyte, myelocyte, metamyelocyte, band cell, and finally developing into a mature neutrophil (Fig. 3) (Görgens *et al.,* 2013; Vietinghoff *et al.,* 2008).

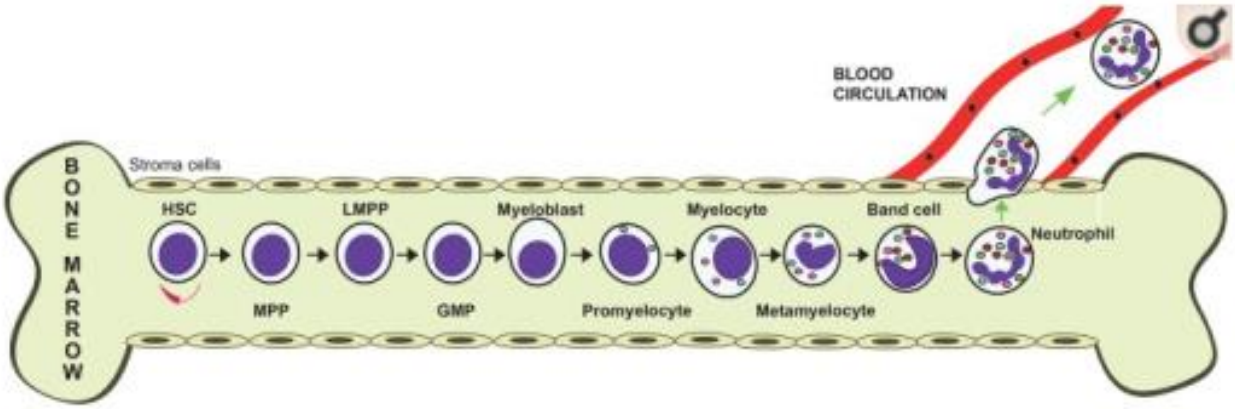


Figure 3-The process of granulopoiesis. The granules are represented by coloured dots. The process of granulopoiesis. The granules are represented by coloured dots. The process of granulopoiesis. The granules are represented by coloured dots Neutrophils are produced in the bone marrow from self-renewing hematopoietic stem cell (HSC) into multipotent progenitor cells (MPP). The MPPs produce lymphoid-primed multipotent progenitors (LPMP) which differentiate into granulocyte-monocyte progenitors (GMP). The GMPs generate neutrophil by turning into myeloblast under the control of colony-stimulating factor (G-CSF) which as a result follow the maturation process of promyelocyte, myelocyte, metamyelocyte, band cell and finally develop into a neutrophil (Görgens et al., 2013).

During the maturation and differentiation process the evolving neutrophil changes the shape of its nucleus from round into banded emerging with a lobulated morphology. The expression of many receptors changes during development including the chemokine receptor (CXCR2) and Toll-like receptor 4 (TLR4) which are up regulated, whereas the integrin α4β1 (VLA4) and the CXCR4 are downregulated ***(Häger et al., 2010).*** In order to maintain the progenitor cells

in the bone marrow, the stroma cells express the vascular cell adhesion molecule 1 (VCAM1) which is a ligand for VLA4, and the chemokine stromal-derived factor-1/SDF-1 (CXCL12) which is a ligand for CXCR4. Neutrophils contain many secretory vesicles and granules which are developed at precise differentiation stages (Häger *et al.,* 2010). The azurophilic (primary granules) are developed during the transformation phase of myeloblast to promyelocyte; the specific (secondary granules) granules are found during the transformation stage of myelocyte to metamyelocyte, and gelatinase (tertiary) granules are found in banded cells and ***secretory*** vesicles are found in mature neutrophils (Fig.3). Granules store a collection of matrix metalloproteinases, antimicrobial enzymes, elastase, and myeloperoxidase cathelicidins and defensins to kill ingested pathogens (Görgens *et al.,* 2013) (Table 2).

Table 2-The different types of neutrophil **granules** (Faurschou *et al.,* 2003; Borregaard et al*.,* 2007).

Granule	*Granule type content*
Primary granules-Azurophilic granules	Myeloperoxidase Neutrophil Elasatse Cathepsin G Lysozyme Defensins
Secondary or specific granules	Lactoferrin Lysozyme
Tertiary or gelatinase granules	Store metalloproteases such as gelatinase.
Secretory granules	Carry cytokines

1.3.3. The exit of neutrophils from the bone marrow.

Neutrophils leave the bone marrow and enter into blood circulation depending upon their maturity levels. Mature neutrophils are released into the circulation under basal conditions. These neutrophils have a short half-life of 6.5hr in the blood are destroyed in the bone

marrow, spleen, and liver. During infection or inflammation neutrophils release from the bone marrow supply is significantly increased and this process is facilitated by the co-ordinated actions of chemokines and cytokines (Furze *et al.,* 2008).

1.3.4. The process of neutrophil migration

The movement of neutrophils from the circulatory system to the site of inflammation in the body is defined as neutrophil diapedesis (Wright *et al.,* 2010; Butterfield *et al.,* 2006). This process is highly organised and controlled by many different communications with the vascular endothelium. During diapedesis, integrins selectins and adhesion molecules are involved for neutrophils to adhere to the endothelial wall (Fig. 4) In addition, chemotaxis to the site of inflammation or lifespan of the priming agents activate transcription factors and certain cytokines and receptors are expressed (Wright *et al.,* 2010). L-selectin expressed on the surface of neutrophils permits loose binding to ligands expressed on the surface of the endothelial cells (E and P selectin and P-selectin glycoprotein ligand) causing slowing down from blood transit and rolling along the endothelium (Fig. 4). This in response produces conformational alterations in integrin molecules involving very late antigen-4 (VLA-4; α4β1-integrin, (CD49d/CD29b), lymphocyte function-associated antigen-1 (LFA-1; αLβ2-integrin, CD11a/CD18) and macrophage antigen-1 (MAC-1; αMβ2-integrin, CD11b/CD18), which recognise ligands located on the surface of the endothelial cells, intercellular adhesion molecule (ICAM)-1 and -2, vascular cell-adhesion molecule-1 (VCAM-1) and mucosal vascular cell-adhesion molecule-1 (MADCAM-1), leading to firm adherence. The procedure of rolling arrest is facilitated by binding of IL-8 (chemoattractant) to neutrophil receptors (Ley *et al.,* 2007). Following rolling arrest neutrophils migrate into the tissues via the junctions located

between neighbouring endothelial cells (paracellular migration) utilising the surface ligands such as ICAM-2, platelet endothelial-cell adhesion molecule-1 (PECAM-1) and proteins of the junctional adhesion molecule (JAM) family (Woodfin *et al.,* 2009). High ICAM-1 expression is needed for transcellular transmigration and density; small fractions of neutrophils infiltrate and pass through the pores in the cytoplasm of endothelial cells (Yang *et al.,* 2005).

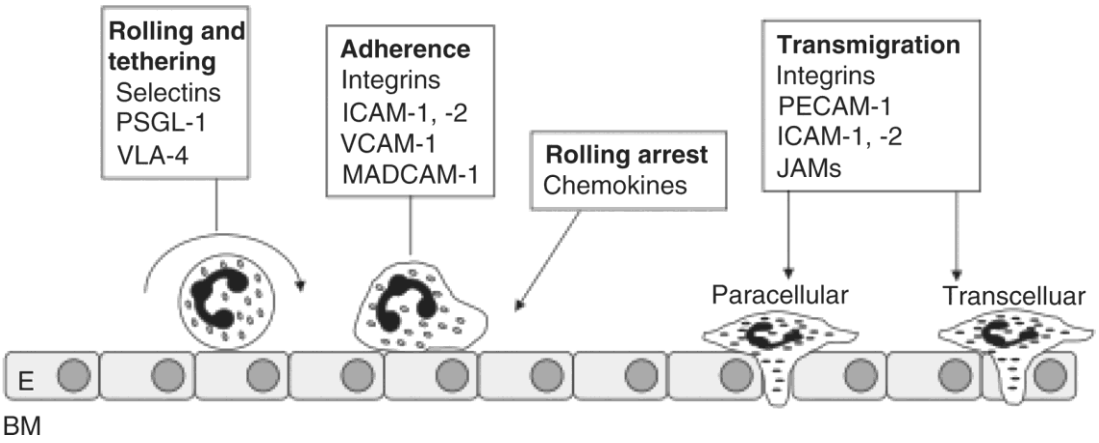


Figure 4-The migration of neutrophils from the peripheral blood to the site of inflammation. To pass from the peripheral blood to the site of inflammation the neutrophil sticks to the endothelial wall by utilising adhesion, selectins and integrins molecules. This is followed by transmigration via the endothelial lining of the blood vessel through chemotaxis to the inflamed site (Wright *et al.,* 2010).

Upon leaving the circulation**,** neutrophils pass through the endothelium and migrate towards the site of inflammation along a chemotactic gradient, via attractants such as chemokines, N-formylmethionyl-leucyl-phenylalanine (fMLP) and complement component 5a (C5a) that encourage cellular polarisation of chemoreceptors and actin rich pseudopodia at the leading

edge of the cells is produced (Servant *et al.,* 2000). At the site of inflammation membrane receptors for the complement proteins and immunoglobulins distinguish and bind to opsonised bacteria allowing the formation of pseudopodia, phagocytosis of the microbe, leading to the destruction of the pathogen within the intracellular phagosome and in response producing ROS (Wright *et al.,* 2010).

Upon activation, neutrophils produce or display many different chemokines, cytokines and angiogenic factors. The production of these molecules is highly controlled by regulatory mechanisms that act at different levels such as mRNA transcription, stability or translation via microRNA-facilitated targeting, and protein secretion (Cassatella *et al.,* 1999; Mantovani *et al.,* 2011; Yamada *et al.,* 2011) In relation to protein excretion, important fractions of B cell-activating factor (BAFF), TNF-associated apoptosis inducing ligand (TRAIL), CXCL8, CC-chemokine ligand 20 (CCL20) and IL-1 receptor antagonist (IL-1RA) are not immediately released after synthesis but are collected in intracellular pools. These cytokines are only released when neutrophils are stimulated by secretagogue-like molecules (Scapini *et al.,* 2008). Neutrophils are involved in the resolution of active initiation of inflammation by the release of pro-resolving lipid mediators such as such as lipoxins, resolvins and protectin (Mantovani *et al.,* 2011).

1.3.5. The clearance of neutrophils

In inflammatory sites most neutrophils undergo the process of apoptosis and are cleared by macrophages **(via efferocytosis)** and dendritic cells. In blood the expression of CXCR4 is

upregulated on senescent neutrophils which mediates a return to the bone marrow for clearance (Martin *et al.,* 2003). Apoptosis is vital for controlling neutrophil production in the bone marrow (Stark *et al.,* 2005). During resolution an anti-inflammatory process is triggered during the phagocytosis of apoptotic neutrophils which is characterised by a decline in IL-23 by macrophages, a reduced amount of IL-17 and less G-CSF production which leads to reduced granulopoiesis (Stark *et al.,* 2005).

1.4. The function of neutrophils in an inflammatory response

1.4.1. The process of neutrophil phagocytosis.

The three main functions of neutrophils in response to pathogens are phagocytosis, production of reactive oxygen species and the production of neutrophil extracellular traps (NETs) (Fig.5). Phagocytosis is a vital cellular mechanism that involves binding, ingesting, degrading and killing bacteria or fungi (Hellebrekers *et al.,* 2017). The engulfed pathogen is transported in the cell to the phagolysosome providing a highly toxic environment essential in killing mechanism (Nauseef 2007). Bacteria are recognised by receptors for immunoglobulins (FcγR receptors such as CD32 and CD16) or complement factor C3 (complement receptor (type3 such as C3aR) and CD11B/CD18 and Mac-1) binding to the bacteria surface via a mechanism known as opsonisation (Kessel *et al.,* 2014). This further involves killing bacteria via the respiratory burst.

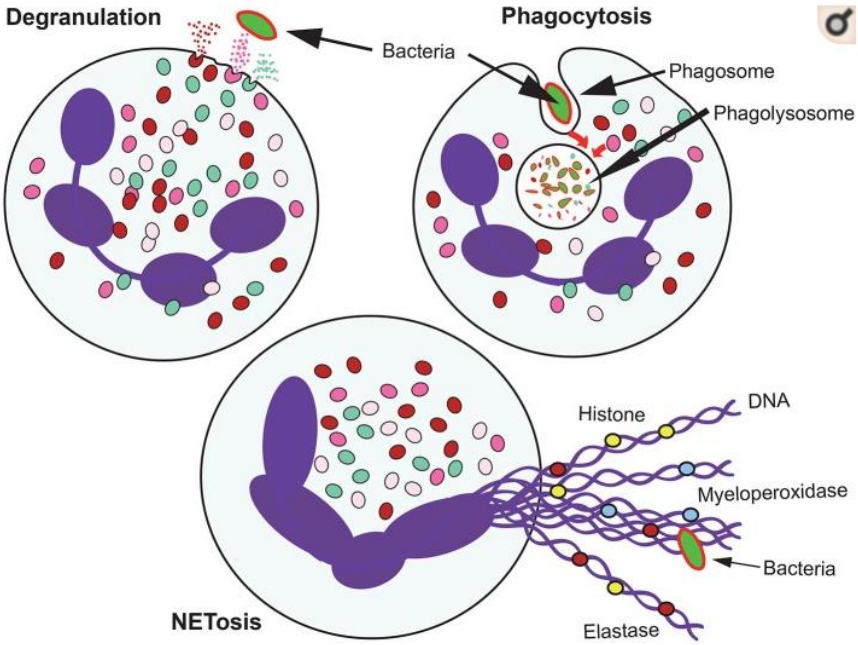


Figure 5-Illustration of the 3 main functions of neutrophils such as phagocytosis, production of ROS and NETosis involved in antimicrobial mechanisms. When neutrophils recognise microbial pathogens, they display phagocytosis, ROS production and NETosis. During phagocytosis microorganisms are ingested into a phagocytic vacuole that becomes phagolysosome upon maturation. The microorganisms are further destroyed by low pH and destroying enzymes. Neutrophils degranulate and release the content of their granules into their environment. Neutrophils can produce neutrophil extracellular traps (NETs) which are formed by DNA proteins and fibres from the granules and released from the cell (Rosales 2018).

1.4.2. The process of respiratory burst in neutrophils

One of the main hallmarks of antimicrobial and inflammatory activity of neutrophils is the activation of a powerful oxidative burst and production of reactive oxygen and nitrogen species are produced which have the potential for an antimicrobial mechanism (Jaillon *et al.,* 2013) (Fig. 5 and **6**). Stimulation of primed neutrophils by phagocytosis is characterised **by the** rapid production of ROS via nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase complex NADPH oxidase accumulates in the phagolysosome membrane following activation (Winterbourn *et al.,* 2016). In order to produce superoxide ions oxygen is reduced by NADPH to form the superoxide radical ($O2^-$) which respond with water molecules to form molecular oxygen and hydrogen peroxide, a reaction catalysed by superoxide dismutase. Hydrogen peroxide (H_20_2) is further converted to hypochlorous acid (HOCl) through a catalysis reaction by myeloperoxidase (MPO), from the azurophilic granules) (Fig.**6**) Hydroxyl ions and nitrogen oxygen species produced also contribute to the oxidative burst. Nitrogen oxide radicals are derived from the transformation of the amino acid L-arginine to L-citrulline by nitrogen oxygen synthase (NOS). Isoforms of NOS falls into distinct categories 1) constitutive NO synthases (NOS_1 and NOS_3) that are reliant on $Ca2^+$/calmodulin 2) inducible NOS (NOS_2), increased by cytokines and other inflammatory stimuli, independent of Ca^{2+} (Fialkow *et al.,* 2007). These products are released into the phagolysosome and along with granule components also released destroy the ingested bacteria. The build-up of oxidative stress and the production **of** oxygen radicals may cause DNA damage, oxidation of lipids, lipoproteins and proteins and, can also cause mutations in **immunoglobulins** (Wright *et al.,* 2010). NADPH oxidase and granules can also fuse with the plasma membrane and release of ROS and granule

components, including elastase and MPO can damage neighbouring cells in addition to extracellular bacteria (Chen *et al.,* 2012). Tissue damage due to the neutrophil response is characteristic of anti-neutrophil cytoplasmic antibody associated vasculitides and systemic lupus erthymatosus (Johansson *et al.,* 2016; Padmanabhan *et al.,* 2012).

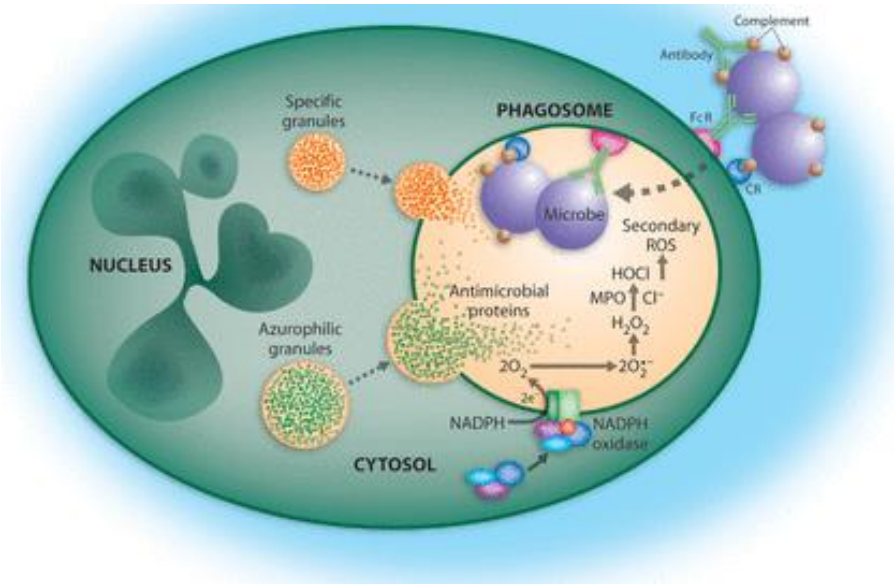


Figure 6-The initiation of ROS (and NADPH oxidase) in phagocytosis (Quinn *et al.,* 2006). The Complement and antibody receptors (CRs and FcRs) stimulate the process of up taking micro-organisms by neutrophils, which as response triggers degranulation and production of ROS. Superoxide ions are produced by the reduction of oxygen by NADPH to form superoxide radicals (O2-). The superoxide radicals respond with water to form molecular oxygen and hydrogen peroxide. Hypochlorous acid (HOCl) is produced via the conversion of hydrogen peroxide via myeloperoxidase (MPO) and this further leads to production of ROS.

1.4.3. Production of Neutrophil Extracellular Traps

The third function of neutrophils in inflammation is the generation of neutrophil extracellular traps (NET) (Brinkmann *et al.,* 2007). NET are composed mainly of DNA (histones) and proteins from neutrophils granules, primary granules (neutrophil elastase and myeloperoxidase), secondary granules (lactorferrin and pentraxin 3) and tertiary granules (matrix metalloproteinase 9) (Brinkmann *et al.,* 2004) (Fig. 7). NETs are formed by activated neutrophils to trap bacteria leading to their destruction (Jaillon *et al.,* 2013).

NET formation allows a well-orchestrated cell death programme of neutrophils defined as NETosis. During NETosis, the nucleus delobulates and the granules ruptoure followed by membrane vesiculation. Once the nucleus is disintegrated and before the cell ruptures the chromosomes expand. This allows successful contact between granular and cellular components. As a result, the cytoplasmic membrane breaks and NETs are released into the extracellular space. The intracellular membrane reorganisation allows the proteins and chromatins to connect to form NET (Sollberger *et al.,* 2018).

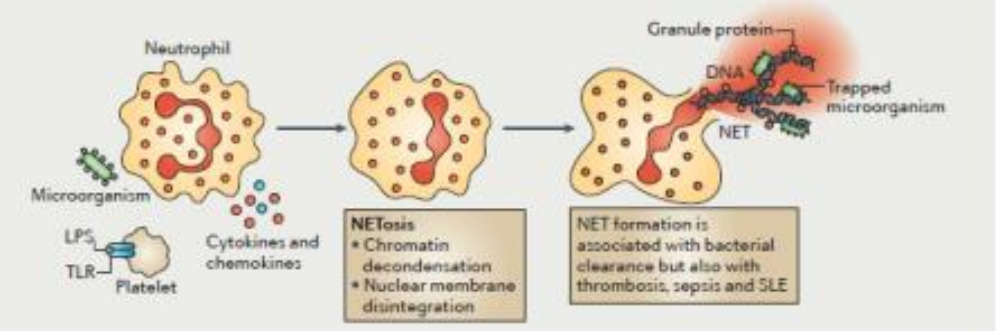


Figure 7-Formation of neutrophil extracellular traps in systemic inflammation. The autoantibodies specific for antimicrobial peptides present in NETs allow the transport of DNA into plasmacytoid dendritic cells through CD32 and interferon-α production in a Toll like receptor supported manner (Mantovani *et al.,* 2011).

1.4.3.1. NADPH oxidase dependent NETosis.

During suicidal NETosis NADPH oxidase derived ROS act as second messengers by promoting regular separation and loss of the nuclear membrane which further dissociates into small separate vesicles. Chromatins dissolve throughout the cytoplasm and get mixed with cytoplasmic proteins and granule toxins (Delgado-Rizo 2017). This formation of NET is highly dependent upon elastase and myeloperoxidase transportation of granules to the nucleus. Lastly, chromatin is released outside the cell via the membrane pores and cellular cell death (Delgado-Rizo 2017). This form of NETosis is dependent upon the production of ROS for histone citrullination by PAD4 involved in chromatin decondensation.

1.4.3.2. NADPH oxidase independent NETosis.

During vital NETosis (pathogen-derived stimulation of neutrophils) neutrophils release NETs without showing a loss of nuclear or plasma membrane (within 5-60 minutes). This process

arises without utilising ROS, rather induction of the Raf/MERK/ERK pathway (Delgado-Rizo 2017). Nuclear DNA is released through three morphological changes. The first change is described as the growth of the nuclear envelope and vesicle **release.** Secondly, nuclear decondensation occurs and finally, the nuclear envelope disrupts (Manely *et al.,* 2018). Vital NETosis is activated by the recognition of stimuli through Toll-like receptors (TLRs) and the complement receptor for C3 protein. In addition, interaction of glycoprotein Ib in platelets with β2 integrin (CD18) in neutrophils may allow NET formation via activated , **extracellular signal-regulated kinases** (ERK), **class I phosphoinositide 3-kinase** (PI3K), and **proto-oncogene tyrosine-protein kinases** (SRC kinases). Finally, once the nucleus is released, neutrophils are still to phagocytose pathogen (Kubes 2013).

1.4.3.3. Mitochondrial neutrophil extracellular traps

Activation of viable neutrophils with granulocyte/macrophage-colony stimulating factor (GM-CSF) and TLR4 receptor agonist or C5a can lead to release of mitochondrial DNA to form NET (Yousefi *et al.,* 2009). The dynamic and active discharge of mitochondrial DNA by viable neutrophils involves the release of nuclear DNA, proteases, histones leading to the production ROS that a result lead to cell damage (Köckritz-Blickwede *et al.,* 2009). Even though, the primed release of mitochondrial DNA by viable neutrophils is also ROS-dependent, this process does not lead to cell death (Yousefi *et al.,* 2008).

1.5. The heterogeneity of neutrophils

Neutrophils are classified as differentiated cells that develop from immature neutrophils in the bone marrow to circulating mature neutrophils. Upon priming or stimulation mature neutrophils can migrate into tissues and exert effector functions discussed above (Deniset *et*

al., 2018). Neutrophils were previously considered to be a homogenous population of differentiated cells with a well distinct and conserved function, based on the basis of short life span, their limited capacity to produce a vast amount of cytokines, failure to re-circulate from the tissue into the blood stream and the absence of neutrophil proliferation (Silvestre-Roig., *et al.,* 2019). However, neutrophils quickly alter their characteristics and behaviour upon activation, age or whilst entering a new environment. In addition, stimulation or priming of neutrophils by meditators in acute or chronic inflammatory conditions can increase their life span and as a result provide an enhanced opportunity to polarise and produce other effector molecules (Kennedy *et al.,* 2008; Summers *et al.,* 2010; Pillay *et al.,* 2010).

Neutrophils respond to many different innate responses such as sterile injury, infection, cancer and autoimmunity in different ways by utilising different effector functions or mechanisms. As neutrophils display divergent functions in different settings, this has encouraged the field to discover and explore both phenotypic and functional heterogeneity especially to identify different neutrophil subsets as observed for other immune cells lineages (Chorny *et al.,* 2016; Horckmans *et al.,* 2017). The different neutrophil populations are proposed on many factors such as function, cell surface markers, maturity levels, localisation and buoyancy under pathophysiological and homeostatic conditions (Deniset *et al.,* 2018).

Neutrophil heterogeneity was established by numerous studies in the 1970s-1980s highlighting the functional differences of neutrophils with regards to density and the biosynthesis of ribonucleic acid (Broxmeyer *et al.,* 1980; Harvath *et al.,* 1982). Further advances in this area investigated subpopulations by using monoclonal antibodies which showed 5% of the neutrophils were $CD10^{-ve}$ (Clement et al. 1983; McCormack *et al.,* 1987).

Gallian (1984) clearly showed the aspect of neutrophil heterogeneity but the biological importance remained unanswered.

While the importance of neutrophils as effectors within the innate immune response are well described, they are now recognised as also having regulatory function (Garley *et al.,* 2018). Increasing scientific evidence has suggested a considerable phenotypic heterogeneity and functional flexibility of neutrophil populations (Table 3).

Table 3-Different types and functions of heterogenous population of neutrophils. (Garley *et al.,* 2017).

Neutrophil subset	*Immunophenotype*	*Functional properties*	*References*
Mature/classic	HNA-1 (FccRIIIb, CD16)	-	Bux (1999)
	HNA-2 (CD177)	-	Bux (1999)
	HNA-3 (CTL2)	-	Bux (1999)
	HNA-4 (CD11b/CD18; Mac-1, CR3)	-	Bux (1999)
	HNA-5 (CD11a/CD18)	-	Bux (1999)
Long-living	HLA-DR, CD80, CD49d	Production of IL-8, IL-1Ra, IL-1-b	Chakravarti *et al.,* (2009)
Aged	$CD62L^{low}CXCR4^{high}CD11B^{high}$ $CD49^{high}$	Phagocytosis, NETosis	Casanova-Acebes *et al.,* (2013), Rankin (2010), Zhang et al. ,(2015)
NBH		BAFF, APRIL	Puga *et al.,* (2011)
TCR+	TCRab	IL-8 inhibition of apoptosis	Puellmann *et al.,* (2006)

PMN-I	TLR2/TLR4/TLR5/TLR8 $CD49d^{+}CD11b^{-}$	IL-12, CCL3	Tsuda *et al.* (2004)
PMN-II	TLR2/TLR4/TLR7/TLR9	IL-10, CCL2	
PMN-N	TLR2/TLR4/TLR9 CD49d-$CD11b^{-}$		
mPR3+	$mPR3^{high}$	Outbreak or progression of chronic inflammatory	Witko-Sarsat *et al.* (1999)
	$mPR3^{+}$ $CD177^{+}$	Wegener's granulomatosis	Bauer *et al.* (2007)
CD177	High, low, negative expression		Wu *et al.* (2016)
LDNs	CD66b, $CD11b^{+}$ $CD16^{+}$ and/or $CD11b^{low/-}CD16^{low/-}$	immature	Deng *et al.,* (2016), Giallongo *et al.,* (2015)
LDNs/G-MDSCs	$CD66b^{+}CD15^{+}CD14^{-/dim}CD33^{dim}$ $HLADR^{-}$	Immunosuppressive	Jiang *et al.,* (2014), Mandruzzato *et al.,* (2016),
LDGs	$CD15^{+}/CD14^{low}$, $CD10^{+}/CD14^{low}$, $CD16^{high}/CD86^{-}$	IFN-c, TNF-α proinflammatory	Denny *et al*., (2010)
Bone marrow-derived, immature neutrophils	$CD10^{low/-}CD16^{low}$		Manz and Boettcher (2014)
MDSCs	$CD11c^{bright}/CD62L^{dim}/CD11b^{bright}/CD16^{bright}$	Inhibition of T lymphocytes proliferation	Pillay *et al.*, (2012)
MDSCs in cancer	$CD14^{+}$ $CD33^{?}$ $HLA\text{-}DR^{-}$	Inhibition of T lymphocytes function	Almand *et al.,* (2001), Hoechst *et al.,* (2008),
N1	$CD11b^{+}/Ly6G^{+}$	Anti-neoplastic activity	Fridlender *et al.,* (2009), Fridlender and Albelda (2012)

N2		Pro-neoplastic propties, VEGF, MMP-9	Schmielau and Finn (2001)
Proangiogenic	CXCR4^{high}/VEGFR1^{high}	MMP9	Christoffersson *et al.,* (2012)

B-cell-helper neutrophils (NBH), Membrane proteinase 3 (MP3), polymorphonuclear leukocytes I, II (PMN I,II), polymorphonuclear leukocytes normal (PMN-N), Low density neutrophils (LDN), granulocytic (G-MDSCs), myeloid suppressor cells (MDSCs), N1 neutrophils (N1), N2 neutrophils (N2), Tumour necrosis factor alpha (TNF-a), Interferon c (IFN-c), chemochine receptor 4 (CXCR4), Vascular Endothelial Growth Factor receptor 1 (VEGFR1), Interleukin 8 (IL8), Human neutrophil antigen1,2,3,4,5 (HNA-1,HNA-2,HNA-3,HNA-4 and HNA-5), Interleukin-1 receptor antagonist (IL-Ra), Interleukin 1 beta (IL-1-b), Interleukin 12 (IL-12), Interleukin 10 (IL-10), Chemokine ligand 3 (CCL3),Chemokine ligand 2 (CCL2), B-cell activating factor (BAFF), A proliferation-inducing ligand (APRIL), Chemokine ligand 2 (CCL2), Toll-like receptor 2,4,5,8 (TLR2,4,5,7,8)

1.5.1. The neutrophil maturation states.

Neutrophils go through phenotypic changes that can be dictated by their maturation stage and the local environment in the peripheral tissues and circulation. The immature pool of neutrophils can be considered as precursors differing from mature neutrophils based upon their nuclear morphology, protein expression, capacity to proliferate and increased transcriptional activity (Theilgaard-Monch *et al.,* 2005; Kim *et al.,* 2017). These populations can be further characterised by several surface markers. In human, immature neutrophils express CD15 and CD11b, followed by increased expression of CD16 and CD10 on reaching maturity (Terstappen *et al.,* 1990; Marini *et al.,* 2017). The function of the immature neutrophils is to generate mature neutrophils under homeostatic conditions. However, during inflammation immature neutrophils expand in bone marrow and are released into the circulation and are associated with pathogenesis of different diseases. Singhal *et al.,* showed

that immature neutrophils were able to differentiate into an antitumor phenotype in response to reduced levels of GM-CSF and IFN-γ, as observed in the tumour microenvironment (Singhal *et al.,* 2016). Mature neutrophils in the circulatory system coming towards the end of their life, may obtain an 'aged' neutrophil phenotype, with reduced expression of CD62L and the presence of CXCR4 (Casanova-Acebes *et al.,* 2013; Zhang *et al.,* 2015). Studies in mice have demonstrated that CXCR4 expression is involved in homing to the bone marrow, which is important for neutrophil clearance (Suratt *et al.,* 2001; Furze *et al.,* 2008). However, an influence of the microbiome via bacterial products priming of TLRs and Myd88 drives the aged phenotype, and at the transcriptome level indicated an activated phenotype (Zhang *et al.,* 2015). This contributes to increased recruitment and phagocytic capacity during an inflammatory response (Uhl *et al.,* 2016). This particular phenotype may also promote neutrophil-mediated tissue damage. A correlation between mature neutrophil phenotype and disease pathogenesis in experimental endotoxemia and sickle cell disease has been demonstrated (Zhang *et al.,* 2015).

Immature neutrophils can differentiate into different types of cells due to the presence of transcriptional proteins that are not found in mature neutrophils and, this indicates different subsets. There is a probability that certain molecules can stimulate neutrophils to express different proteins allowing single population to display different activated phenotypes (Deniset *et al.,* 2018).

1.5.2. The transient functional subsets of neutrophils

Studies of more than 50 years ago proposed marginated groups of neutrophils adhered to blood vessels or are outside the blood circulation which can be mobilised by stimulation

(Athens *et al.,* 1961). Many studies have performed human experiments by radiolabelling neutrophils which identified the spleen, liver and bone marrow as reservoirs (Peters *et al.,* 1985; Ussov *et al.,* 1995). Whether these tissues are genuine reservoirs or due to longer transit times through these tissues is still an issue. Studies have suggested that a longer transit time through the lung is highly dependent upon whether the cells are activated (Hogg *et al.,* 1995; Summers *et al.,* 2014). Neutrophils roll along the blood vessel walls of the skin forming a marginated pool due to the basal expression of endothelial selectins. It is unclear why some but not all neutrophils roll along the blood vessels however, this may indicate a specific population of neutrophils.

Recent studies using intravital microscopy have confirmed the spleen and the lung to be the reservoirs of neutrophils and evidence now suggests that neutrophils found within these organs may have specialised roles (Deniset *et al.,* 2017). One population of neutrophils that seem to exist in the pulmonary capillaries was originally defined to be stuck as they moved through the lung microcirculation (Yipp *et al.,* 2017). Recent images have shown that in response to sterile injury in the liver neutrophils were shown to 1) move around and patrol 2) the cells may become immobilised or, 3) detach to re-enter the blood stream Wang *et al.,* 2017).

1.5.3. Circulating neutrophil populations

The mature neutrophil populations in the circulatory system have been defined in humans based on the expression of CD177 which is a **glycophosphatidylinositol** (GPI) associated glycoprotein and is expressed in the plasma membrane and on the membranes of specific neutrophil granules (Goldschmeding *et al.,* 1992). The function of CD177 is to act as membrane receptor and control the activity of the granule proteinase 3 (PR3) on the plasma

membrane, which can be an autoantigen in systemic vasculitis. The percentage of $CD177^+$ neutrophils differs among different individuals and 1-10% of individuals do not express $CD177^+$ cells at all, dependent upon the allelic frequency of a mutation in exon7 of the protein (Goldschmeding *et al.,* 1992; Wu *et al.,* 2016).

Olfactomedin-4 (OLFM4) is a glycoprotein found within specific granules of neutrophils (Clemmensen *et al.,* 2012;). OLFM4 mRNA is observed in precursor neutrophils and the protein is only detected in 20-25% of the circulatory neutrophils indicating expression controlled by transition membranes (Clemmensen *et al.,* 2012). Early investigations of OLMF4+ and OLMF4- neutrophils in individuals did not show significant differences in apoptosis, phagocytosis or migration to site of injury or inflammation, however, OLFM4+ neutrophils generated different qualities of NETs (Welin *et al.,* 2013).

A proangiogenic neutrophil (PAN) population has been identified in both healthy donors and wild type mice. PAN are defined by markers such as $CXCR4^+$, $VEGFR1^+$ and $CD49d^+$ and form a small proportion of total circulating neutrophils (3%) in human and mice (Massena *et al.,* 2015). The recruitment of PAN is dependent upon VEGF-A and CD49d is essential for retaining neutrophils at sites of hypoxia (Massena *et al.,* 2015). PANs have higher expression matrix metalloproteinase-9 in-comparison to pro-inflammatory neutrophils, which contributes towards the remodelling of the ECM (Christoffersson *et al.,* 2012). Studies have demonstrated that inhibition of PAN leads to delayed vascular development in an experimental hypoxia model (Massena *et al.,* 2015). This may have vital inferences for cancer as neutrophil derived MM9 has been associated with tumour angiogenesis (Nozawa *et al.,* 2006; Deryugina *et al.,* 2014). It is vital to understand whether PAN phenotype observed

during homeostasis is resulting from progenitor subsets or from the VEGF polarisation remains to be known.

1.5.4. The N1/N2 neutrophils

The M1 and M2 titles have been commonly used to describe pro-inflammatory and anti-inflammatory macrophage polarisation states in response to different stimuli. Fridlender *et al.,* first applied this idea to neutrophils to identify the pro-tumoural and anti-tumoural roles for tumour-associated neutrophils (TAN). Evidence suggested that TGF-β will induce mature TAN to change their function from a protumoural phenotype (N2) to an antitumuoral (N1) subset (Fridlender *et al.,* 2009). Other polarisation signals include angiotensin-II, type I IFNs and the proto-oncogene MET have the aptitude to drive N2 or N1 subsets from mature neutrophils to protumoural or antitumoural phenotypes Jablonska *et al.,* 2010; Shrestha *et al.,* 2016). Molecular analysis at transcriptomic level further confirmed that N1 and N2 neutrophils signify different populations (Shaul *et al.,* 2016) and N2 neutrophils vary from circulating PMN-MDSCs in tumour bearing mice (Fridlender *et al.,* 2012). A recent study reported that TGF-β mediated polarisation of mature high-density neutrophils to mature low density neutrophils in mice and allowed these populations being known as N1c and N2c (Sagiv *et al.,* 2015).

Markers of M2 macrophages are present on N2 including mannose receptor and chitinase Ym1 which may define this population. N2 have been implicated in cardiovascular disease and in stroke where PPAR-gamma agonist mediated protection in the brain in a mouse model was reliant on neutrophils and large numbers of N2 were observed (Cuartero *et al.,* 2013).

1.5.5. Low and normal density neutrophils (LDN and NDN) in diseases

Centrifugation of blood in a density gradient permits the separation of two distinct populations, low and normal density neutrophils, also known as high density neutrophils (Garley et al., 2018). LDN are found in the PBMC layer while NDN are found in the PMN section which sediments on top of the red blood cell pellet of the gradient (Fig. 8). Neutrophils have a half-life of few hours in the blood circulation but they successfully attain phenotypic heterogeneity before migrating into tissues. It has been suggested in vivo neutrophils alter their phenotype and morphology (Garley et al., 2018). LDN can be divided into different subtypes such as immature neutrophils, pro-inflammatory LDG and immune suppressive G-MDSC.

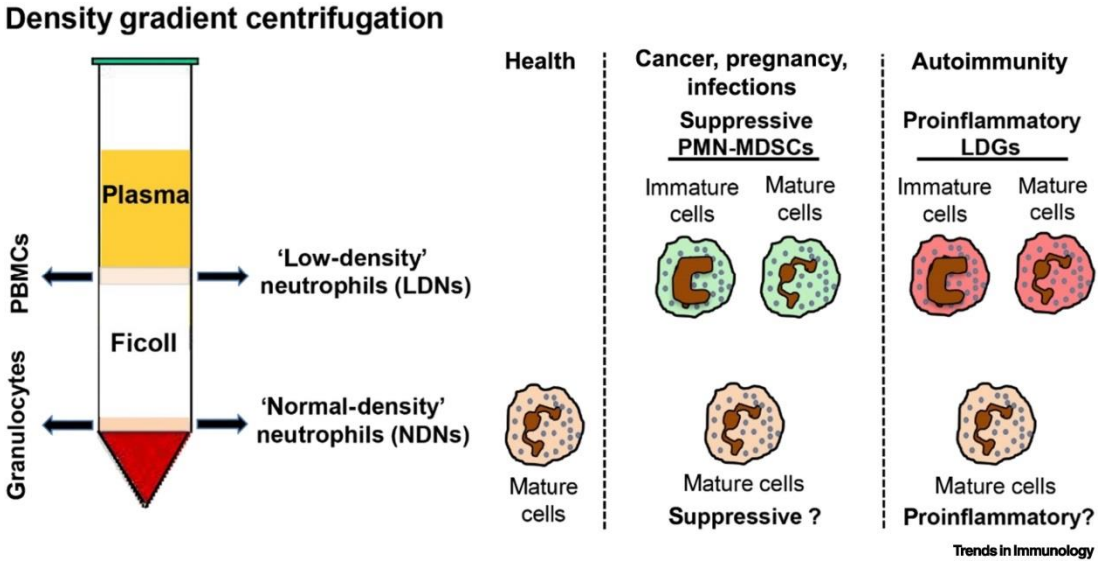


Figure 8--Ficoll-hypaque gradient of the main LDN populations 1) myeloid-derived suppressor cells (MDSCs), 2) low-density granulocytes (LDGs) (Silvestre-Roig *et al.,* 2019).

1.5.5.1. The immature phenotype of LDN

Increased numbers of immature neutrophils ($CD66b^{+}CD10^{-}$) in the circulation occurs during emergency granulopoiesis, artificially induced inflammation (e.g stimulation with lipopolysaccharide (LPS)) or during a disease state for e.g., sepsis, infection or cancer) (Lawrence *et al.,* 2018). Despite their immature state, these cells show functions such as chemotaxis and antimicrobial peptide production functions (Scapini *et al.,* 2016; Leliefeld *et al.,* 2018; Grinsven *et al.,* 2018). Leliefeld *et al.,* (2018) showed that like neutrophils with a segmented nucleus, the banded subset demonstrated effective migration throughout narrow pores, therefore, the nucleus did not prevent the banded subset from reaching an infection site. During acute inflammation hyper segmented ($CD16^{bright}/CD62L^{dim}$) and banded $CD16^{dim}$ neutrophils phagocytose bacteria. Grinsven *et al.,* (2018) demonstrated that $CD16^{low}$ neutrophil subset (immature neutrophils) did not show reduced migration in in vitro models of transendothelial and interstitial migration.

The findings suggested a neutrophil heterogeneity in their antimicrobial capacity and the presence of neutrophil subsets with a diversity of function during acute inflammation.

Neutrophils isolated from males that exhibit an immature phenotype are less activated and show a reduced capacity of ant-microbial responses such as NET formation in comparison to cells from females. These differences in maturity of the neutrophils between females and males has been suggested to contribute towards the exaggerated immune responses of females whilst beneficial with regards to microbial clearance being offset by increasing their susceptibility to autoimmune disease (Blazkova *et al.,* 2017).

Immature neutrophils can exhibit functional plasticity and immunoregulatory functions such as cytokine production by, and modulation and proliferation of $CD4^+$ and $CD8^+$ T cells, (Lang et al., 2018; Guerin *et al.,* 2014). Whether immature neutrophils signify a distinct neutrophil subpopulation remains an open question (Silvestre-Roig *et al.,* 2019).

1.5.5.2 Low density granulocytes (LDG)

Most of the knowledge with regards to neutrophil heterogeneity in chronic inflammation is derived from studies performed in autoimmune diseases such as systemic lupus erythematosus (SLE). The LDN derived from autoimmune diseases have been identified as low-density granulocyte according to their pro-inflammatory properties (Chatfield et al., 2018) and promotion of Th17 cell proliferation and differentiation (Wu et al., 2016).

Genetic analysis of purified neutrophil fraction of lupus LDGs expressed several different genes to other neutrophils groups (Rivera et al., 2013). Analysis showed that 302 genes were differentially expressed in LDG from lupus patients compared to cells from healthy controls, and 281 genes were altered when comparing each patient's LDG to their autologous lupus

neutrophils. Canonical pathway analysis of the altered genes showed actin cytoskeleton, macropinocytosis, clathrin-mediated endocytosis, and integrin signalling pathways in lupus LDG increased compared to normal density neutrophils.

Non-suppressive LDN may display pro-inflammatory function in thrombocytopenia syndrome patients showing severe fever (Scapini *et al.,* 2016; Li *et al.,* 2019). The proinflammatory phenotype of LDG in SLE was verified by their ability to release different cytokines such as necrosis factor alpha (TNF-α), interferon gamma (IFN)-γ and type I IFN which have been implicated in the pathogenesis of SLE (Denny et al., 2010).

In a second study up regulation of various genes, in LDG from patients, associated with serine proteases, bactericidal proteins, and other molecules present in azurophilic granules was observed in lupus LDG. By comparison, genetic analyses of lupus normal density neutrophils reveal no differences in gene regulation when comparing to cells from healthy control individuals (Villaneuva 2011).

A study by Wright *et al.,* showed that Rheumatoid Arthritis (RA) LDG were functionally different from RA neutrophils. $CD14^{pos}/CD15^{hi}$ are markers of mature neutrophils although mRNA transcripts showed an immature neutrophil characteristic (Wright *et al.,* 2016.) The expression of transcripts for granule proteins and cell-cycle genes and cytokine receptors especially TNFR, showed a lower expression in RA LDG compared to RA neutrophils, resulting in a reduced response to TNF-α in culture, which may a have a significant consequence for disease in patients with RA, as TNF-α is involved in disease progression and anti-TNF therapy is used to treat severe RA (Wright *et al.,* 2017).

In mice, neutrophil heterogeneity has been linked to the nature and severity of the insult or pathogen (Tsuda *et al.,* 2004). In methicillin-resistant *S. aureus* infection pro-inflammatory, $CD11b^{-}CD49d^{+}IL^{-}12^{+}$; anti-inflammatory, $CD11b^{+}CD49d^{-}IL^{-}10^{+}$ cell populations have been reported (Tsuda *et al.,* 2004).

Neutrophils with antigen-presenting activities are produced during a microbial infection and after stimulating tissues with pathogens such as *S. aureus* a subpopulation of $CD11b^{high}$ $CD62L^{low}$ $CXCR2^{low}$ neutrophils were able to promote adaptive immunity (Hampton *et al.,* 2015).

Sterile injury or infection induce a $CD54^{+}$ neutrophil subpopulation that was able to retro-transmigrate from the affected tissue to the bloodstream, and enter other organs causing further damage, but also showing an elevated ability to attack pathogens (Woodfin *et al.,* 2016).

1.5.5.3 Granulocytic myeloid derived suppressor cells (G-MDSCs)

While the nomenclature is still confusing LDN can be separated into the myeloid derived suppressor cells (MDSC) subpopulation which are immunosuppressive (Scapini, *et al.,* 2016). G-MDSCs are classified as immunoregulatory due to their ability to suppress T cell proliferation in autoimmune disease, infection, and metabolic diseases (Bowers *et al.,* 2014). G-MDSC display immunosuppressive mechanisms by surface expression of many different checkpoint inhibitors such as programmed death-ligand 1 (PD-L1), programmed death-ligand 2 (PD-L2), and CD73. The suppressor cells also release many different enzymatic or chemical mediators such as (arginase-1 [Arg1] and nitric oxide synthase (Solito *et al.,* 2014).

1.5.5.4 Normal density neutrophils

Systemic inflammation induces a population of mature hyper-segmented neutrophils within the NDN population. Like LDN, in NDN a subpopulation of mature activated suppressive PD-L1^{+} neutrophils that inhibit T cell functions has been reported (Bowers *et al.,* 2014). CD10^{+} neutrophils that constrain T cell functions by releasing ARG1 controlled by CD11b have been observed in LDN and NDN blood segments of patients diagnosed with HIV-1 infection (Marini *et al.,* 2017). The immunosuppressive properties include inhibiting T cell proliferation via production of ROS in an CD11B-dependent manner (Hellebrekers *et al.,* 2018). Together the data supports a mature activated suppressive population within the NDN fraction (Silvestre-Roig *et al.,* 2017).

1.6. Ocular disease

1.6.1. Ocular immune privilege

The eye is an immune privileged site due to several different protective mechanisms to protect and prevent any complications with regards to visual activity (Zhou *et al.,*2010; Caspi 2010). The protective mechanisms include the physical barriers encountering efficient blood-retina barrier and lack of efferent lymphatics. The physical barriers control entry and exit of cells and large molecules into and out of the eye. An immunosuppressive ocular microenvironment is sustained by cell bound and soluble TGF-β, alpha-melanocyte-stimulating hormone (α-MSH) and vasoactive intestinal peptide, within the eye which as a result inhibits the role of immune-competent cells. Damage to these mechanisms by inflammatory processes can damage the highly organised and complex structures of the eye

(Fig.9), resulting in visual distortion of the whole visual axis through the anterior chamber, lens and vitreous body to the retina (Zhou *et al.,* 2010; Caspi 2010).

1.6.1.1. The anatomy and physiology of the eye

The eye is classified as on the most complex organs of the human body and consists of three distinct layers. The outer region contains the cornea and the sclera. The function of the cornea is to deflect and transmit light to the lens, provides protection against infections and prevents structural damage to the deeper origins (Willoughby et al., 2010, Müller et al., 2007). The connective tissue knowns as the sclera is formed to protect the eye from internal and external forces, is involved in retaining its shape and is connected to the cornea via the limbus. The visible part of the sclera is a transparent mucous membrane known as conjunctiva. The intermediate layer of the eye is made up of the iris, ciliary body, and the choroid. The function of the iris is to control the size of the pupil and therefore the amount of light reaching the retina (Willoughby et al., 2010). The ciliary body regulates the power and shape of the lens and is responsible for production of aqueous humour. The choroid is defined as a vascular layer that delivers nutrients to the outer retinal layers.

The inner part of the eye, the retina, is a complex layered structure of neurons that encapsulates, and processes light delivered through the lens. Rod and cone cells process the light and transfer the signal to neural cells which pass the signal via the optic nerve to the optical chiasma, where the Image is defined. The retina is protected by the vitreous fluid which helps to maintain retinal structure (Willoughby *et al.,* 2010).

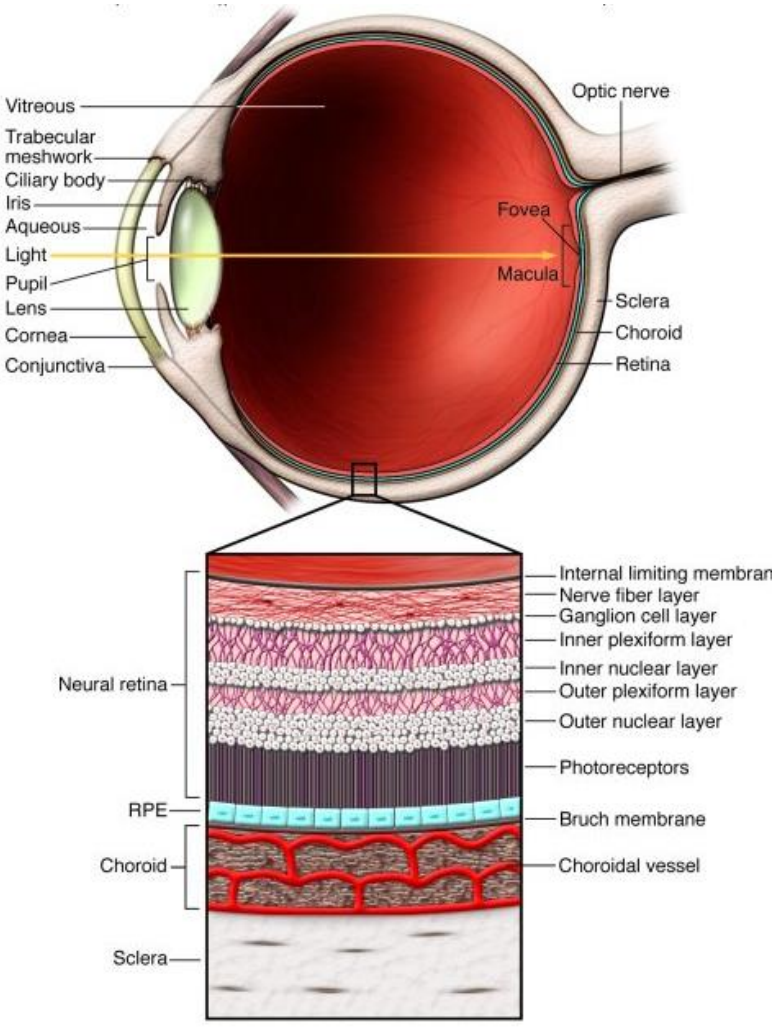


Figure 9-The anatomical structure of the eye. An expanded section of retina and uvea displaying anatomical layers. Light passes via ocular media and concentrates on the area of retina accountable for colour and vision. The photoreceptors cells sense the signals and transmit the signals to the brain through the optic nerve. Therefore, any damage to the ocular

structures along the visual axis lead to visual deficit. Retinal pigment epithelium (RPE). (Caspi 2010).

1.6.2. Behçet's Disease.

1.6.2.1. The background of Behçet's Disease

Behcet's Disease (BD) is a complex systemic inflammatory disorder, consisting of oral aphthous ulcers, genital ulcers, papulopustular and erythema nodosum-like skin lesions, uveitis, retinal vasculitis, thrombophlebitis, arterial aneurysms, and arthritis. The disease is found primarily along the ancient Silk Route from the Mediterranean Basin across Asia to Japan. The prevalence of BD in UK is around 0.64/100,000, whereas in Istanbul 370/100,000 individuals are affected by this disease. A BD-like condition was first described by Hippocrates in the fifth century. In recent times the first case of BD was presented to the Medical Society of Athens in 1930 by Dr Benediktos Adamantiades as relapsing iritis with hypopyon, and oral ulceration In 1946, it was discovered that thrombophlebitis as the fourth main sign of BD disease (Verity *et al.,* 1999a; Zouboulis, 2002).

Dr Hulusi Behçet formally published the clinical symptoms of BD in 1936 by reporting a sequence of three medical cases (Verity *et al.,* 1999a). He originally illustrated a triad of BD symptoms as genital ulcers, oral aphthae, and hypopyon uveitis and named it as Behçet's triple complex syndrome (Gray, 1950). The term Behçet's Disease was formally accepted at the International Congress of Dermatology in 1947 (Verity *et al.,* 1999a).

Currently Behçet's Disease is characterised by mucosal inflammation, oral, genital and gut ulceration, and infiltration of immune privileged sites including the eye, brain and synovial joints (Zeidan *et al.,* 2016). BD can induce severe manifestations in the cardiovascular system,

causing systemic vasculitis connected with increased morbidity and mortality, most common in males with early age onset (Mendes *et al.,* 2009). BD disease can develop before puberty but is most likely to develop between the third and fourth decades of age.

There is no diagnostic tool, blood or serum biomarker to detect or measure the severity of BD (Zeidan *et al.,* 2016). Earlier age onset is associated with more acute clinical manifestations and mortality (Verity *et al.,* 1999a). Currently, no specific treatment is designated for BD and those available can only relieve symptoms in order to control disease progression. To manage the symptoms of the disease, systemic anti-inflammatory and/or immune-modulating drugs are prescribed to patients (Sfikakis *et al.,* 2007).

1.6.2.2. Clinical manifestations of BD

Chronic lesions of the mucus membranes are considered to be the hallmark of BD. This includes oral ulcers (in the tongue, pharynx and mucosal membranes) genital ulcers (on scrotum in men and vulva or vagina in women). Oral ulcers occur in 98% of BD patients and genital aphthae occur in 60-65% of patients and together are indication of the diagnosis of BD (Lancet *et al.,* 1990). The cardiovascular manifestations of BD encounter symptoms of vascular inflammation including the arterial and venous systems; however, venous disease is more common with patients with BD and it occurs in 30% of the cases. Patients diagnosed with BD exhibit arthralgia and arthritis in 45% of cases, affecting mainly knees and ankles (Lancet *et al.,* 1990). Other clinical manifestations may include neurological involvement in 20-40% of the cases by showing parenchymal and non-parenchymal lesions (Wechsler 2002). Patients can present with gastrointestinal involvement commonly affecting the terminal ileum and the caecum, but can affect any site from the oesophagus to the anus. These lesions

form penetrating ulcers that have the capacity to perforate. The symptoms of gastrointestinal involvement are acute abdominal pain, constipation, and diarrhoea (Skef *et al.,* 2015). A few BD patients exhibit otological symptoms leading hearing loss and disequilibrium (Greco *et al.,* 2018).

1.6.2.3. Diagnosis of BD.

Presently, BD is defined with the vasculitides with severe inflammation of the blood vessels that may involve nearly all of the tissues of the body, especially the eye, mucous membranes, musculoskeletal, vascular and central nervous systems (Escudier *et al.,* 2006; Evereklioglu, 2005). Cardiovascular, gastrointestinal and neurological symptoms are most associated with the highest mortality and morbidity (Verity *et al.,* 1999b; Talarico *et al.,* 2012).

The diagnosis of BD is mainly based upon clinical assessment and therefore, standardising the diagnosis of BD is vital for precise international studies with regards to treatment, epidemiology, and the development of the disease. As a result, sixteen various diagnostic and categorisation criteria have been recommended to date to standardise and classify BD the first by Curth in 1946 (Curth 1946). Today, two out of sixteen criteria, the result of international collaboration are mainly used for BD diagnosis (Lancet, 1990; Davatchi *et al.,* 2012)

Researchers in the UK, follow the diagnostic and classification criteria described by the International Study Group (ISG) to regulate the diagnosis of BD for research projects and to certify that only well-defined cases are included in research (Lancet, 1990). The criteria discovered was a result of collaboration between different countries (France, Iran, Japan, Turkey, UK and USA) and as a result developed a consensus on BD classification and diagnosis.

In order to diagnose by the ISG criteria, oral ulceration and at least two of extra manifestations need to be present. ISG has a sensitivity of 92% and specificity of 97%) (ISG, 1992, Lancet, 1990) (Table 4).

Table 4-The ISG diagnostic and classification criteria of BD (ISG,1990).

The main criteria include frequent ulcerations on the oral mucosa
Extra Criteria
Skin involvement
Ocular involvement
Frequent episodes of genital ulceration
Positive pathergy reaction

international Study Group (ISG)

A more current criteria known as the International Criteria for Behçet's disease (ICBD) was recommended in 2006 and reported 2010 (Davatchi *et al.,* 2010a). The ICBD was a result of collaboration of countries such as China, Austria, Egypt, Japan, Jordan, Pakistan, Azerbaijan, Libya, Morocco, Saudi Arabia, Taiwan, Singapore, Spain, Taiwan, Turkey, USA and Thailand. Originally, there were two different types for the ICBD; 1) the traditional organisation, 2) the classification tree and only the traditional organisation is currently acknowledged. According to the traditional format a straightforward scoring system is applied and patients scoring 3 or more points are classified as having BD (Table 5).

Table 5-The diagnostic criteria proposed by ICBD (2006)

The criteria of scoring one point:
1) Skin involvement
2) Vascular involvement
3) Pathergy positive test
4) Oral ulcers
The criteria of scoring two points:
1) Genital ulcers
2) Ocular Manifestation

The International Criteria for Behçet's Disease (ICBD)

ICBD criteria were developed to cover patients who may have BD-like disease but without oral ulceration. While of use in certain countries according to the author's opinion the ICBD criteria can lead to over diagnosing BD.

Currently, a specific recognised laboratory test for BD is not established. The pathergy test which evaluates reactivity of the skin to a sterile injection or needle prick forms an element in both the ISG and ICBD. Therefore, the procedure of the pathergy test is carried out by puncturing the skin with 25 or 21-gauge needle. Certain centres use three tests such as 25-gauge needle enclosing intradermal injection with sterile saline, 21- and 25-gauge needle without injection (Davatchi *et al.,* 2010a). The reaction occurred is measured after 24 and 48 hours. However, the pathergy test is used more in some countries with BD, including Turkey and Iran, but is not commonly assessed in the UK.

In order to diagnose BD Greco *et al.,* suggested that at least two major symptoms should be present to make differential diagnosis. These signs include oral mucosa, genital ulcerations, uveitis and aphthous-like ulcerations (Kaneko *et al.,* 2014; Greco *et al.,* 2018).

An activity scoring system, Behçet's disease current activity form, was established as a result of the International Scientific Committee. BDCAF scores genital ulcers, skin involvement, oral ulcers, joint involvement, gastrointestinal involvement, fatigue and headache relating to the time of symptoms. The index recordings with regards to large vessel and CNS involvement and Behçet's oculopathy are also taken. The opinions of patients and clinicians on the disease activity are rated on a 7-point scale (Bhakta *et al.,* 1999).

Laboratory studies have demonstrated a higher production of C-reactive proteins (CRP), serum neopterin and erythrocyte sedimentation rate (ESR) in patients with active BD in comparison to patients with inactive BD or healthy controls. However, these markers are increased in other inflammatory and infectious disorders and therefore, are not specific for BD (Coskun *et al.,* 2005). Total homocysteine and soluble necrosis factor receptor (TNFR) levels and endothelial cells in the circulation have also been linked to disease activity in BD but are also not specific to this condition (Sarican *et al.,* 2007; Kutlay *et al.,* 2008; Turan *et al.,* 2008). Currently, a specific international consensus on activity markers does not exist to diagnose BD, and as such diagnosis continues to be based on activity criteria.

1.6.2.4. Epidemiology of BD

The highest prevalence of BD patients is found along the Silk Road, or their descendants lived in countries through which the Silk Road passed, including Turkey, Iraq, Iran, China and Japan as well as including other countries. The "Silk Road" is an historical land trading route that connects East, South and West Asia with Mediterranean countries, European countries and North and East Africa, and by a sea route from the Red Sea to East Africa, and Southeast Asia (Ohno *et al.,* 1982; Verity *et al.,* 2003). BD is most predominant in Turkey (420 per 100,000

individuals are affected), while the UK has a prevalence of (0.3 to 0.6 per 100,000 individuals) (Davatchi *et al.,* 2010a; Leccese *et al.,* 2017).

Table 6-The prevalence rate of BD worldwide (Mohammad *et al.,* 2013 and Leonardo *et al.,* 2015).

Country	***Prevalence***	***Reference***
United Kingdom	0.3-0.6 per 100,000	Leccese *et al.,*2017
Turkey	420 per 100,000	Davatchi *et al*., 2010a
USA	0.38 per 100,000	CALAMIA *et al.,* 2009
Iran	80 per 100,000	Mohammad *et al.,* 2013
Saudi Arabia	20 per 100,00	Mohammad *et al*., 2013 and Leonardo *et al*., 2015
Iraq	17 per 100,00	Mohammad *et al.,* 2013
Israel	15.2 per 100,00	Mohammad *et al.,* 2013
Japan	13.5 per 100,00	Mohammad *et al.,* 2013 and Leonardo *et al.,* 2015
France	7.1 per 100,00	Mohammad *et al.,* 2013 and Leonardo *et al.,* 2015
Sweden	4.9 per 100,00	Mohammad *et al.,* 2013 and Leonardo *et al.,* 2015
Germany	2.26 per 100,00	Mohammad *et al.,* 2013 and Leonardo *et al.,* 2015
Portugal	1.53 per 100,00	Mohammad *et al.,* 2013 and Leonardo *et al.,* 2015

BD usually affects young individuals between second and fourth decade of life but the onset can occur any stage of life (Table 4) (Mundy *et al,* 1978; Kone-Paut *et al.,* 2011).The countries such as Iran, Japan, China, Korea and Germany have a national survey for BD and declare the mean age of onset of BD is 26.2, 35.7, 33.8, 29, and 26 years respectively (Zouboulis *et*

al., 1997; Davatchi *et al.,* 2010a; Davatchi *et al.,* 2010b). BD was originally classified as being more predominant in men than women along the "Silk Road countries" (Zouboulis *et al.,* 1997) however, the trend is now reversed in Western Europe and the USA (Evereliogl u, 2005). Currently, there seems to be almost no gender differences **(in mean values)** in most populations diagnosed with BD (Table 7).

Table 7-Age and gender ratio of individuals diagnosed with BD worldwide (Oguz *et al.,* 2017).

Country	*Age of onset*	*Male/female ratio*	*Reference*
Turkey	37.2–38.02	0.69–1.03	Gul, 2005; Türsen *et al.,* 2003
Japanese	36.8	0.74	Kirino *et al.,* 2016
Iran	26.2	1.4	Davatchi *et al.,* 2010
Israel	34.9	1.22	Krause *et al.,* 1998
Netherlands	43	0.64	Kappen *et al.,*
Senegal	32	1.6	Ndiaye *et al.,* 2015
Tunisian	29.12	2.1	Hamzaoui *et al.,* 2014
Germany	24.5–27.4	1.51–1.38	Zouboulis *et al.,* 1997 ; Bonitsis *et al.,* 2015
Saudi Arabia	29.3	3.4	al Dalaan *et al.,* 1997
China	35.8	1.4	Zhang *et al.,* 2013
Italy	33	1	Salvarani *et al.,* 2007
England	32	0.96	*Chamberlain et al., 1997*
USA	29.25	0.3	Davari *et al.,* 2016

United states of America (USA)

1.6.2.5. Ocular involvement in BD

In patients diagnosed with BD, 70% cases show ocular involvement and substantial morbidity. Anterior uveitis in ***BD patients is represented as hypopyon***, a visible white thick exudate in the lower part of the anterior chamber of the eye. Persistent attacks of hypopyon can lead to cataract or glaucoma (Davatchi *et al.,* 2010b, Soloway *et al.,* 1996). Persistent posterior uveitis and vasculitis of the retinal veins can lead to visual loss in BD patients. The ocular association in BD involves chronic, relapsing bilateral non-granulomatous uveitis that includes the anterior segment, posterior segment or both (Mendes *et al.,* 2009). Ocular complications of BD include cataract, glaucoma, virtutis, retinitis and retinal detachment.

1.6.2.6. Pathogenesis Behçet's Disease

1.6.2.6.1. The genetic theory

The geographic distribution **of** BD correlates with the prevalence of an allele of the major histocompatibility complex (MHC) locus (HLA-B*51), and is the strongest genetic association with BD (Verity *et al.,* 1999; Remmers *et al.,* 2010). There is a stronger association of HLA-B*51 with Turkish and Japanese patients in comparison to Europeans (Verity *et al.,* 1999). Many studies have identified that a clear association of HLA-B*51 with BD and it concluded that more than 60% patients diagnosed with BD are positive for HLA-B*51 (Bodis *et al.,* 2018).

Other genes situated in the MHC locus such as HLA-B*5701, or in close proximity, *MICA*, *TNF*, have been associated with BD however, their contribution is believed to be linked to disequilibrium with HLA-B51 gene (Marshall 2004). Many other genes that **are** located outside the MHC region that are evaluated to be involved in the pathogenesis of BD. GWAS studies identified common variations in *IL10*, *IL23RIL12B2* genes with BD (Remmers *et al.,* 2010). IL-

23 is a proinflammatory cytokine and is involved in stimulating Th17 proliferation, linked to neutrophil release from the bone marrow, and expression of IL-23 p19 mRNA in erythema nodosum-like skin lesions in patients with active BD has been reported (Lew *et al.,* 2008). Deep phenotyping and imputation of GWAS data identified *CCR1, STAT4, KLRC4, IL1A/ILB* as new associations with BD (Kirino *et al.,* 2013; Takeuchi *et al.,* 2017). Kirino *et al.,* further reported variants in *ERAP1* associated with BD and a **haplotype** based on these variants was reported as a strong risk factor for BD (Kirino *et al.,* 2013; Takeuchi *et al.,* 2017). Studies on Turkish and Iranian patients identified mutations in FUT2, encoding a protein involved in fucosylation in the gut, with BD, as result validated by in a second study on Turkish patients (Xavier et al., 2015; Takeuchi *et al.,* 2017). Analysis of the genetics of BD show that most genes involved with the condition can be classed as immunoregulatory. However, while a few mutations such in factor V Leiden gene have been associated with specific manifestations of BD, thrombosis and ocular symptoms, the majority do not (Verity *et al.,* 1999; Deng *et al.,* 2018).

1.6.2.6.2. Infectious theory of BD

Bacterial and viral pathogens have been implicated as environmental triggers of BD. *Streptococcus sanguis* association was suggested by clinical studies that showed BD patients had a higher incidence of tonsillitis, dental caries and treatment with antibacterial drugs showed positive effects on patients diagnosed with BD (Lehner *et al*., 1991; Mumcu *et al*., 2007).

Polymerase chain reactions (PCR) detected Herpes simplex virus type-1 (HSV) in saliva, intestinal ulcers, and genital ulcers in BD patients (Lee, 1996; Sohn 1998). A BD-like mouse

model was developed by injecting HSV into mouse earlobes, and HSV DNA sequences were found in ulcerative injuries (Sohn *et al*., 2001). However, to date there is no consistent evidence of a specific infectious agent in BD pathology.

1.6.2.6.3. Immunologic theory of BD

Blood vessel endothelial cells are targeted in vasculitic conditions Anti-endothelial cell antibodies (AECA) have been identified in some patients with BD, with enolase as a possible target (Lee *et al*., 2003). AECA binding to FcRgIII cooperate to enhance neutrophil binding although this has not been proven in BD (Florey *et al*., 2007). T lymphocytes have been implicated in the pathogenesis of BD and have an activated phenotype and many inflammatory cytokines are produced (Alipour *et al*., 2017). IL-17 expression by $CD8^+$ and $\gamma\delta^+$ T cells was higher in BD compared to healthy controls, as was IFN-γ production by $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells. As stated, *IL23R* gene mutations associated with BD could be related to IL-17 production, which in turn can induce neutrophilia, also seen in BD (Deniz *et al* 2017; Pineton 2012). MHC class I molecules such as HLA-B*51 mediaite natural killer cell activity via interaction with killer-inhibitory receptors (KIR) association with BD (Petrushkin *et al.,* 2015). Recent analysis of KIR3DL1 identified haplotypes both for susceptibility and resistance to BD, although functional studies have yet to confirm these effects (Petrushkin *et al*., 2019).

1.6.2.7. The treatment of BD

The current standard treatment for BD is as monotherapy or combined with immunosuppressant drugs (Greco *et al*., 2018).Corticosteroids are used a topical therapy to treat ocular and mucocutaneous disease or systemically dependent on organ involvement (Evereklioglu 2005) (Table. 8). Recently drugs classified as biologicals have been used in

patients with BD (Table. 9). Most common biological treatment is TNF blockade which protects against vasculitis and leukocyte infiltration of tissues (Park *et al.,* 2018). Interleukin-1 inhibitors, anakinra, canakinumab, and gevokizumab have been trialled in small studies with mixed results (Bettoli *et al*., 2019). Interferon 2α is used if corticosteroids and immunosuppressants fail. Treatment increased T-reg numbers but not IL-10 production, decreased Th17 and increased monocyte activity, although there are significant side-effects (Albayrak *et al*., 2019) To date, treatment regimens remain guided by clinical assessment as there are no genetic or laboratory biomarkers to aid selection.

Table 8-The types of topical treatment used for BD (Mendes *et al.,* 2009; Scherrer *et al.,* 2017).

Type of treatment	*Dose*	*Indications*
Prednisone	Local systemic: 0.5–1 mg/kg	Uveitis, Mucocutaneous involvement, Neurological involvement, Refractory arthritis, and Gastrointestinal ulceration
Colchicine	0.5–1.5 mg/day	Skin involvement, Arthritis
Dapsone	100 mg/day	Mucocutaneous involvement and Arthritis
Azathioprine	2–3 mg/kg/day	Uveitis
Pentoxifylline	1200 mg/day	Mucocutaneous involvement
Sulfasalazine	1–3 g/day	Mucocutaneous involvement, Arthritis
Thalidomide	100–200 mg/day	Refractory mucocutaneous involvement
Cyclosporine	3–5 mg/kg/day in divided doses	Uveitis, Mucocutaneous involvement
Methotrexate	7.5–15 mg/week	Arthritis, uveitis (rarely)
Cyclophosphamide	750 mg/m 2/mo IV	Life-threatening involvement (vasculitis, neurological)

Behçet's Disease (BD)

Table 9-The types of biologics used for BD.

Type of treatment	*Dose*	*Indications*
Infliximab (TNF-a blocker)	5 mg/kg/day	ocular inflammation
Fusion protein, etanercept (TNF-a blocker)	25 mg / twice a week	Oral ulcers and nodular and papulopustular lesions
Adalimumab (TNF-a blocker)	40mg/week	Uveitis
Anti-IL-1 (IL-1 blocker)	-	vasculitis

Adapted from Daele *et al.,* (2009), Sfikakis *et al.,* (2004) and Botsios *et al.,* (2008) Behçet Disease (BD) , Tumour necrosis factor (TNF-a), Interleukin 1 (IL-1)

1.6.2.8. The role of neutrophils in BD

A study by Eksioglu-Demiralp *et al.,* (2001) showed that neutrophils are active in vivo in BD. Oxidative burst and phagocytic function of neutrophils was measured in blood from patients diagnosed with BD and expression of neutrophil activation markers such as CD10, CD14 and CD16 was evaluated by flow cytometry. The results showed a reduced production of ROS, unchanged phagocytic index, while markers of activation were elevated in BD patients. It was suggested that neutrophils in BD may have a pre-activated state and contribute towards the pathology of the disease (Eksioglu-Demiralp *et al.,* 2001).

The enzyme myeloperoxidase (MPO) is situated in the azurophil granules and is a key element of neutrophil phagocytic system. MPO levels in plasma and in supernatants of neutrophil cultures from patients diagnosed with BD, showed enhanced production of MPO suggested that hyperactivity of neutrophils is an important factor in the pathology of vasculitis associated to BD (Accardo-Palumbo *et al*., 2000).

Whether neutrophil hyperactivity or activation affects fibrinogen modification and thrombosis in patients with BD, was addressed by measuring oxidative markers and

evaluating fibrinogen function and structure. The results of the study showed that circulating neutrophils from patients with BD patients generated a higher production of ROS and NADPH compared to healthy controls. The study further demonstrated that these two findings significantly correlated with fibrinogen clotting ability and results provide a valid link between neutrophil activation and thrombosis in BD (Becatti *et al*., 2016).

Neutrophil-to-lymphocyte (NLR) ratios or values are used to predict or indicate enhanced inflammation. NLR, platelet-to-lymphocyte ratio (PLR), and mean platelet volume (MPV) in patients with BD were investigated. The results showed that NLR and PLR were significantly higher in BD patients in comparison to healthy individuals. However, in comparison to other ratios investigated, multivariant analysis defined NLR as an independent parameter **of** BD, and proposed NLR as a diagnostic biomarker for BD; a conclusion needs to be supported with larger studies (Alan *et al*., 2015). (CD64), a marker of activation is upregulated on neutrophils in reaction **to** proinflammatory cytokines and microbial wall components specifically in patients with BD, or infectious disease in comparison to patients with inactive BD and healthy controls. The authors suggested that neutrophil CD64 expression is increased during exacerbation of BD as a non-specific inflammatory response and not just in response to pathogens (Ureten *et al*., 2005). This indicated that hyperactivation of neutrophils is a pure disease affect as opposed to an inflammatory response triggered by a living micro-organism.

Anterior uveitis is the most common ophthalmological finding in BD and an investigation of 68 patients with BD and anterior uveitis, showed an increase in NLR in patients compared to healthy individuals (Avci *et al*., 2017).

Acute iridocyclitis with hypopyon is a feature of the active phase of BD which relapses naturally. Hypopyon compromises of inflammatory cells penetrating the eye with neutrophils as being the main component. Investigation of neutrophil death in BD showed that spontaneous apoptosis of neutrophils was reduced in the remission phase of uveitis despite the stimulation with LPS, anti-TNFα antibody or Fas:Fc fusion protein. Why circulating neutrophils in patients BD are more resistant to apoptotic cell death in the remission phase of uveitis in BD patients remains to elucidated (Fujimori *et al*., 2007).

A role for neutrophil extracellular traps in vasculitis in patients with BD has recently been proposed. Isolated neutrophils, from Middle Eastern patients with active BD, produced more extracellular DNA *in vitro* without stimulation, compared to healthy controls. In sections of skin lesions from patients' cell-free DNA was identified around affected blood vessels. Overall, the findings suggested that NETs contribute towards vasculitis in BD (Safi *et al*., 2018).

In a second study serum from BD patients contained significantly higher cfDNA levels and MPO-DNA complexes compared to healthy controls and patients with inactive BD and healthy controls. Thrombin generation in BD plasma was elevated and positively correlated with levels of MPO-DNA complexes and cfDNA positively correlated with thrombin generation (Le Joncour *et al*., 2018).

The results suggested that NET production and NET markers in BD contribute towards the procoagulant state. Therefore, future research is needed in terms of targeting NETs which may signify a potential therapeutic target for the reduction or prevention of thrombotic risk in BD patients. These studies will be discussed in more detail in future chapters.

The subsequent lack of tissue impairment in sites such as the anterior chamber of the eye and in joints where the arthritis is non-erosive, contrasts that in vascular tissue where inflammation caused by the release of oxidants and proteases leads to severe outcomes may be due to neutrophil function, but the mechanisms need to be addressed.

1.6.3. Ocular Mucous Membrane Pemphigoid

1.6.3.1 Background of Ocular Mucous Membrane Phemogoid

Mucous membrane pemphigoid (MMP) is described as a diverse group of autoimmune subepidermal blistering disorders that affects mucous membranes at the cavities, including the ocular, oral, nasopharyngeal, tracheal, oesophageal, anogenital, and genitourinary (Ong *et al*., 2018; Saw *et al.,* 2008). The prevalence rate of OcMMP in the UK is 0.8 per 100,000 and occurs in elderly individuals (60-80 years) (Kourosh *et al*., 2011). Cicatrising conjunctivitis (CC) is an unusual sight-threatening group of disorders which is commonly found in patients with OcMMP. A study by Radford *et al*., (2012) showed that 61% of the CC cases is caused by OcMMP equating to an incidence of 0.8, 0.2, and 0.2 per million in the United Kingdom.

Ocular mucous membrane pemphigoid (OcMMP) describes ocular involvement of MMP which causes chronic dry eye and permanent cicatrisation of mucosal surfaces of the conjunctiva and cornea (together forming the ocular mucosa) that can ultimately lead to blindness, due to ocular surface failure. OcMMP is characterised by subepithelial fibrosis, symblepharon, (adhesion of the tarsal conjunctiva to the bulbar conjunctiva), trichiasis (eyelashes growing inwards to the eye) fornix shortening, scar formation and keratinisation (Kirzhner *et al.,* 2011). Ocular mucous membrane pemphigoid is one the most difficult ocular

surface conditions to manage and a result; early corrective treatment can prevent irreversible blindness in OcMMP patients (Saw et al*.,* 2008; Dart 2017).

1.6.3.2. The pathogenesis of OcMMP

The pathogenesis of OcMMP remains unclear. The major gene polymorphism associated with the condition is HLA-DQB1*0301 (Xu *et al*., 2013). The heritance of the HLA-DQB1*0301 for OcMMP is dominant and may contribute to the production of autoantibody against basement membrane antigen (Ahmed *et al*., 1991). Type 2 hypersensitivity reactions against the basal epithelial membrane of the conjunctiva play a role in the pathophysiological mechanism of OcMMP (Pflugfelder *et al*., 2004).

The cause of scarring in OcMMP patients results from persistent inflammation including T cells, macrophages and dendritic cells (DC) (Sacks *et al*., 1989). In a diseased tissue the levels of pro-inflammatory cytokine such as TNF, IFNγ, Interleukin 5 (IL-5), Interleukin 13 (IL-13) and Interleukin 17 (IL-17) and levels of profibrotic cytokines (TGF-beta (TGF-β) and Interleukin-4 (IL-4) are increased. It was also demonstrated that OcMMP fibroblasts encounter a profibrotic phenotype in vitro which may result from inflammation encountered with the activity of chronic profibrotic fibroblast (Ahadome, *et al*., 2016a).

The conjunctiva is the ocular surface mucosa combined of superficial epithelium overlying loose connective tissue. The epithelium is non-keratinised, stratified, secretory epithelium. In ocular disorders such as OcMMP the non-keratinised condition of the conjunctival epithelium vital for its health, is lost undergoes squamous metaplasia and changed to a non-secretory, keratinized epithelium. Furthermore, loss of goblet cells amplified cellular stratification, expansion of superficial cells and reduced limbal stem cells (Pflugfelder *et al* 2004). It is

suggested that in the conjunctiva that fibrosis eventually leads to blindness in OcMMP. The secondary fibrogenic role of DCs via T cell activation and inflammatory cell recruitment is well known. A study by Ahadome *et al*., (2016b) showed that a direct induction of fibrosis by DCs. This study exhibited that classical $CD11b^+$ DCs in the ocular mucosa demonstrated an increase in the aldehyde dehydrogenase (ALDH). The enzyme ALDH is required for the synthesis of retinoic acid (RA), RA is a vital biological molecule involved in vision and encounters a immunologic function. The role of RA is also described in fibroblast growth, proliferation and production of extracellular matrix proteins such as collagen. The results in this study further showed that $CD11b^+$ DCs derived ALDH was related with 9-cis-retinoic acid ligation to retinoid x receptor (RXR), which encouraged conjunctival fibroblast activation. *In vivo* the results showed that stimulating RXR controlled rapid onset of ocular mucosal fibrosis and inhibiting ALDH activity in DCs or reducing DCs depleted fibrosis.

Pemphigus diseases (PD) are also mucocutaneous disorders categorised by blistering lesions of the mucous membranes of the skin, oral cavity and ocular mucosa (Bystryn *et al.,* 2005). PD and MMP are characterised by the presence of autoantibodies that react with antigens located on the cell surface of keratinocytes, causing intraepithelial and subepidermal blister formation (Broussard *et al*., 2015).

Fundamental disease mechanisms in OcMMP are driven by autoantibodies to the hemidesmosome subunits. This includes the epiligrin (the subunit of laminin 5), bullous pemphigoid antigen 1 [BP230], and bullous pemphigoid antigen 2 [BP180]) at the basement membrane zone (BMZ) (Rice *et al.,* 1990; Bernauer *et al*., 1993; Chan *et al*., 2002). Th2 $CD4^+$ cell infiltration and production of TGF-β and IL-13 contribute to antibody production. In

severe conjunctival inflammation, there is an increase in the CD4:CD8 ratio, a response also seen in murine models of Sjögren's syndrome (Bernauer *et al*., 1990; Rice *et al*., 1990; Stern *et al*., 2002).

1.6.3.2.1. Immunologic theory of OcMMP

Immunopathological development of OcMMP involves 3 different stages, the injury phase, the acute inflammation, proliferation and fibrosis phase. During the injury phase autoantibodies (IgG and IgA) against BP180, laminin 332 (laminin 5), collagen VII, and α6β4 integrin (the BM antigens) are present (Kasperkiewicz *et al*., 2012). These autoantibodies activate the classical complement pathway in the subepithelial tissue (Bernauer *et al*., 1993). In acute inflammation phase, complement-mediated damage to epithelial, BMZ and connective tissue initiates vasodilatation and accumulation of inflammatory cells such as neutrophils, macrophages, antigen-presenting cells, mast cells, platelets, and T cells (Chan *et al.,* 1999; Georgoudis *et al*., 2019). In the fibrosis stage, activation of conjunctival fibroblasts is observed leading to proliferation and production of cytokines, such as IL-1, IL-17, TNFα, and TGFβ, and extracellular matrix (ECM) deposition, Vascular endothelial cells multiply to induce subconjunctival scarring and fibrovascular granulation tissue (Georgoudis *et al*., 2019). It is proposed that expression of HLA-DR4, HLA-DQw3, and HLA-DQβ*10301 alleles are associated with antigen presentation to T cells which contribute to OcMMP (Zakka, *et al*., 2011). However, in most cases no initiating factor is evident (Georgoudis *et al*, 2019).

1.6.3.3. Clinical manifestations of OcMMP

The phenotype of OcMMP is a sight-threatening disorder characterised by conjunctival inflammation, progressive conjunctival fibrosis, leading to loss of goblet cells, keratinisation,

limbal epithelial stem cell failure and subsequent corneal neovascularisation, scarring. In patients diagnosed with long-lasting OcMMP low-grade subconjunctival inflammation supports fibrosis and penetration of the tarsal and bulbar conjunctiva leading to symblepharon and ankylopblepharon (Thorne *et al*., 2004).

Progressive conjunctival scarring leads to obstruction of the main lacrimal gland duct, goblet cell damage and meibomian gland dysfunction leading to severe dry eye affecting aqueous, mucus and lipid components of the tear film (Mondino *et al*., 1981; Kirzhner *et al*., 2011). Persistent epithelial impairment, corneal ulceration is also observed in OcMMP patients (Dart 2017).

Differential phenotype is dependent on early and late presentation as well as age. It is documented that patients with early onset of OcMMP encounter a severe ocular surface inflammation in comparison to those with more established disease (Williams *et al*., 2011). In addition, younger patients diagnosed with OcMMP show more severe ocular and systemic symptoms (Rauz *et al*., 2005).

1.6.3.4. The histopathology and diagnosis of OcMMP.

One of the techniques to detected serum autoantibodies is indirect immunofluorescence (IF) microscopy technique on salt-split skin. However, questions have been raised regarding the sensitivity and specificity of this technique (Dart 2017). Biopsy samples of bulbar perilesional conjunctival allow histopathological examination and validation of MMP diagnosis via direct immunofluorescence (DIF) testing (Grau *et al*., 2013). Overall, it was proposed that OcMMP can be diagnosed if DIF is negative and IF is positive and in cases where IF is negative when other reasons of cicatricial conjunctivitis have been ruled out (Ong *et al*, 2014). Cicatricial

conjunctivitis can be as stationary/slow when the disease is controlled or when a particular drug is withdrawn (Saw *et al.,* 2008). A precise diagnosis of OcMMP is complicated by other causes of cicatrising conjunctivitis, for instance certain drugs such as phospholine iodide or Pilocarpine (Dart 2003).

1.6.3.5. Treatment of OcMMP

OcMMP is managed by controlling the immune mediated inflammatory response, inhibiting fibrosis and treating the ocular surface disease. The first international consensus on MMP with ocular involvement suggested that systemic immunosuppressive treatment, prednisolone any cyclophosphamide should be prescribed, with mycophenylate effective for moderately active disease (Chan *et al*., 2002; Saw *et al.,* 2008). The effectiveness of treatment is defined as a white eye, without the development of cicatrisation. If inflammation continues whilst patients are on treatment, the dose of the drug can be increased and/or addition systemic corticosteroid is considered. However, if the symptoms continue and treatment progression is not observed application of new agents are considered (Table. 10). Intravenous immunoglobulin or plasmapheresis with immuno-adsorption can be affective in decreasing the number of circulating autoantibodies and enhancing the treatment provided (Langenhan *et al*., 2014).

Failure to attain long lasting remission and the existence of serious side effects such as hypogammaglobulinemia, has led to alternative treatment strategies. Rituximab (anti-CD20) provided as monotherapy or in combination with immuno-adsorption and/or immunoglobulin infusion and/or conservative immunosuppressive agents, has been an effective in inducing complete remission in refractory patients (Ahmed *et al*., 2015a). However, patients with IgA-dominant disease MMP are less responsive to Rituximab (Horvath

et al., 2012; Joly *et al*., 2017; He *et al.,* 2015). This is because further evaluation of circulating plasma cells showed a mucosal phenotype, demonstrating that their precursor B cells are mucosal resident, and are not inhibited by Rituximab (Lamberts *et al*., 2018). A study showed a fast-positive response in MMP patients with Infliximab, (anti–TNF-α) agent and concluded that Infliximab is an effective therapy for MMP patients (Tavakolpour 2016).

Table 10-The summary of drugs used in therapy of OcMMP (Sobolewska *et al*., 2013).

Drug	*Mechanism of action*
Mycophenolate mofetil MMF	Inhibitor of inosine of inosine monophosphate dehydrogenase (IMPDH) in purine biosynthesis (specifically guanine synthesis)
Antimetabolite drugs	
Methotrexate (MTX)	Inhibitor of metabolism of folic acid
Azathioprine	Purine analog that inhibits DNA and RNA synthesis
Sulfonamide antibiotics	
Dapsone or Sulfapyridine	Anti-inflammatory and immunomodulatory properties
Alkylating drugs	
Cyclophosphamide	Alkylation of the guanine base of DNA
Biologic agents	
Intravenous immunoglobulin	Anti-inflammatoryimmunomodulatory
Anti-TNF-a drugs	Neutralization of thebiological activity of TNF-a
Rituximab	Chimeric monoclonal antibody against the CD20 antigen

Tumour necrosis factor alpha (TNF-a), Ribonucleic acid (RNA) and Methotrexate (MTX)

1.6.3.6. Epidemiology of OcMMP

OcMMP is suggested to be a leading cause cicatrizing conjunctivitis in many different developed countries. In the UK the incident rate of OcMMP is 0.8 per million with 60% of the cases representing cicatrizing conjunctivitis. A similar percentage of cases have been observed in France (1.13 per million) and Germany (0.87 per million). It suggested that OcMMP can affect individuals of any race, however, it is more common the Caucasian population (Radford *et al.,* 2012).

1.6.3.7. The role of neutrophils in ocular surface disease

The tear film maintains a continuous population of neutrophils. Tear washes collected on awakening contained a substantial neutrophil population that were alive, but did not respond (oxidative burst or receptor upregulation) to LPS, PMA or fMLP, unlike blood neutrophils from the same individual (Gorbet *et al.,* 2015). Neutrophils accumulate in the closed eye during sleep with numbers being higher after seven hours compared to one hour. Over time surface receptor expression including CD16, CD11b and CD14 was decreased, making these neutrophils less phagocytic, but potentially more immunosuppressive (Postnikoff *et al.,* 2017a) To address these findings blood neutrophils were incubated in conditions that mimic a closed eye environment, hypoxia, co-culture with human corneal epithelial cells and artificial tear solution. Hypoxia and ATS induced a significant increase in expression of CD11b and CD66b, a marker of degranulation in response to LPS and fMLP. That blood-derived neutrophils respond differently to closed-eye environments such as tear film derived cells may be due to a lack of extravasation which has significant effects on receptor expression. Resident populations in mouth, lung and peritoneum also show a refractory phenotype and higher expression of CD66b (Postnikoff *et al.,* 2017b). On awakening dry eye subjects had

significantly greater numbers of leukocytes in tear washes with a significant neutrophil to lymphocyte ratio, suggesting that the cellular component of tears is not simple diffusion from the blood (Postnikoff *et al.,* 2018) Conjunctival impression cytology samples in patients with dry eye disease transferred glass and analysed by confocal microscopy showed cDNA, NET and neutrophils. NET consisted of histones, antimicrobial peptides, and neutrophil elastase. Nuclease activity in tears from patients with dry eye was diminished, whereas cfDNA was significantly increased, compared to healthy controls (Sonawane *et al.,* 2012). Serum eye drops used to **treat** DED may restore nuclease activity and destruction of DNA and NET.

Human blood PMN exposed to prostaglandin E2 alter eicosanoid production from leukotriene B4 and 5-lipoxygenase, to lipoxin A4 which inhibits neutrophil infiltration into tissues (Levy *et al.,* 2001). In dry eye disease PMN are the cells that generate the anti-inflammatory LXA4 in the cornea, a process mediated by oestrogen. In mice LXA4 PMN were present in the corneas of both sexes' in disease, however desiccating stress induces a significant decrease in lymph node PMN and LXA4 formation only in females. This correlated with increased Th1 and Th17 cells, reduced numbers of Tregs, and increased tissue damage. Female mice treated with LXA4 showed reversal of T cell subsets and reduced tissue damage (Gao *et al.,* 2018). In a second study, dietary docasahexonoic acid (DHA) is protective in DED. Administration to mice increased LXA_4 PMN in the cornea and lymph nodes and inhibited DED (Gao *et al.,* 2018). These results may contribute to the biased female prevalence of dry disease.

A murine model of OcMMP showed that neutrophil elastase induced sub-epithelial blisters, and mice that were elastase-deficient were resistant to such blistering (Liu *et al*., 2000). The study showed irregular high levels of caseinolytic activity, constant with neutrophil elastase was observed in extracts of skin lesions and blister fluid in mice injected with anti-BP180 IgG.

The pathogenic anti-BP180 IgG failed to induce subepidermal blistering in NE-null ($NE^{-/-}$) mutant mice. $NE^{-/-}$ mice reconstructed wild-type (WT) neutrophils (from mice) became vulnerable to experimental Bullous pemphigoid. Furthermore, WT mice induced with NE inhibitors (α1-proteinas and Me-O-Suc-Ala-Ala-Pro-Val-CH2Cl), but not with cathepsin G/chymase inhibitors (α1-antichymotrypsin or Z-Gly-Leu-Phe-CH2Cl), were unaffected by pathogenic activity of anti-BP180 antibodies. As a result, incubation of murine skin with NE induced BP-like epidermal-dermal detachment (Liu *et al*., 2000).

The best evidence for a role for PMN in DED comes mainly from our laboratory studies. Using ocular surface cytology populations of neutrophils have been reported in patients with Stevens-Johnson Syndrome, (SJS) a form of DED, where conjunctival inflammation was graded as absent/mild. There was an increase in $CD45^{INT}$ $CD11b^{+}$ $CD16^{+}$ $CD14^{-}$ neutrophils, and a concomitant reduction of CD8αβ (+) T cells in these patients. Neutrophil numbers inversely correlated with disease duration, and persistence of neutrophils suggests an unresolved innate-inflammatory process. As Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (SJS-TEN) is suggested to have an OcMMP like conjunctival phenotype, neutrophils may play a role in the pathogenesis of OcMMP. In support of this concept neutrophils were elevated in cytology samples from patients with OcMMP compared to healthy controls (Williams *et al*., 2013). Over 12 months follow-up progression of disease was associated with higher neutrophil numbers at baseline. Eyes with no observable infiltration but raised conjunctival neutrophils were more likely to progress and have greater tissue damage than those patients with low neutrophil counts (Williams *et al*., 2016) A study by Arafat *et al*., showed elevated levels neutrophil collagenase, matrix metalloproteinase-8 and 9 and myeloperoxidase in tears of patients with OcMMP and SJS-TEN (Arafat *et al*., 2014). The

specific role of conjunctival neutrophil population is not clear but could be linked changed ocular surface microbiome due to cicatrization, autoimmune-driven complement activation of neutrophils, or immunosuppression (Williams *et al*., 2016).

Similarly, in model of pulmonary fibrosis higher neutrophil counts and neutrophil elastase was observed in cryptogenic fibrosing alveolitis, while neutrophil-elastase deficient animals were resistant (Obayashi *et al*., 1997).

1.8. Hypothesis and aims

Against this background it is hypothesised that neutrophils activated by mucosal inflammation are involved in the pathogenesis of these ocular inflammatory disorders.

Therefore, the following aims will be addressed,

- To assess neutrophil function in patients with ocular inflammatory diseases in relation to disease activity and response to treatment.
- To analyse the phenotype of neutrophils in ocular inflammatory disease in relation to activity and response to treatment.

CHAPTER 2

MATERIAL AND METHODS

2.0. Material and Methods

2.1. Materials

All reagents and antibodies used in this project are evident in Table 11-15

Table 11-The details of all regents used in this project.

Reagents	*Usage*	*Company*	*Cat No*
BD Facs Lysing Solution	Red blood cell lysis	FACS	349202
Dextran	Neutrophil Isolation	Pharmacosmos	551005009007
EasySep ™	Direct Human Neutrophil isolation kit	Stem Cell	19666
EasySep ™	EasySep ™ Direct RapidSpheres ™ used for purification	Stem Cell Stem Cell	50300
Ficoll® Paque Plus	Isolation of LDN and NDN	GE Healthcare	17-1440-03
Fluoromount media	Visualisation of NETs	Sigma-Aldrich	F4680-25ML
Giemsa Stain	Neutrophil purity check	Sigma-Aldrich	GS500-500ml
Heat-inactivated foetal calf serum (FCS)	Media	Lab tech	FCS-SA
L-glutamine, penicillin, streptomycin solution (GPS)	Media	Sigma	085M4757V
Methanol	Neutrophil purity check	Sigma-Aldrich	322415-2L
Paraformaldehyde (4% in PBS)	Visualisation of NETs	Sigma-Aldrich	P6148-500G
Percoll	Neutrophil Isolation	GE Healthcare	17544501
PhagoBurst Kit	Determination Reactive oxygen species	Glycotope Biotechnology	341060

PhagoTest Kit	Determination of Phagocytic capacity	Glycotope Biotechnology	341058
Phorbol 12-myristate 13-acetate (PMA)	Production of NETs	Millipore	524400-5MG
Phosphate-buffered saline (PBS)	Buffer multiple uses	Oxoid	BR0014G
REAG B of PhagoTest kit	Production of NETs	Glycotope Biotechnology	341058
RPMI	Assay media	Gibco	21875-034
Sodium Chloride	Neutrophil Isolation	Sigma-Aldrich	S3014-500g
Sytox Green Dye	Visualisation of NETs	Thermoscientific	S34860
Triton X-100	Visualisation of NETs	Sigma-Aldrich	X100-500ML
Trypan blue (1:1)	Cell Viability	Sigma-Aldrich	93595-50ML
Viability dye	Flow cytometry	eBioscience	65-0863-14
λ-DNA	Analyse the production of cDNA	ThermoFischer Scientific, UK	

Cell free DNA (cDNA) lambda-deoxyribonucleic acid (λ-DNA)

Table 12-Clone selected for single and multiple subtype analysis in all neutrophils.

Antigen	*Fluorophore*	*Antibody*	*Company*	*Cat Number*	*Dilution*	*Stock Concentration*
CD15	PE/CY7	W6D3	Bio-Legend	323030	1/1000	200 µg/ml
HLA-DR	PE	L243	Bio-Legend	307606	1/2000	0.2 mg/ml
CD54	APC	HA58	Bio-Legend	353112	1/2000	100 µg/ml
CD66B	FITC	G10F5	Bio-Legend	305106	1/1000	40 µg/ml
CD16	APC/CY7	DREG-56	Bio-Legend	302018	1/200	200 µg/ml
CD11B	BV711	ICRF44	Bio-Legend	301344	1/1000	100 µg/ml
CD62L	PE	DREG-56	Bio-Legend	304806	1/100	40 µg/ml
CD14	BV605	M5E2	Bio-Legend	301834	1/100	100 µg/ml
CXCR2	FITC	5E8/CXCR2	Bio-Legend	320704	1/500	200 µg/ml
CD33	APC	P67.6	Bio-Legend	366606	1/500	100 µg/ml

Table 13-Isotopes selected for single and multiple subtype analysis in all neutrophils.

Antigen	*Fluorophore*	*Isotype control*	*Cat Number*	*Company*	*Dilution*	*Stock Concentration*
CD15	PE/CY7	PE/Cy7 Mouse IgG1, κ Isotype Ctrl	400126	Bio-Legend	1/1000	200 µg/ml
HLA-DR	PE	PE Mouse IgG2a, κ Isotype Ctrl	400212	Bio-Legend	1/1000	0.2 mg/ml
CD54	APC	APC Mouse IgG1, κ Isotype Ctrl (FC)	400122	Bio-Legend	1/1000	200 µg/ml
CD66B	FITC	FITC Mouse IgM, κ Isotype Ctrl	401606	Bio-Legend	1/1000	0.5 mg/ml
CD16	APC/CY7	APC/Cy7 Mouse IgG1, κ Isotype Ctrl	400128	Bio-Legend	1/1000	200 µg/ml
CD11B	BV711	Brillant Violet 711™ Rat IgG2b, κ Isotype Ctrl	400168	Bio-Legend	1/200	100 µg/ml
CD62L	PE	PE Mouse IgG1, κ Isotype Ctrl	400112	Bio-Legend	1/100	40 µg/ml
CD14	BV605	Brilliant Violet 605™ Mouse IgG2a, κ Isotype Ctrl	400270	Bio-Legend	1/100	100 µg/ml

CXCR2	FITC	FITC Mouse IgG1, κ Isotype Ctrl	400108	Bio-Legend	1/1000	200 µg/ml
CD33	APC	APC Mouse IgG1, κ Isotype Ctrl (FC)	400122	Bio-Legend	1/500	200 µg/ml

Allophycocyanin Conjugate (APC) Phycoerythrin: Cy-7 Tandem Conjugate (PE/CY7) Fluorescein Isothiocyanate Conjugate (FITC) BD Horizon Brilliant™ Violet 605 (BV605) , Phycoerythrin Conjugate (PE), BD Horizon™ Brilliant Violet™ 711 (BV711) and Allophycocyanin: Cy-7 Tandem Conjugate (APC/CY7), Immunoglobin G 2a kapa (IgG2a, K) and Immunoglobin G1 kappa (IgG1, K)

Table 14-Antibodies used for single and multi-subtype analysis in LDN and NDN.

Antigen	*Fluorophore*	*Antibody*	*Company*	*Cat Number*	*Dilution*	*Stock Concentrations*
CD15	PE/CY7	W6D3	Bio-Legend	323030	1/1000	200 µg/ml
HLA-DR	PE	L243	Bio-Legend	307606	2/2000	0.2 mg/ml
CD66B	FITC	G10F5	Bio-Legend	305106	1/1000	40 µg/ml
CD14	BV605	M5E2	Bio-Legend	301834	1/100	100 µg/ml
CD33	APC	P67.6	Bio-Leged	366606	1/500	200 µg/ml

Allophycocyanin Conjugate (APC), Phycoerythrin: Cy-7 Tandem Conjugate (PE/CY7) Fluorescein Isothiocyanate Conjugate (FITC), BD Horizon Brilliant™ Violet 605 (BV605) and Phycoerythrin Conjugate (PE)

Table 15-Isotopes used for single and multiple subtypes' analysis in LDN and NDN.

Antigen	*Fluorophore*	*Isotype control*	*Cat Number*	*Company*	*Dilution*	*Stock Concentrations*
CD15	PE/CY7	PE/Cy7 Mouse IgG1, κ Isotype Ctrl	400126	Bio-Legend	1/1000	200 µg/ml
HLA-DR	PE	PE Mouse IgG2a, κ Isotype Ctrl	400212	Bio-Legend	1/1000	0.2 mg/ml
CD66B	FITC	FITC Mouse IgM, κ Isotype Ctrl	401606	Bio-Legend	1/1000	0.5 mg/ml
CD14	BV605	Brilliant Violet 605™ Mouse IgG2a, κ Isotype Ctrl	400270	Bio-Legend	1/100	100 µg/ml
CD33	APC	APC Mouse IgG1, κ Isotype Ctrl (FC)	400122	Bio-Legend	1/500	200 µg/ml

2.2 Ethical approval

Patient sampling was undertaken following ethical approval in accordance with the Declaration of Helsinki (Birmingham East, North and Solihull Ethics Committee: Inflammation in Ocular Surface Disease IOSD 08/H1206/165, UKCRN 7448). All requirements with regards to Control of Substances Hazardous to Health (COSSH) were successfully completed before starting this study. In addition, ethically approved participant (patient and healthy) information sheets and consent forms were used to recruit healthy individuals and patients for this study.

2.3. Patients

The patient samples used in this study were obtained from the relevant clinics at the Birmingham Eye Centre (BMEC). Behçet's disease and OcMMP samples used in this study were used as disease controls for each other. Age matched healthy controls were recruited from volunteers and cataract patients who had no evidence of ocular or systemic inflammatory disease or diabetes. The demographics of patients and healthy individuals is displayed in table 16.

Table 16-The demographics of patients and healthy controls recruited in this study.

	Healthy controls (N=48)	*BD patients (N=73)*	*OcMMP patients (N=63)*
Gender M/F	23/25	22/51	21/42
Age	65 (20-88)	45 (16-66)	69 (35-87)
Ethnicity			
Asian	12	4	0
White	33	59	54
Middle Eastern	2	0	0
Any other background	1	10	9
Treatment			
No Treatment	0	20	6
Conservative	0	11	1
Topical/local	0	20	0
Conventional immunosuppression	0	22	56

Age represented as mean values and the age range is displayed in brackets, Bechet's (BD) and Ocular mucous membrane pemphigoid (OcMMP)

2.4 Isolation of neutrophils

2.4.1 Isolation of all neutrophils using Percoll density gradients

Blood samples were collected in vacutainer tubes containing Heparin, and processed within 4 hours, from the relevant clinics or from the University of Birmingham. Under sterile conditions (sterile hood) blood was dispensed into a 50 ml falcon tube (Corning UK) and 2% dextran (Pharmacosmos) was added at a ratio of 1ml dextran: 6ml blood and the tubes were inverted 5 times. The tubes, with a loosened lid, were left for 30-40 minutes, for yellow coloured buffy coat to appear. 5 mls of 56%, Percoll was added into a 15 ml falcon tube, then 2.5 ml of 80% Percoll was layered underneath to form a gradient. Blood sample buffy coats were carefully added to the gradients spun (Heraeus Megafuge 16, Thermoscientific) at 136 x g for 20 minutes at room temperature with one acceleration and zero de-acceleration. Two distinct layers of cells (Fig.10) were observed. The top layer represented the peripheral blood mononuclear cells (PBMCs) and therefore was discarded. The second layer containing neutrophils was collected and placed into a new 15 ml falcon tube containing RPMI 1640 medium+ Foetal calf serum (10% FCS)+ L-Glutamine–Penicillin–Streptomycin solution (1% L-GPS) and spun for 10 minutes at 287 x g at room temperature with one acceleration and zero de-acceleration. Neutrophils were purified (negative selection) using the EasySep Human Neutrophil Isolation kit (Stem Cell Technologies). The neutrophils were re-suspended in 0.25-6.5 ml media ($5x10^7$ neutrophils/ml) in a 15 ml falcon tube. 50 µl/ml of the isolation cocktail (a combination of monoclonal antibodies in PBS) was added, and the sample was incubated for 5 minutes at 2-8^oC. The rapid spheres (a suspension of magnetic particles and monoclonal antibodies in PBS) were vortexed for 30 seconds and was added (40 µl/ml) and incubated for 3 minutes at 2-8^oC. The preparation was made up to 5ml by adding RPMI media and the sample was placed within the Easy Sep magnet without the lid on the falcon tube for 3

minutes at room temperature. The solution was poured in a new 15 ml falcon tube, without disturbing the cell suspension. All neutrophils were purified according to manufactures instructions. Once the samples were centrifuged the supernatant was poured off and the pellets were re-suspended in 1-5 mls of RPMI 1640 (Gibco) +10% FCS + and 1% L-GPS (Sigma-Aldrich) and cells counted using a haemocytometer (Sigma-Aldrich) and trypan blue (Sigma-Aldrich)

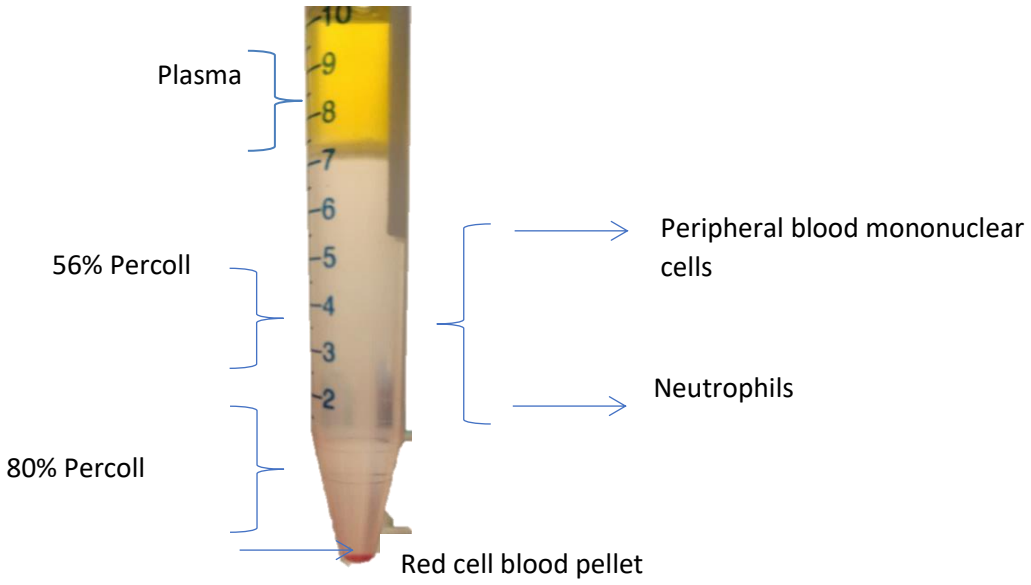


Figure 10- The representation of Percoll density gradients. Due to neutrophil buoyancy, neutrophils can pass through the 56% but not through 80% layer. 56% percoll is layered over the 80% percoll. The neutrophil population is represented as the fluffy layer whereas the PBMCs are found below the plasma and a pellet of red blood cells are found at the bottom of the gradient.

2.5 The Isolation of low-density neutrophils (LDN) and normal density neutrophils (NDN)

2.5.1 Isolation of LDN and NDN using Ficol-hypaque gradient

All blood samples were processed within 4 hours once obtained from the relevant clinics or from the University of Birmingham. The blood samples were collected in a micro vacutainer (containing Heparin). The Ficoll-Hypaque (GE Healthcare) solution under sterilised conditions was placed into a 50 ml falcon tube in the ratio of 2 ml of Ficoll-Hypaque: 1 ml of blood. The diluted (with equal volume of PBS) blood was slowly layered over the Ficoll-Hypaque solution and was further centrifuged (Heraeus Megafuge 16, Thermoscientific) centrifuge for 40 minutes at 400 x g at 22^{o}C with 0 break. After centrifugation the LDN, from the peripheral blood mononuclear cell layer) located at the interface between the upper layer (plasma) and the Ficoll-Hypaque (bottom) layer was removed (Fig.11). The cells were transferred to a 15 ml falcon tube containing 10 ml of PBS and centrifuged for 10 minutes at 400 x g at 4^{o}C. After centrifugation the supernatant was discarded, the wash was repeated, and the cells were used as required. The NDN layer (the granulocyte and fluffy layer) located at the top of the Ficoll-Hypaque was removed and 100 µl of the cell suspension was transferred to 15 ml falcon tube. The cell suspension was lysed with red blood lysis buffer (BD Facs) in the ratio of 100 ul:1 ml (red blood lysis buffer). The cells were vortexed and incubated in the dark for 20 minutes. The cells were further centrifuged at 250 x g for 5 minutes at room temperature. After centrifugation, the supernatant was discarded and the cells were purified.

The LDN (from the PBMC layer) and NDN (from the granulocyte layer) were purified using the the EasySep Human Neutrophil Isolation Kit (replaces Neutrophil Enrichment Kit by Stem Cell Technologies). The methodology was followed according to manufactures instructions. The LDN and NDN were resuspended at 5x10^{7} neutrophils/ml 0.25-6.5 ml media in a 15 ml falcon

tube. 50 µl/ml of the isolation cocktail was added, and the sample was incubated for 5 minutes at 2-8^{o}C. The rapid spheres were vortexed for 30 seconds and was added (40 µl/ml) to the sample and the sample was incubated for 3 minutes at 2-8^{o}C. The solution was made up to 5 ml by adding RPMI media and the sample was placed within in the Easy Sep magnet without the lid on the falcon tube for 3 minutes at room temperature. The tube and the magnet were inverted in one continuous motion and the solution was poured in a new 14 ml falcon tube. This was performed without shaking or pouring the cell suspension. The inversion was repeated twice before the isolated LDN and NDN were purified.

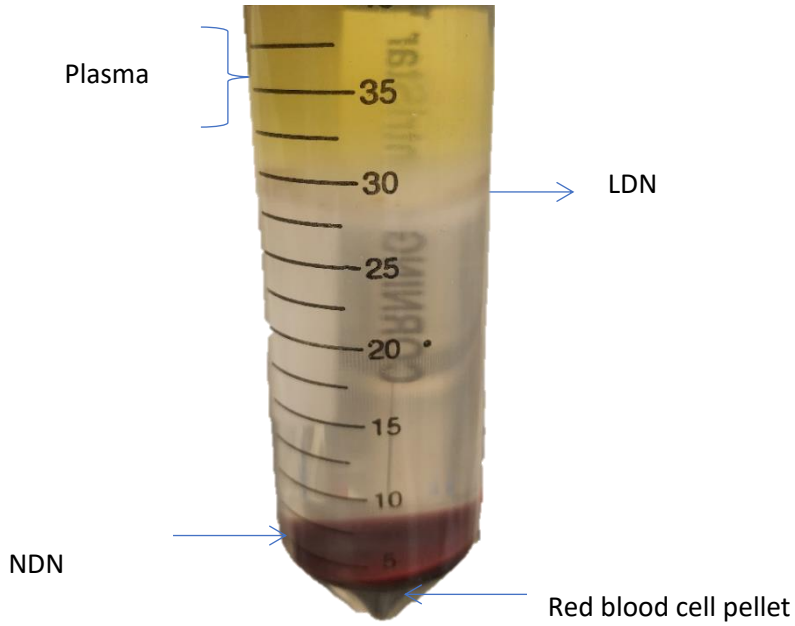


Figure 11-The isolation of LDN and NDN using Ficol-hypaque gradient. The low density neutrophils (LDN) are found in the PBMC layer located underneath the plasma and normal density neutrophils (NDN) sediment on top of the red blood cell pellet. The LDN are identified as immature neutrophils and NDN have a phenotype of mature neutrophils.

2.6. The determination of cell viability

In order to determine the cell viability (cells/ml) 10 µl isolated and purified neutrophils were suspended with 10µl of trypan blue and the following equation was used;

Average of viable cells (viable cells observed in all 4 outer corners of the haemocytometer) X2 (the dilution factor of cells suspension and Trypan blue (Sigma-Aldrich) was 1:1 (Fig.12).

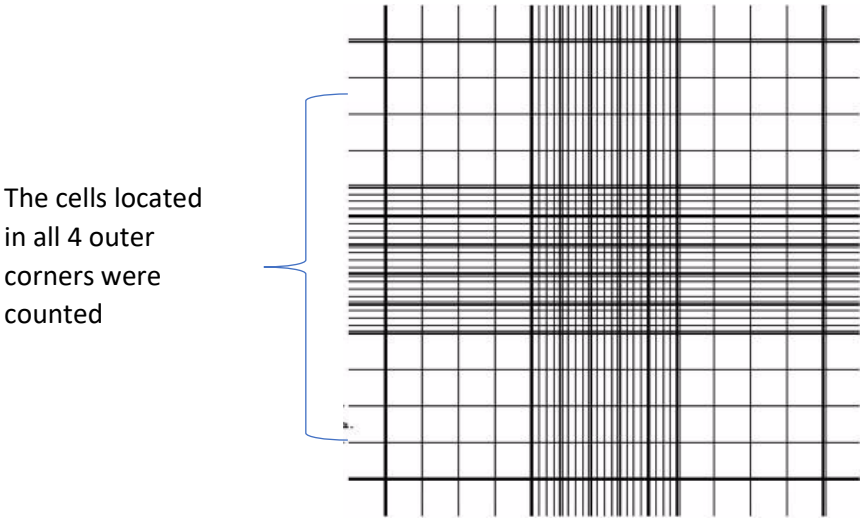


Figure 12-The representation of the grid in the haemocytometer used to count viable cells.

The cells were counted in the 4 corners by using trypan blue that allowed the identification of viable (unstained) and dead cells (blue). The viability was also used to investigate the concentration of the cells used in the experiment.

2.7. The determination of cell purity.

To determine the purity of neutrophils obtained, cells were re-suspended in 50µl of RPMI 1640+FCS and GPS and the slides (Fisher Scientific) were constructed. 50µl of the sample was added into the funnel (Simport and Shandon EZ single cytofunnel with white filter cards and caps) and spun in the cytospin centrifuge (Cytospin 3, Thermo Shandon) at 10 x g for 10 min. Post spinning, the slide was left to air dry for 30 min., dipped into cold methanol (Sigma-Aldrich) for 30 secs and left to air dry for 10 min. A small drop of diluted Giemsa (1/20) (Sigma-Aldrich) was added for 30 min., then the slide was rinsed thoroughly with distilled water and left to air dry for 1 hour. The slide was visualised using Zeiss video-microscope using X400 magnification. All samples used for further experimental work encountered more than 99.9% purity.

2.8. Quantitative analysis of neutrophil phagocytosis and oxidative burst in whole blood samples using the Phagotest and Phagoburst kits.

A quantitative analysis of neutrophil phagocytosis and oxidative burst activity in whole blood samples was quantified by using Phagotest and Phagoburst kits manufactured by Glycotope Biotechnology.

2.8.1. Phagocytic assay

The whole blood samples were collected in micro vacutainers (containing Heparin) and aliquoted (100 µl) into pre-labelled Fluorescence-activated cell sorting (FACS) (BD) tubes. 20 µl of fluorescein isothiocyanate (FITC)-labelled *Escherichia coli* (*E.coli*) (pre-cold) was added to all control and test samples. Control samples were incubated on ice and the test samples were vortexed for 5 seconds and were further incubated at 37^{o}C for 10 minutes, then placed on ice. Trypan blue (100 µl) was added (on ice) to all samples (controls and test) and vortexed for 5 seconds. The samples were washed with 3 mls of washing buffer and vortexed, and then centrifuged at 4^{o}C for 6 minutes at 250 x g. The supernatant was removed without disturbing the pellet formed.

2.8.2. Production of reactive oxygen species (ROS)

Whole blood samples for Phagoburst were collected in micro vacutainers (containing Heparin) and where aliquoted (100 µl) into pre-labelled FACS tubes. A control sample was set up by adding 20 µl of the wash buffer for each different blood sample, and 20µl of non-labelled *E.coli* was added to test samples for oxidative burst analysis. The samples were incubated at 37^{o}C for 10 minutes. After incubation, 20 µl of the Phagoburst substrate (dihydrorhodamine 123) was added all tubes. The tubes were flicked to re-suspend the cells and incubated at 37^{o}C for 10 minutes.

2.8.3. Analysis of Phagoburst and Phagotest assays

The lysing solution (2 mls) was added to all Phagotest and Phagoburst samples (control and test samples) vortexed for 5 seconds and incubated for 20 minutes in the dark at room temperature, then centrifuged for 5 minutes at 250 g at 4^{o}C. The resultant supernatants were removed (100 µl of the residual volume was attained) and the cells were re-suspended in 100 µl of the residual volume. All samples were washed with 3mls of wash buffer and centrifuged at 250 x g for 5 minutes at 4^{o}C. The resultant supernatant was discarded (100 µl of the residual volume was retained) and the cells were re-suspended in 100 µl of the residual volume. All samples were stained with 200µl of DNA staining solution, vortexed for 5 minutes and incubated on ice for 10 minutes. Samples were analysed using Accuri flow cytometer within 1 hour of preparation and 10,000-15,000 neutrophils were counted per sample (Glycotope Biotechnology b, 2016). The gating strategy used is illustrated in Figure 13. The percentage of neutrophils performing phagocytosis and the percentages of cells having produced reactive oxygen metabolites and accompanying mean fluorescent intensity was measured.

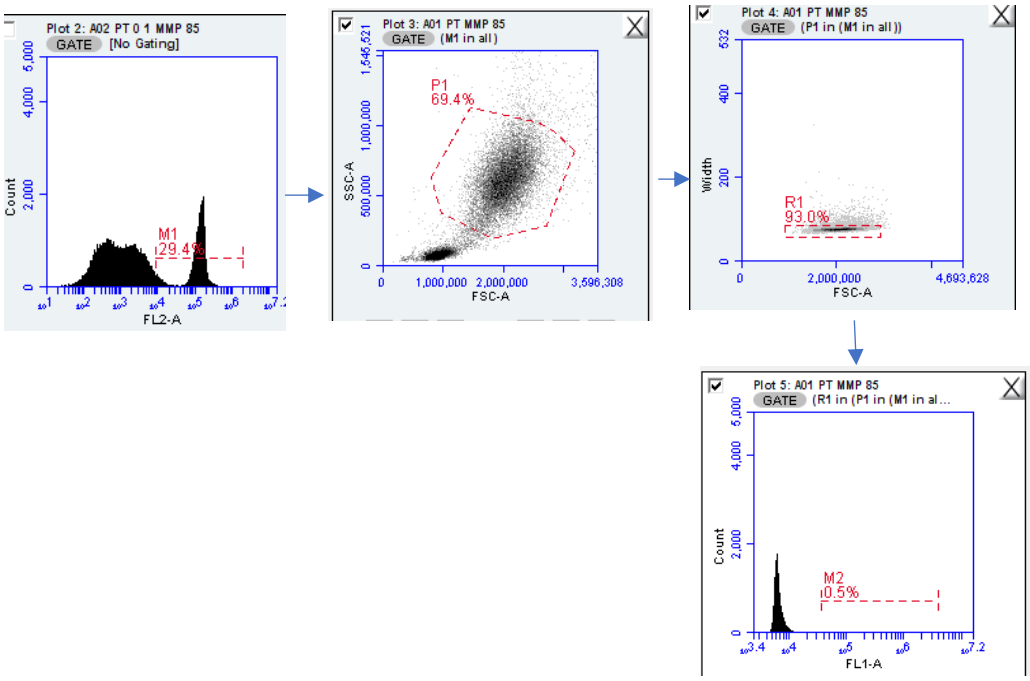


Figure 13-Gating strategy used to determine the phagocytic capacity and ROS production in all neutrophils, LDN and NDN. In order to determine phagocytic capacity the leukocyte population was gated (M1), set on the granulocytes population (P1), singlet cells (R1) within the granulocyte population was gated and positive population was identified (M2).

2.9. Neutrophil extracellular traps (NET)

2.9.1. The generation of Neutrophil Extracellular Traps in all neutrophils.

Isolated neutrophils ($2x10^6$ cells/ml) were seeded (200 µl) into wells of clear flat bottomed 96 well plates (Corning, UK), and incubated alone or with 25nM PMA (Sigma-Aldrich) for 3 hours at 37^{o}C and 5% CO_2 atmosphere. PMA was diluted from stock solutions with RPMI-1640 (Gibco) with 2mM L-glutamine 100U/ml, penicillin and 100µg/ml streptomycin (Sigma-Aldrich). After incubation the supernatant was removed from the wells, transferred into 600ul Eppendorf tubes (BD) and centrifuged at 2200 x g for 10 minutes at 4^{o}C. 100 ul of cell free supernatant was removed and placed into black 96 well plates (Corning) and incubated with 1uM SYTOX Green Dye (Invitrogen) for 10 minutes in the dark at room temperature. Florescence was measured using BioTek Synergy 2 fluorometric plate reader (BioTek) with excitation at 485nm and emission at 528 nm. All samples were analysed in duplicates. A calibration step was performed using the cell free supernatant from unstimulated neutrophils and buffer controls were analysed in duplicate. To calibrate the samples λ-DNA (0.3 µg/µl was diluted 1:20 in PBS) standard curve ranging from 1000 ng/ml to 0 ng/ml. The top standard was serially diluted 7 times with a final dilution of 1:15 with 1uM SYTOX Green Dye (Invitrogen). The same protocol assay was followed using opsonised *E.coli* (1-2 x 10^9 bacteria per ml).

2.9.2. Visualisation of Neutrophil Extracellular traps in total neutrophils.

Neutrophils isolated from a Percoll gradient ($2x10^6$ cells /ml) were re-suspended in 2ml of RPMI- 1640 (GiBco) with 2 mM L-glutamine 100U/ml, penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) (assay media). The isolated neutrophils were seeded onto 8 well chamber slides (ThermoFisher) and incubated for 30 minutes at 37^{o}C in a 5% CO2 atmosphere to accommodate cell adherence, then stimulated with 25nM PMA (Millipore) or opsonised *E.coli* (1-2 x 10^9 bacteria per ml) for 3 hours at 37^{o}C in a 5% CO_2. All agonists were prepared using the stock solutions in assay media which also served as a buffer control. Post stimulation, cells were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) (200 µl) for 30 minutes at 37^{o}C in 5% CO_2. Post fixation, the slides were washed in sterile Phosphate-buffered saline (PBS) (Oxoid) 3 times at room temperature. The slides were further washed with 0.1% Triton X-100 (to lyse the cells and extract protein) (Sigma-Aldrich) at room temperature for 1 minute followed by a 5-minute wash in sterile PBS at room temperature. The cells were incubated (5 minutes) with 1µM SYTOX green Dye at room temperature followed by a 5-minute wash in sterile PBS at room temperature. The slides were then mounted in Fluoromount medium (Sigma- Aldrich) and imaged using a LEICA DMI 6000B microscope. In separate experiments, LDN and NDN prepared from Ficoll gradients were prepared and treated and analysed by the same procedure.

2.10. Heterogeneity of all neutrophils LDN and NDN using single and multicolour flow cytometry.

The neutrophils were isolated using percoll density gradients and the concentration of the isolated neutrophils was adjusted to $1X10^6$/ml. The isolated cells (100 µl) were added to appropriate FACS tubes (BD) (isotype controls, compensation controls and sample) and compensation and negative controls were also set up simultaneously. The compensation controls were single staining of the isolated cells with each antibody used in the panels (Table 2.2 and 2.3). The isotype controls were also set up for each antibody. The samples were further centrifuged at 448 x g for 5 minutes at room temperature. The resultant supernatants were removed (leaving 100µl of the residual volume) and the cells were re-suspended. The titrated concentration (Table 2.2 and 2.3) of different antibodies was added to each relevant tube including the compensation and isotype controls. The samples, compensation and isotypes controls were vortexed for 5 minutes and were further incubated for 30 minutes at 2-8^{o}C (protected from light). Post-incubation 4mls of FACS buffer (1% PBS, 0.4% EDTA (v/v) and 0.5% BSA (w/v)) was added to all samples (including compensation and isotype controls) and all tubes were centrifuged for 448 x g for 5 minutes at room temperature.

The samples were fixed by adding 100 µl of 4% PFA to each sample tube (compensation and isotype controls). The samples were further protected from light and were incubated for 15 minutes at room temperature. 4 mls of FACS buffer was added to all samples and the samples were centrifuged for 5 minutes at 448 x g at 37^{o}C. The supernatants were removed (leaving 1000 µl of residual fluid) and the cells were re-suspended in 100 µl of the residual fluid. 200µl of FACS buffer was added to all samples (including compensation and isotypes controls) and the samples were stored in 2-8^{o}C until acquired. In separate experiments, LDN and NDN from Ficol-hypaque gradients were prepared, treated and analysed by using the same protocol in

order to evaluate the expression of surface markers displayed in Table 14 and 15. The gating strategies used to evaluate the expression of surface markers are illustrated in Fig.14 and 15.

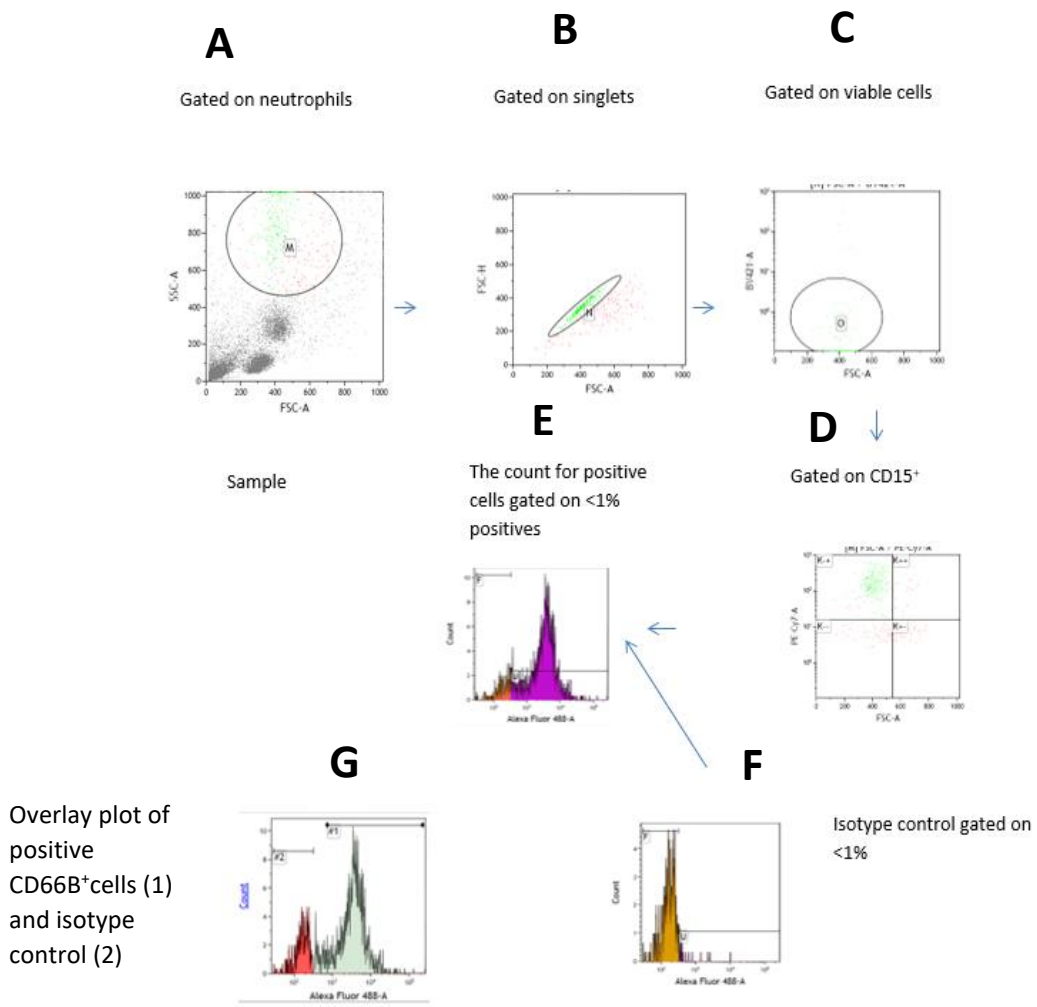


Figure 14-The gating strategy used in order to evaluate the expression of single neutrophil surface marker in all neutrophils, LDN and NDN. A represents total neutrophil population, B represents singlets, C represents viable cells, D represents $CD15^{+}$ (neutrophil marker), E represents positive cells based upon isotype control, F represents isotype control gated upon less than 1%, G represents the overlay of test (CD66B) and isotype control . The same gating strategy was used to assess the expression of all surface makers in all neutrophils, LDN and NDN displayed in Table 12-15

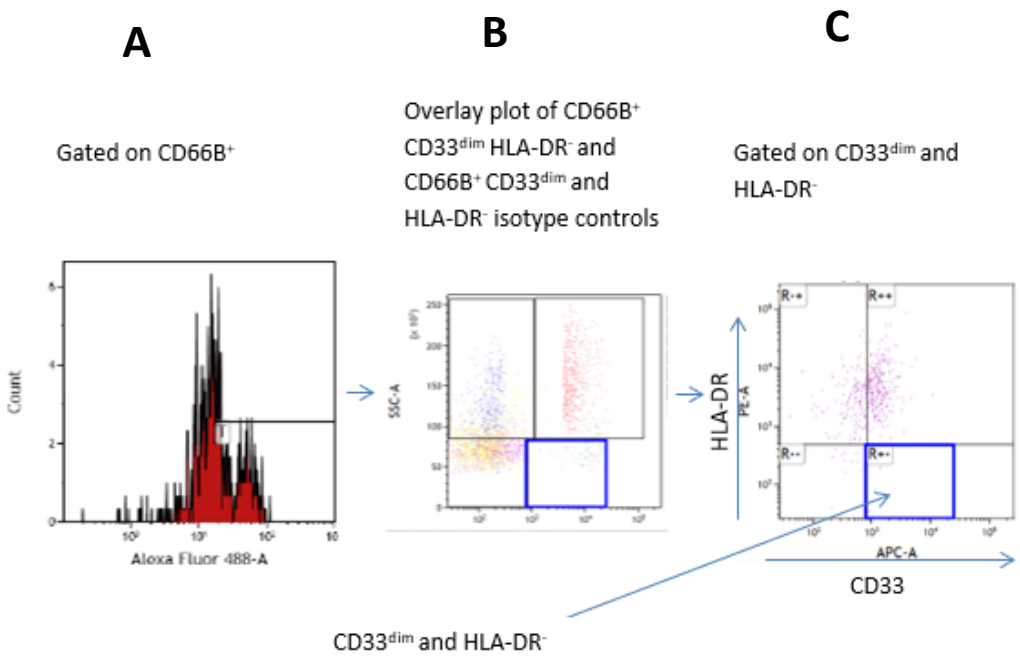


Figure 15-The gating strategy used to evaluate the expression of multiple subtypes in all neutrophils, LDN and NDN. The same gating strategy (A represents $CD66B^{+}$population B, represents $CD33^{dim}$ and $HLA\text{-}DR^{low}$ population based on isotope controls and C, represents the overlay plot of test samples and isotype controls) was used to assess the expression of multiple subtypes in all neutrophils, LDN and NDN displayed in Table 14,15.

2.11. Statistical analysis

A Mann-Whitney U (non-parametrical test) test was performed to statistically analyse non-parametrical data using GraphPad Prism for a comparison between 2 different groups. All data was presented in scatter vertical plots with mean and standard error mean by using GraphPad Prism version 5.04. The data was statistically analysed using non-parametrical tests such as the Kruskal-Wallis test, Dunn's Multiple Comparison Test (Post-Hoc) for a comparison between 3 different groups (if required) by using GraphPad Prism version 5.04

CHAPTER 3

FUNCTION OF TOTAL NEUTROPHILS IN BD AND OCMMP PATIENTS

3. Introduction

Neutrophils are a major component of the immune cell population (40-60%) in the blood, present in a resting state in healthy individuals. They play a dynamic role within the inflammatory response. Neutrophils are phagocytic cells that ingest infectious agents such as bacteria and fungi that are destroyed by the production of reactive oxygen species (Quinn *et al.,* 2006).

Another process of protection utilised by the neutrophils is known as NETosis. Neutrophil extracellular traps (NETs) are web-like structures composed of cytosolic granular proteins bound to DNA, that protect against infection by pathogens (Papayannopoulos *et al.,* 2018).

Behçet's disease (BD) is a multisystemic auto-inflammatory disorder and is characterised by recurrent oral aphthae, ocular inflammation, genital ulcers, skin lesions and vasculitis and can be associated with HLA-B51 (Mendes *et al.,* 2009; Maldini *et al.,* 2012). This disease was first described by Hulusi Behcet in 1973 however; the cause of the condition remains unknown despite being a strong correlation to HLA-B51. Ocular manifestations include bilateral, recurrent, non-granulomatous uveitis and retinal vasculitis (Avci *et al.,* 2017). The posterior segment inflammation involves occlusion of retinal veins. Occlusion is recurrent and may occur at any location from the central retinal vein to the small branches (Tugal-Tutkun 2009).

Mucous membrane pemphigoid (MMP) is described as a diverse group of autoimmune subepidermal blistering disorders affecting mucous membranes including the eye (Hong-Hu *et al.,* 2013). Ocular mucous membrane pemphigoid (OcMMP) describes ocular involvement of mucous membrane pemphigoid, which causes chronic dry eye and cicatrisation of mucosal surfaces. Ocular mucous membrane pemphigoid is classified as an anterior segment disease

and early corrective treatment is required to prevent irreversible blindness in OcMMP patients (Saw and Dart 2008).

The presence of $CD45^{INT}CD11b^{+}CD16^{+}CD14^{-}$ subset of neutrophils in the conjunctival mucosa of patients with Stevens-Johnson Syndrome/Toxic epidermal necrolysis (SJS-TEN), a form of OcMMP, in the absence of inflammation (Williams *et al.,* 2013) Moreover neutrophil infiltrate was associated with greater progression of disease in patients with OcMMP (Williams *et al.,* 2016). Finally, a recent study showed elevated levels of neutrophil components including collagenase, MMP-8 and 9 and myeloperoxidase in patients with OcMMP and SJS-TEN (Arafat *et al.,* 2014).

Current evidence for a role for neutrophils in BD includes patients treated with colchicine which is a neutrophil inhibitor. Saleh and Arayassi (2014) reported that colchicine was successfully used to treat patients with BD. Neutrophil to lymphocyte ratios are raised in BD and NET production has been recently been reported (Safi *et al*., 2018).

Given all the evidence described above with regards to neutrophil involvement in BD and OcMMP no study to date had fully investigated ROS production, phagocytosis and NET production in neutrophils in BD and OcMMP patients *in vitro*. In this chapter these functions were assessed in total neutrophils from patients.

3.1 Results

3.1.1 The function of total neutrophils in patients with BD and OcMMP in comparison to healthy individuals

3.1.1.1. Phagocytic capacity and production of reactive oxygen species of BD patients in total neutrophils

The phagocytic activity of neutrophils of BD patients (n=10) was significantly lower in comparison to healthy individuals (n=17) 49 ± 4 (%) vs 88 ± 2 (%) (P<0.0001) (Fig.16A). Similarly, patients with BD (n=10) had a significantly reduced ROS production by neutrophils from patients with BD than controls (n=16) 52 ± 5 (%) vs 80 ± 4 (%) (P=0.0005) (Fig. 16B). The MFI observed for phagocytic capacity and ROS production are displayed in Fig.16C and D.

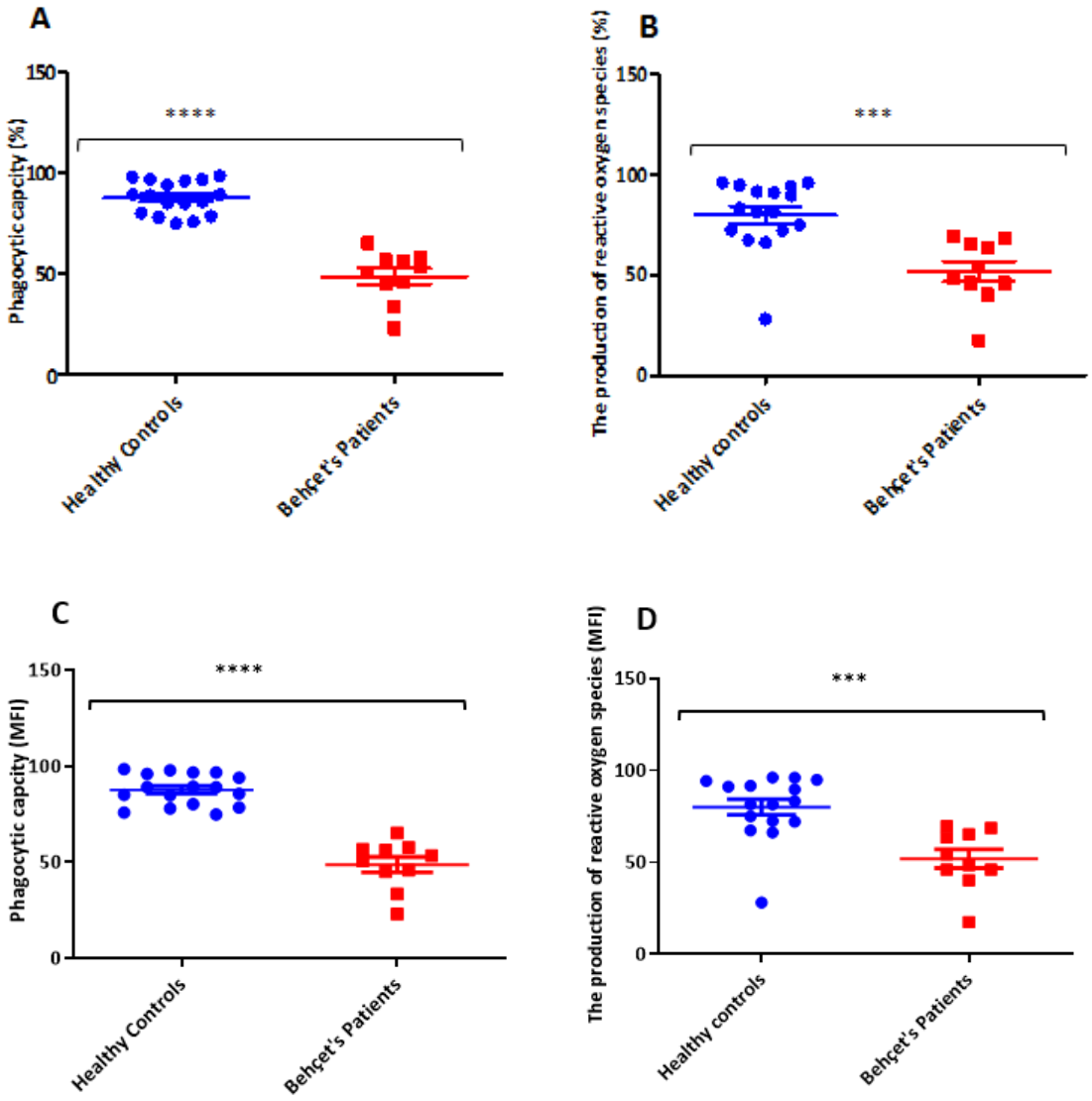


Figure 16-The percentage positive cells and mean fluorescence intensity for phagocytic activity and ROS production in total neutrophils of BD patients and healthy individuals. The phagocytic activity (A, C) was investigated by adding fluorescein isothiocyanate (FITC)-labelled *E.coli* to samples obtained from BD patients (n=10) and healthy controls (n=17). (B, D) ROS production was investigated in patients with BD (n=10) and healthy controls (n=16) by stimulating the samples with non-labelled *E.coli* . ***P <0.001, P<****0.0001

3.1.2.2. Phagocytic capacity and production of reactive oxygen species of OcMMP patients in total neutrophils

Neutrophils from patients with OcMMP (n=16) showed a similar pattern with significantly reduced phagocytic activity and ROS production compared to age-matched controls (n=12), 53 ± 3 (%) vs 91 ± 3 (%) ($P<0.0001$) and 57 ± 4 (%) vs 81 ± 5 (%) ($P=0.0028$) respectively (Fig. 17A and B). The results demonstrates that age did not impact the production of reactive oxygen species or phagocytosis, the results were similar in younger controls (Fig. 17A and B) and older cataract controls (49 ± 4 (%) vs 53 ± 3 (%) and 52 ± 2 (%) vs 57 ± 4 (%)) (Fig. 17A and B). The MFI observed for phagocytic capacity and ROS production is displayed in Fig. 17C and D.

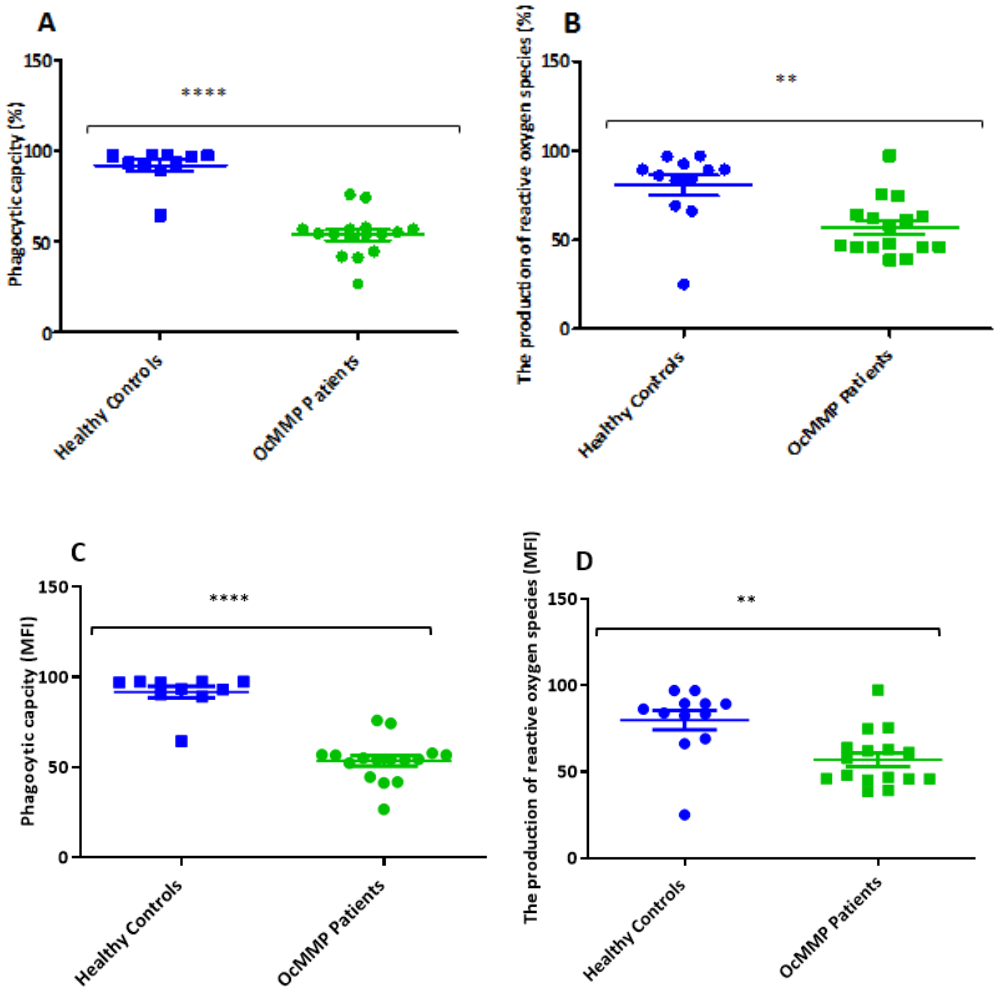


Figure 17-The percentage positive cells and mean fluorescence intensity for phagocytic activity and ROS production in total neutrophils of OcMMP patients and healthy individuals. The phagocytic activity (A, C) was investigated by adding fluorescein isothiocyanate (FITC)-labelled *E.coli* to samples obtained from OcMMP patients (n=16) versus controls (n=12). (B, D) ROS production was investigated in patients with BD (n=10) and healthy controls (n=16) by stimulating the samples with non-labelled *E. coli*. **P<0.01, P<****0.0001

3.1.2.3. The production of NET in all neutrophils from patients with BD stimulated with PMA and *E. coli*

Unstimulated neutrophils from patients with BD showed significantly greater spontaneous cell free DNA release compared to unstimulated cells from healthy controls, 58 ± 2 (ng/ml) vs 31 ± 2 (ng/ml) (p=0.0004). PMA induced significantly greater cfDNA release by neutrophils both from patients with BD (110 ± 2 (ng/ml) vs 58 ± 2 (ng/ml) (p=0.0003) and healthy controls (60 ± 5 (ng/ml) vs 31 ± 2 (ng/ml)) (P=0.0003) compared to unstimulated cells. However, neutrophils from patients with BD responded to stimulus with significantly greater cfDNA release than cells from healthy controls 110 ± 2 (ng/ml) vs 60 ± 5 (ng/ml) (Fig. 18A) (p=0.0002).

In a second analysis spontaneous release of cfDNA was greater in unstimulated neutrophils from healthy controls compared to patients with BD (57 ± 1 (ng/ml) vs 51 ± 1 (ng/ml)) (P=0.002). Stimulation with opsonised *E.coli* significantly increased cfDNA release from both cohorts 115 ± 1 (ng/ml) vs 57 ± 1 (ng/ml) (P=0.0004) and 84 ± 1 (ng/ml) vs 51.3 ± 1 (ng/ml) (P=0.0003) (F1g.18B), however in comparison to PMA stimulation neutrophils from healthy controls produced significantly more than stimulated neutrophils from patients with BD (115 ± 1 (ng/ml) vs 84 ± 1 (ng/ml)) (Fig.16B) (P=0.0002). A comparison of PMA and *E.coli* stimulation is shown in Fig.18C.

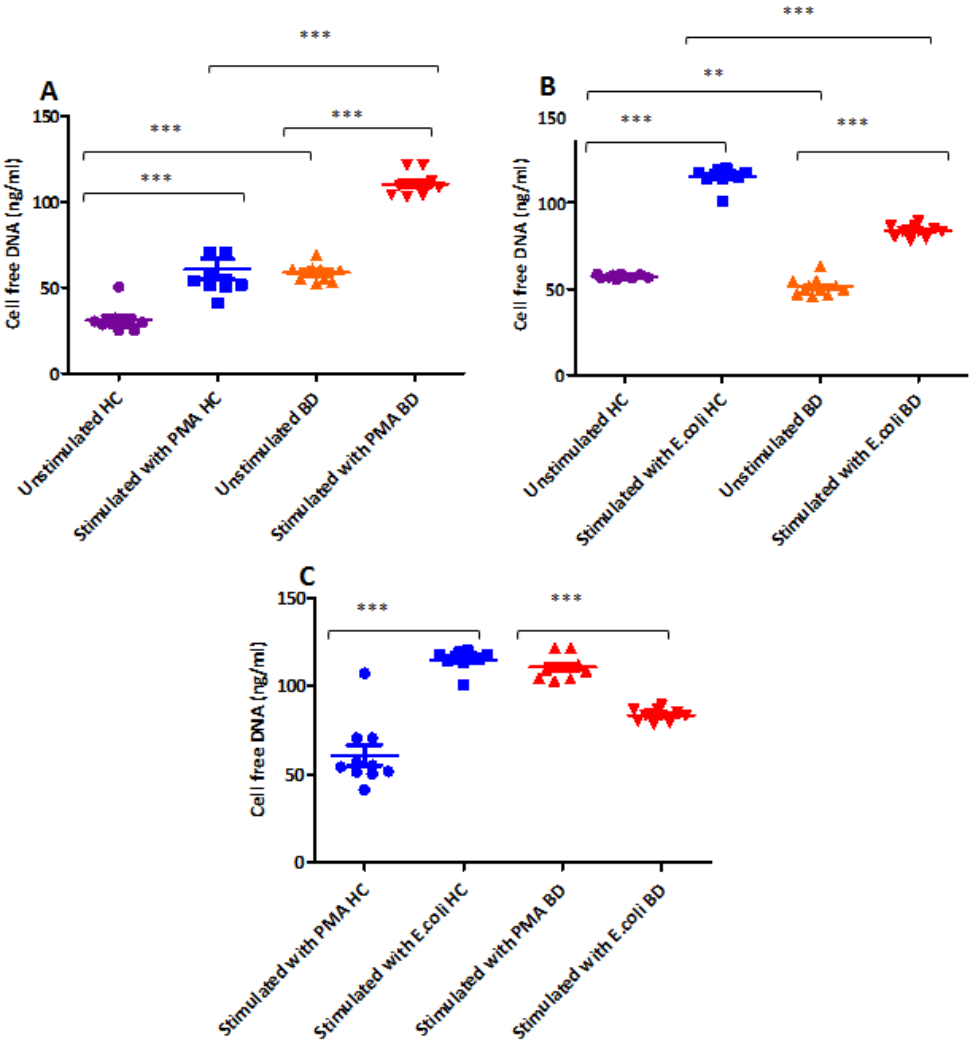


Figure 18-The production of NETs by PMA and E. coli stimulation. (A) The production of NETs in neutrophils stimulated with 25nM of PMA for 3 hours and unstimulated neutrophils from healthy individuals (n=10) and patients with BD (n=10). (B) The production of NETs in neutrophils stimulated with 1-2 x 10^9 bacteria per ml of E.*coli* for 3 hours and unstimulated neutrophils from healthy individuals (n=10) and patients with BD (n=11). (C) The production of NETs neutrophils stimulated with 25nM of PMA and 1-2 x 10^9 bacteria per ml of *E.coli* in neutrophil healthy individuals (n=10) and BD patients (n=1). **P <0.01, ***P <0.001

3.1.2.4. The production of NETs in all neutrophils from patients with OcMMP stimulated with PMA and *E.coli*

There was significant spontaneous NETosis in unstimulated cell cultures of neutrophils from patients with OcMMP compared to neutrophils from healthy controls (66 ± 4 (ng/ml) vs 30 ± 1 (ng/ml)) (Fig.19A) (P=ns). On stimulation with PMA NET production rose significantly in neutrophils from patients with OcMMP compared to match controls (125 ± 1 (ng/ml) vs 60 ± 1 (ng/ml)) (0.0003). Stimulation with *E.coli* induced a significant increase in neutrophils from patients with OcMMP 82 ± 2 vs 57 ± 3 (p=0.002) and healthy (117± 1 (ng/ml) vs 57 ± 1 (ng/ml)) (p=0.002) controls compared to unstimulated cells. Spontaneous NETosis in unstimulated cell cultures of neutrophils from patients with OcMMP (57± 3 vs 57 (ng/ml) ± 1 (ng/ml)) was also observed in the second analysis (Fig.19B), however this was not significantly different from cells from healthy controls (57± 3 (ng/ml) vs 57 ± 1 (ng/ml)) (p=ns)

A higher quantity of cell free DNA was produced in total neutrophils stimulated with *E.coli* (117 ± 1 (ng/ml) vs 60 ± 1 (ng/ml)) in comparison to total neutrophils stimulated with PMA in healthy individuals (p=ns). However, a reduced production of NETs was observed in total neutrophils stimulated with *E.coli* in comparison to PMA stimulation in OcMMP (82 ± 2 (ng/ml) vs 125 ± 1 (ng/ml)) (p=0.0003) patients (Fig.19C).

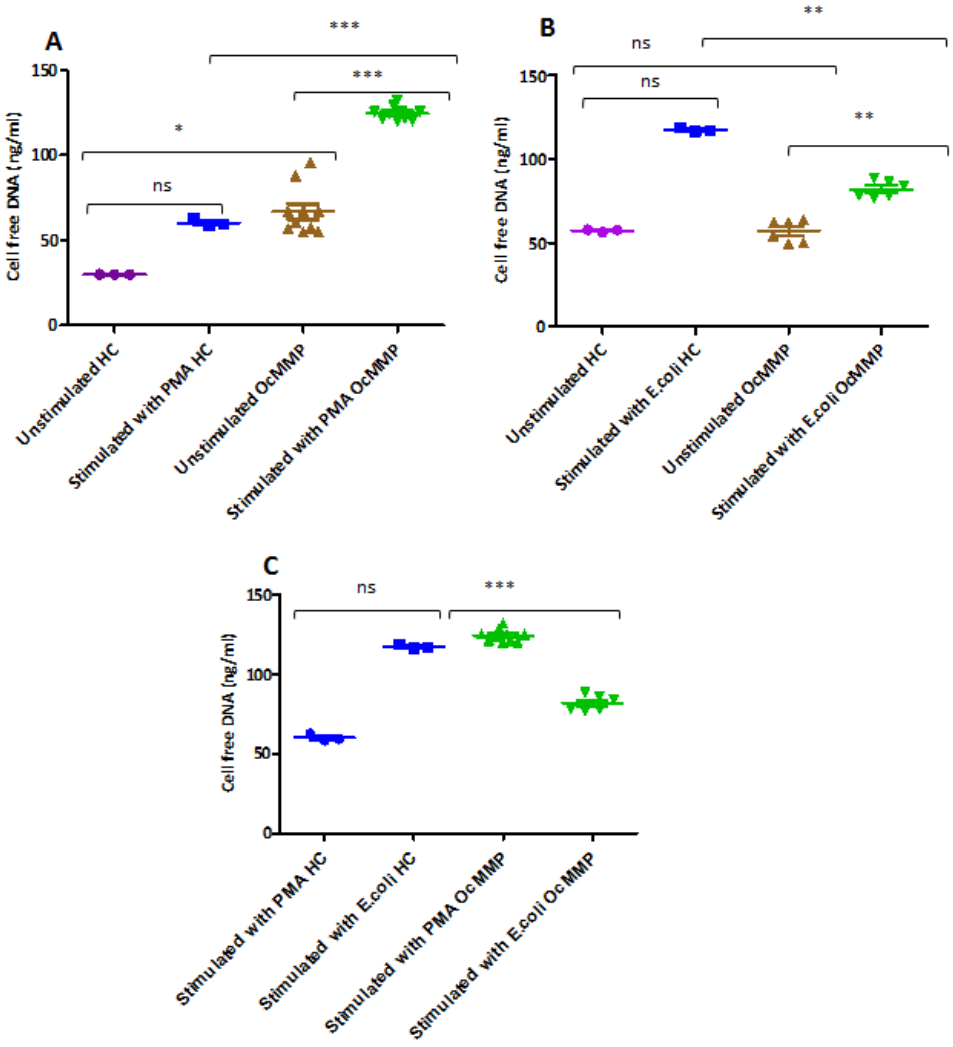
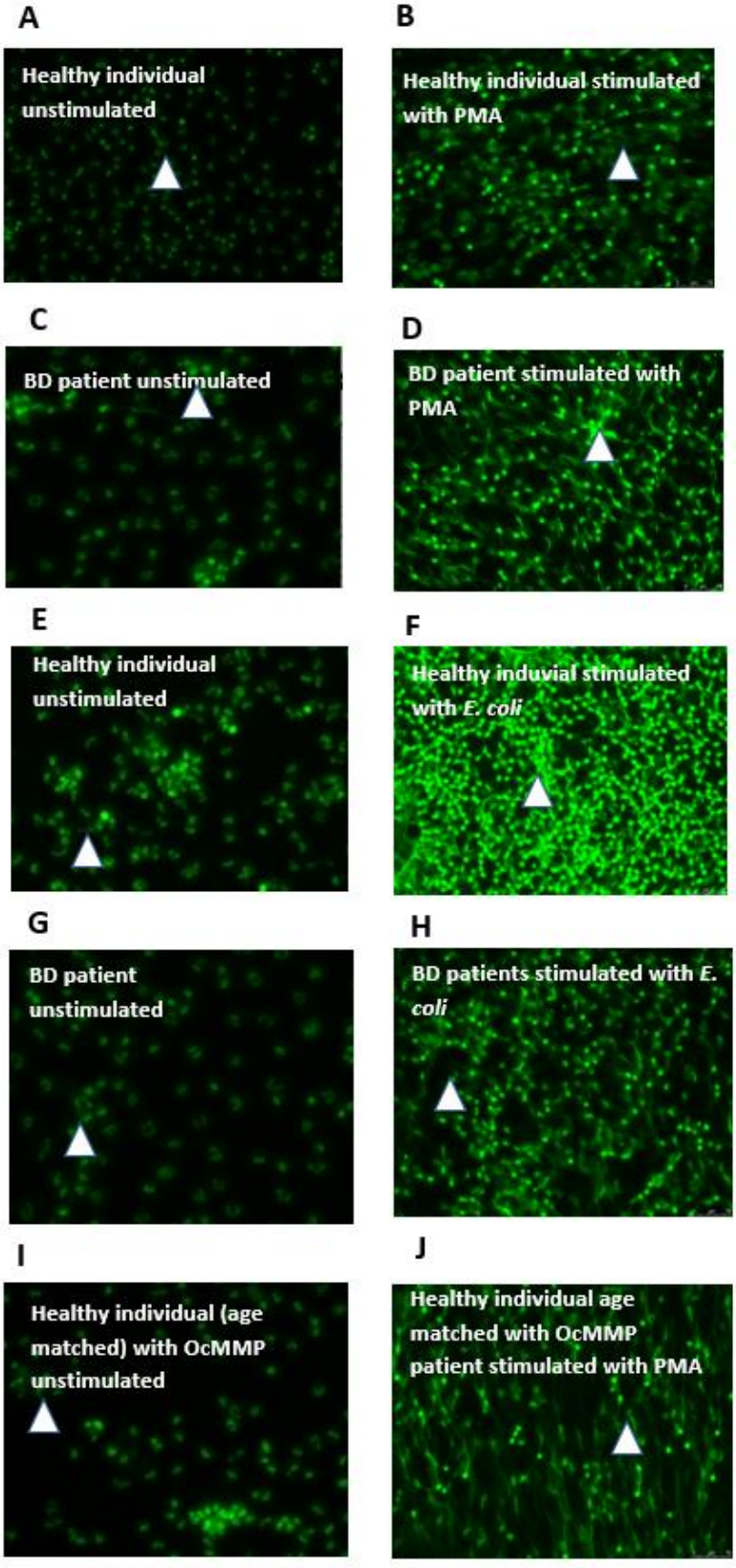


Figure 19-The production of NETs by PMA and E. coli stimulation. (A) The production of NETs in neutrophils stimulated with 25nM of PMA for 3 hours and unstimulated neutrophils from healthy individuals (n=3) and OcMMP patients (n=10). The production of NETs in neutrophils stimulated with 1-2 x 10^9 bacteria per ml of *E. coli* for 3 hours and unstimulated neutrophils from healthy individuals (n=3) and OcMMP patients (n=6). (C) The production of NETs neutrophils stimulated with 25nM of PMA and 1-2 x 10^9 bacteria per ml of *E. coli* in neutrophils of healthy individuals (n=3) and OcMMP patients (n=10). *P< 0.05, **P <0.01, ***P <0.001, not significant (ns)

3.1.2.5. The visualisation of NET in all neutrophils from patients with BD and OcMMP

In order to visualise NET in stimulated neutrophils with PMA and *E.coli* Sytox Green was used. The results were comparable to results obtained for the productions of cell free DNA by all neutrophils from patients with BD in comparison to healthy controls. Figure 20 showed DNA strands in neutrophils stimulated with PMA or *E.coli* from patients with BD (D and H) or OcMMP (L and P) were identified.


A
Healthy individual unstimulated
B
Healthy individual stimulated with PMA
C
BD patient unstimulated
D
BD patient stimulated with PMA
E
Healthy individual unstimulated
F
Healthy induvial stimulated with E. coli
G
BD patient unstimulated
H
BD patients stimulated with E. coli
I
Healthy individual (age matched) with OcMMP unstimulated
J
Healthy individual age matched with OcMMP patient stimulated with PMA

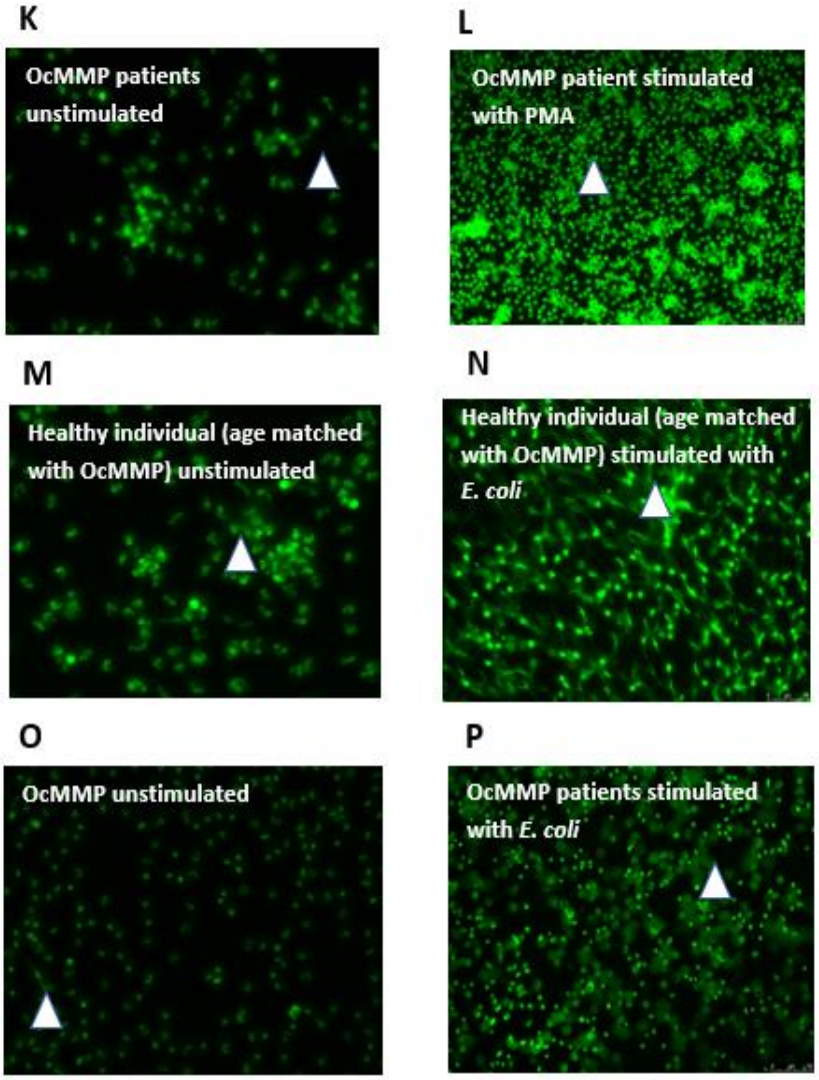


Figure 20-The production of NETs in all neutrophil cultures of BD and OMMP patients. The isolated cell cultures were seeded into 8 well chamber slides and the cells were stimulated by PMA (25nM) and opsonised *E.coli* (1-2 x 10^9 bacteria per ml) for 3 hours and stained with Sytox dye. Images were taken at X20. Arrow- representing formation of NET (strand of DNA).

3.2 Discussion

The neutrophils are known for their functions during an inflammatory response. The aim of this chapter was to investigate the functions of total neutrophils from patients with BD and OcMMP in comparison to healthy controls.

This study showed PMN have a reduced phagocytic capacity and ROS production in both BD and OcMMP patients when compared to healthy controls. A low neutrophil function with regards to phagocytosis indicates a neutrophil deactivation state (Danikas *et al.,* 2008). The results obtained in this study are consistent with a study by Johansson *et al.,* which showed an impaired function of neutrophil phagocytes in anti-neutrophil cytoplasmic antibody associated vasculitides a condition characterised by autoimmune small vessel inflammation (Johansson *et al.,* 2016). A significantly low phagocytic function in BD and OcMMP may be due to continued hyper-stimulation by systemic cytokines and eventually leading to exhaustion.

Similar to phagocytosis, a reduction is ROS production was observed that may be due to constant inflammatory response as a sequential stimulation of neutrophils is taking place. The constant activation of neutrophils may occur due to constant tissue damage in BD patients. Therefore, a re-stimulation activity of pre-stimulated neutrophils results in a reduced production of O_2 radicals in BD and OcMMP. A reduced production of O_2 radicals during the production of ROS shows that neutrophils are previously stimulated in inflammatory diseases (Eksioglu-DImiralp *et al.,* 2001). However, Nesrine Elloumi *et al.,* showed an increase in ROS production in SLE patients by using bacterial stimuli to initiate ROS production (Nesrine

Elloumi *et al.,* 2017). These differences may be due to the heterogeneity of the patient cohorts or the effects of the type of stimuli used.

The data presented in this study showed that neutrophils from age-matched healthy controls overall had a higher ROS production in comparison to BD patients and OcMMP patients. This suggests the results achieved were due to a chronic disease affect. The evaluation of age-associated changes in humans has demonstrated a decrease ROS production in reaction to opsonised bacteria (Fortin *et al.,* 2008). However, the results achieved in this study showed a pure disease affect. Age did not influence the phagocytic capacity and ROS production of neutrophils as healthy donors continued to have a higher phagocytic capacity in comparison to patients diagnosed with BD and OcMMP. The findings clearly support the hypothesis of a pure disease affect.

The results achieved in this study support the hypothesis of neutrophils contributing towards the disease similarly acknowledged by (Williams *et al*., (2013). Williams *et al*., showed an in increase in the percentage of $CD45^{INT}CD11b^{+}CD16^{+}CD14^{-}$ neutrophils and a reverse correlation with the disease duration in patients diagnosed with Stevens-Johnson Syndrome/Toxic epidermal necrolysis (SJS-TEN). Patients with SJS-TEN may have an OcMMP like conjunctival scarring phenotype and morphology. This study showed that neutrophilic infiltration from patients at numerous stages of the disease was observed. Therefore, this associates neutrophil to the progression of conjunctival fibrosis seen in OcMMP patients and disease initiation (Williams *et al.,* 2013).

NET formation may play an important role in the development and preservation of auto immune diseases and organ damage in chronic inflammatory disorders. Results showed increased production of NET in BD patients in comparison to healthy controls. The results

suggested that enhanced production of NET in BD patients may contribute towards the pathology of BD. This is because small vessel vasculitis is a common symptom of BD and NET are proposed to be part of the anti-neutrophil cytoplasmic antibodies. This occurs by the release of, and prolonged exposure to proteins released during NETosis which in response generate antibodies that are involved in small vessel vasculitis and thrombosis (Delgado-Rizo *et al.,* 2017)

A similar phenomenon was observed in OcMMP patients. Results showed increased production of NET in OcMMP patients in comparison to healthy controls. The results suggested that enhanced production of NET contributed towards the initiation of OcMMP. This was supported by a study performed by Chrysanthopoulou *et al*., (2014) that showed NET formation in respiratory mucosa has been associated with lung myofibroblast differentiation and scar formation. Previous studies have identified the production of NET in ocular surface disease. Tibrewal *et al.,* showed that hyperosmolarity induces formation of NETs in dry eye disease. This is because hyperosmolarity leads to death of epithelial cells and to a cascade of inflammatory events, and a result lead to loss of mucin producing goblet cells (Tibrewal *et al.,* 2014). Greater production of NET may also be due to the contribution of extracellular matrix and therefore simultaneous presence of fibronectin may lead to suppression of ROS production and rapid NET production (Stoiber *et al.,* 2015). However, the concept of ROS-NETosis remains controversial and whether ROS is actually required for NET production.

The results showed that overall, total neutrophils from patients with BD and OcMMP stimulated with *E.coli* produced lower quantity of cell free DNA compared to PMA. PMA triggers NET formation though ROS-dependent pathway, while *E.coli* stimulation may include

phagocytosis and/or LPS. There are at least two possible reasons for reduced NET production by *E.coli* one that the cells used are dead and therefore less likely to generate NET-inducing molecules, or two that reduced phagocytosis by neutrophils from patients inhibited the response. Use of live bacteria would be required to address this issue. That said, the results from this study suggest that NET formed by activated neutrophils have the ability to trap Gram^{-ve} bacteria can be considered to form part of the host defence system during BD.

This study suggests that reduced formation of NET by neutrophils with comprised extracellular killing of bacteria by this particular mechanism could have an acute impact on the removal of microbes in BD and OcMMP patients, increasing the chances for the patients to be highly susceptible to the disease. Hashiba *et al*.., showed that patients with sepsis exhibited impaired production of NET in response to *E.coli*. Nevertheless, that stimulation with PMA induced a higher response of NET in patients with OcMMP and BD patients shows that there is no inherent dysregulation in neutrophils from these individuals.

CHAPTER 4

THE IDENTIFICATION AND FUNCTION OF NEUTROPHIL SUBSETS IN BD AND OCMMP PATIENTS.

4. Introduction

An alternative explanation for the results shown in chapter 3 is that patients with BD or OcMMP have different populations of neutrophils in their circulation that may respond differently to stimulation. One of the main aspects of interests that relates to neutrophil heterogeneity is the emergence of mature and immature neutrophils in many different physiological and pathological conditions (Beyrau *et al.,* 2012, Dumitru *et al.,* 2012 and Silvestre-Roig *et al.,* 2016). The majority of the studies discussed in this thesis will focus on immature and mature neutrophil populations. This is because the development of these specific cell populations has been observed to be heterogeneous and have occurred in many conditions such as systemic inflammation, cancer or autoimmune diseases (Scapini *et al.,* 2016). In consequence, in order to understand the processes behind such dynamic heterogeneity it is vital to remember that systemic and chronic inflammation are not only known for the induction of an activated, circulating pool of mature neutrophils, but also induce granulopoiesis, leading to mobilisation of immature neutrophils precursors into the circulation (Hong, 2017). These two features not only occur during sepsis, trauma and burn injuries, but in viral infections, autoimmune disorders and cancer. As a result, one of the important characteristics often neglected is that both immature (LDN) and activated mature neutrophil (NDN) (in vivo) can therefore co-exist in the circulation.

The LDN population are co-purified in the PBMC layer in a Ficoll-density gradient. Further investigation of the nuclear morphology of LDN from a patient with an inflammatory disorder (SLE) revealed a mixture of population of LDN being segmented, banded or appearing as myelocyte-like cells (Fig.21). Transmission electron microscope of purified LDN fractions

showed many different granules in their cytoplasm, less segmented nuclei with their hetero- (dense) and euchromatin (de-condensed) clearly delineated (Carmona-Rivera *et al.,* 2013).

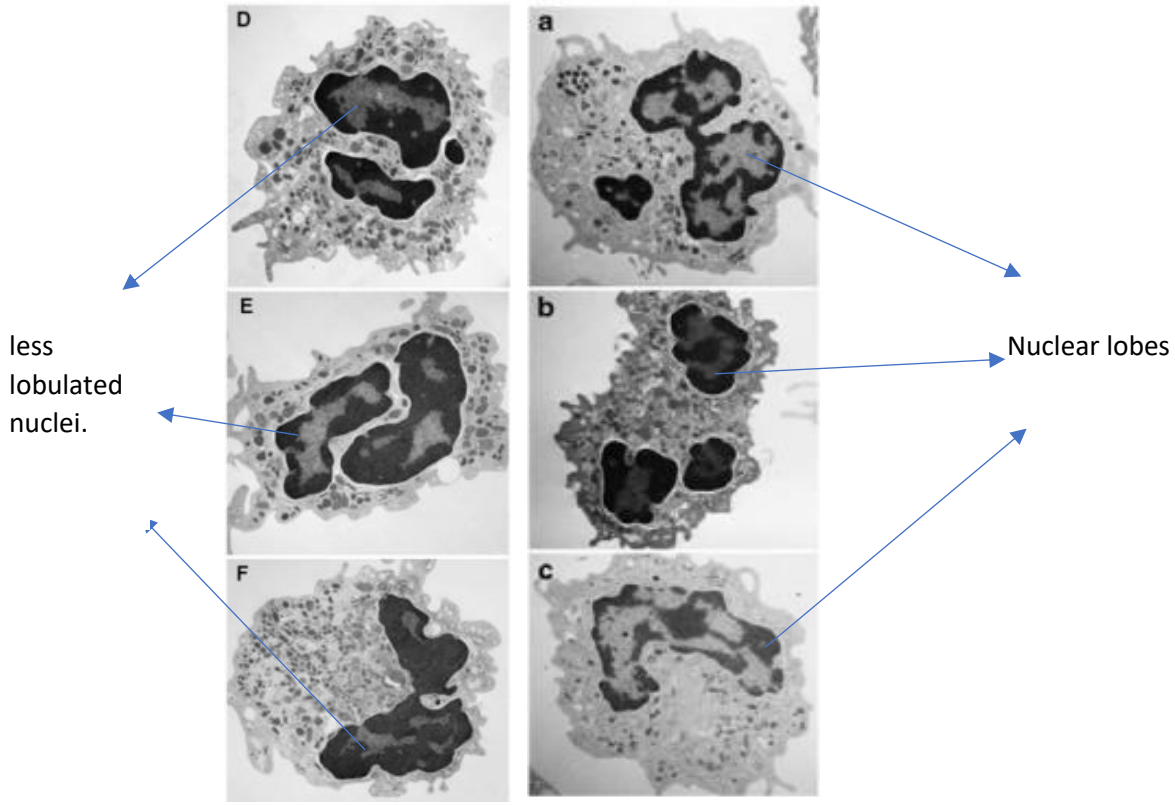


Figure 21-The analysis of LDN and NDN morphology. The LDN morphology (D-F) and NDN (A-C) morphology isolated from a SLE patient using transmission electron microscopy (Rivera and Kaplan 2013). The LDN represent an immature morphology such as banded and NDN represent an mature nuclear lobes. Heterochromatic areas are the darker shaded areas and euchromatic areas are defined as the lighter shaded areas (Carmona-Rivera *et al.,* 2013).

The role of LDN in NETosis is currently being investigated. Rivera *et al.,* suggested that LDN have an enhanced capacity to generate NET. The formation of NET allows LDN to release their intracellular content into the microenvironment including many different molecules LL37, α- and β-defensins and HMGB1 associated with nucleic acids (Carmona-Rivera *et al.,* 2013).

Normal density neutrophils (NDN) comprise both terminally differentiated and in rare case immature neutrophils. In consequence, under normal conditions these cells are the resting state as this particular subset is not stimulated by any external stimuli. It is suggested the composition of immature neutrophils is a small percentage of the peripheral neutrophils. However, *de novo* production of neutrophils during a systemic inflammatory response enhances the number of immature neutrophils in the peripheral circulation during 'emergency granulopoiesis' (Hong, 2017). These immature cells are phenotypically classified as metamyelocytes and band cells. Subsequently, neutrophil differentiation requires granules and based upon granularity the density of these cells is considered to be comparable to that of mature neutrophils.

Normal density neutrophils sediment with the red-blood cells fragment in a Ficoll-density gradient and therefore are classified to be heavier than Ficoll-Paque . It is suggested that due to LDN expression of defensin and their morphology, it can be argued that LDN are progenitors of normal density neutrophils and are prematurely released from the bone marrow during an acute or chronic inflammatory response. It is acknowledged that normal density neutrophils encounter two or more lobes connected with filaments. A study by Carmona-Rivera *et al.,* showed that normal density neutrophils isolated from Ficoll-Hypaque preparations of SLE patients showed segmented nuclei (Fig.21) (Rivera *et al.,* 2013). The genetic analyses of NDN reveal no differences in gene regulation when comparing to healthy controls. This phenomenon was confirmed by Villanueva *et al.,* which showed no significant differences in gene regulation when comparing normal density lupus neutrophils with healthy control neutrophils (Villanueva *et al.,* 2011). The normal density neutrophils can be phenotypically identified as CD15+ population. The 'mature' neutrophil population being classified as NDN are capable of switching and becoming LDN and loss of antitumor

proprieties and the gain of immunosuppressive properties (Sagiv *et al.,* 2015). Given all the evidence described above with regards to neutrophil subsets no study to date had investigated the presence of LDN and NDN subsets and their functional profile in BD and OcMMP patients *in vitro.* In this chapter the presence of LDN and NDN and their functions such as phagocytosis, ROS and NET production were assessed in LDN and NDN to investigate the results observed for total neutrophils from patients in chapter 3.

4.1. Results

4.1.1. The morphology of LDN and NDN

The isolated LDN exhibited a curved nucleus with 2 or fewer nuclear lobes (Fig.22A) providing a representation of an immature neutrophil. The morphological analysis of NDN (Fig.22B) showed mature neutrophils of normal segmented nucleus as also observed in healthy controls.

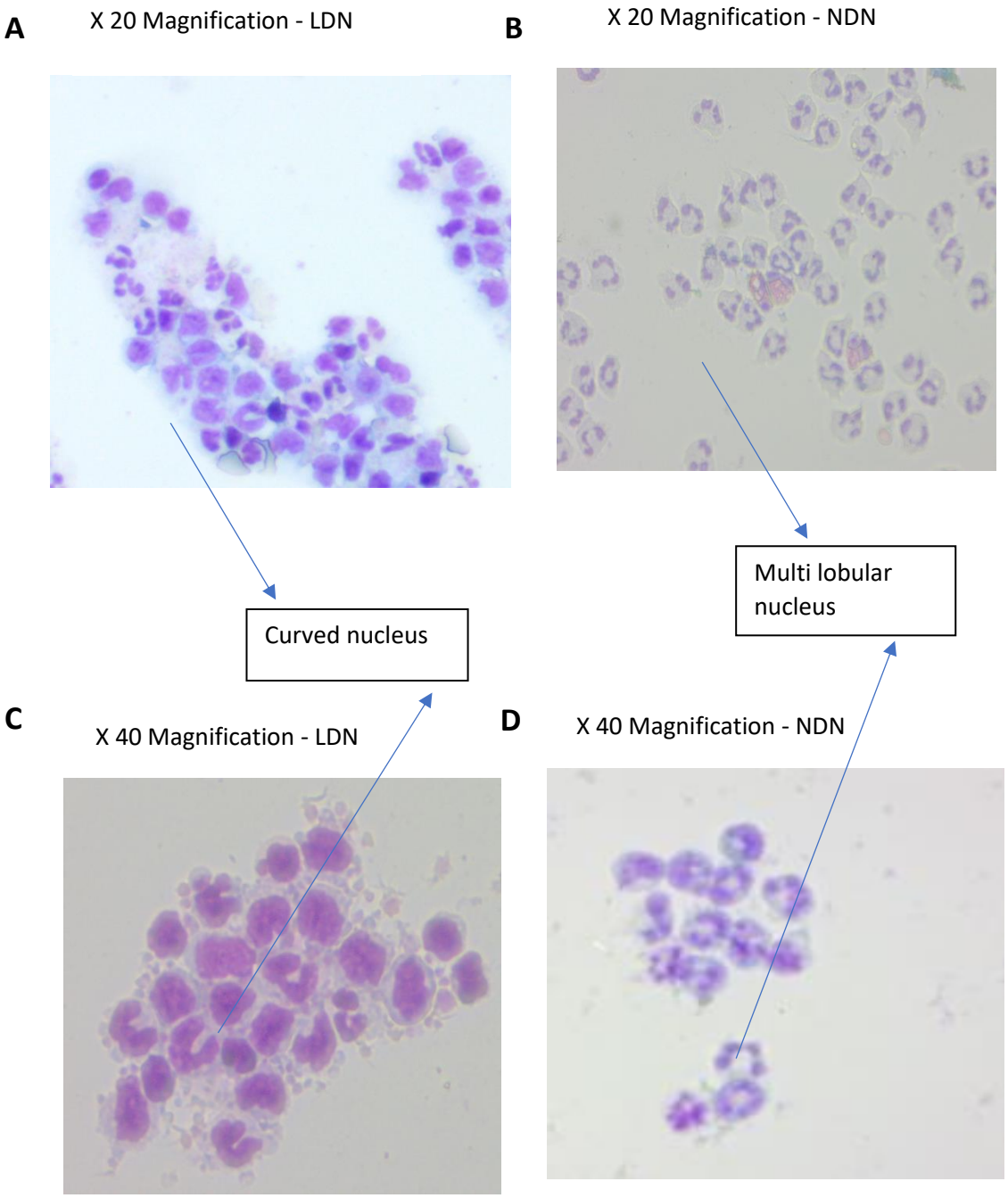


Figure 22-The morphology of different subsets of neutrophils. Low density neutrophils (A, C) and Normal density neutrophils (B, D) were isolated from a healthy individual. The morphology was determined via cytospins and the cells were further stained with Giemsa for 30 minutes. The images were taken at x20 and x40 (for a clear representation).

4.2. LDN and NDN in patients with BD or OcMMP

The results showed that patients with BD showed a significantly higher number of LDN (62 ± 1 (%) vs 17 ± 2 (%)) ($p<0.0001$) and a reduced NDN (38 ± 1 (%) vs 83 ± 2 (%)) ($p<0.0001$) in comparison to healthy controls (Fig.23A). Similar results were observed for patients diagnosed with OcMMP who exhibited a significantly higher number of (68 ± 1 (%) vs 14 ± 1 (%)) ($p<0.0001$) LDN and a reduced NDN (32 ± 1 (%) vs 86 ± 1 (%)) ($p<0.0001$) in OcMMP patients in comparison to age-matched healthy controls (Fig.23B).

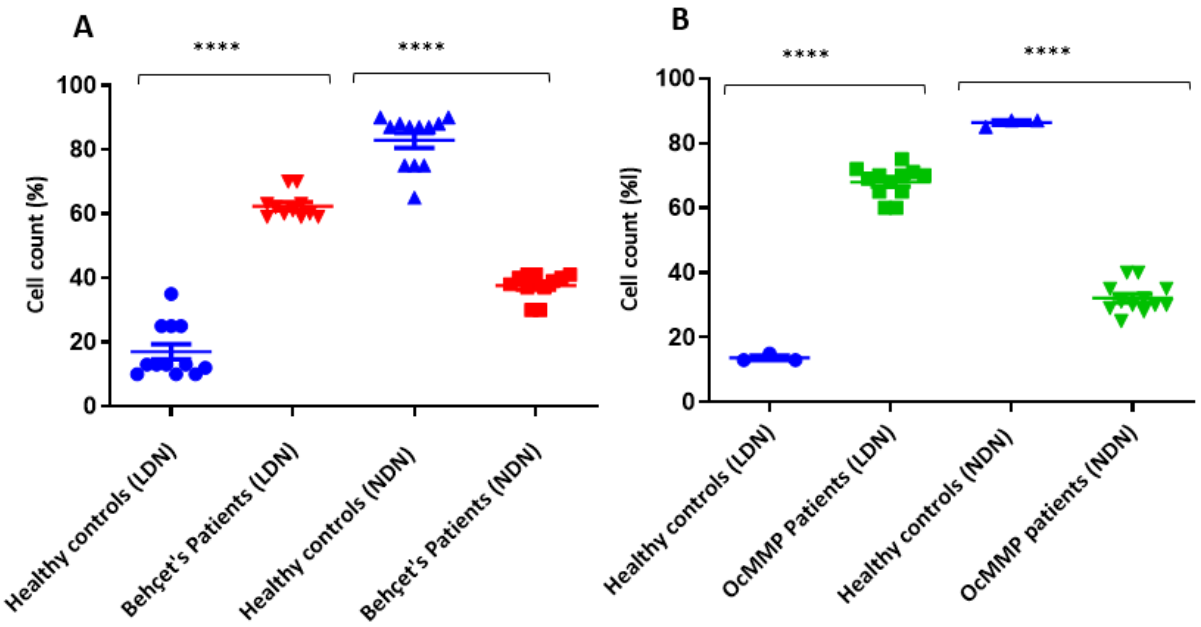


Figure 23-The percentage of LDN and NDN in BD, OcMMP patients and healthy individuals. The percentage (count) of LDN (n=11) and NDN (n=11) in BD patients in comparison to healthy controls (n=12). (B)-The percentage (count) of LDN (n=12) and NDN in OcMMP (n=11) patients in comparison to age matched elderly controls patients (n=3). The percentage of LDN and NDN was investigated based upon the morphology of the isolated cells using Ficol-hypaque gradient. The LDN were isolated from the PBMSC layer and NDN were isolated from the buffy layer on top of red blood cells. ****P<0.0001

4.3 Functions of LDN and NDN

4.3.1 The phagocytic capacity and production of reactive oxygen species in LDN and NDN in BD and OcMMP patients in comparison to healthy controls

The results showed a significantly lower phagocytic capacity of LDN and NDN (Fig.24A) in patients diagnosed with BD (28 ± 5 (%) vs 98 ± 1 (%)) ($p<0.0001$) (28 ± 5 (%) vs 96 ± 3(%)) ($p<0.0001$) in comparison to young healthy individuals. Figure 24B showed that production of ROS by LDN and NDN was significantly lower in BD (32 ± 5 (%) vs 96 ± 1 (%)) ($p<0.0001$) (32 ± 6 (%) vs 91 ± 4 (%)) ($p<0.0001$) in comparison to young healthy individual. The MFI observed for phagocytic capacity and ROS production are observed in Fig.24C and D.

A similar result was seen in LDN and NDN (Fig.25A) from patients diagnosed with OcMMP (42 ± 3 (%) vs 85 ± 1 (%)) ($p<0.0004$) (41 ± 3(%) vs 87 ± 2 (%)) ($p<0.0001$) in comparison to elderly individuals. The production of ROS by LDN (42 ± 3 (%) vs 85± 1 (%)) ($p<0.0001$) and NDN (40 ± 3 (%) vs 74 ±3 (%)) ($p<0.0003$) was significantly lower in OcMMP in comparison to elderly healthy individuals (Fig.25B). The MFI observed for phagocytic capacity and ROS production are observed in Fig.25C and D.

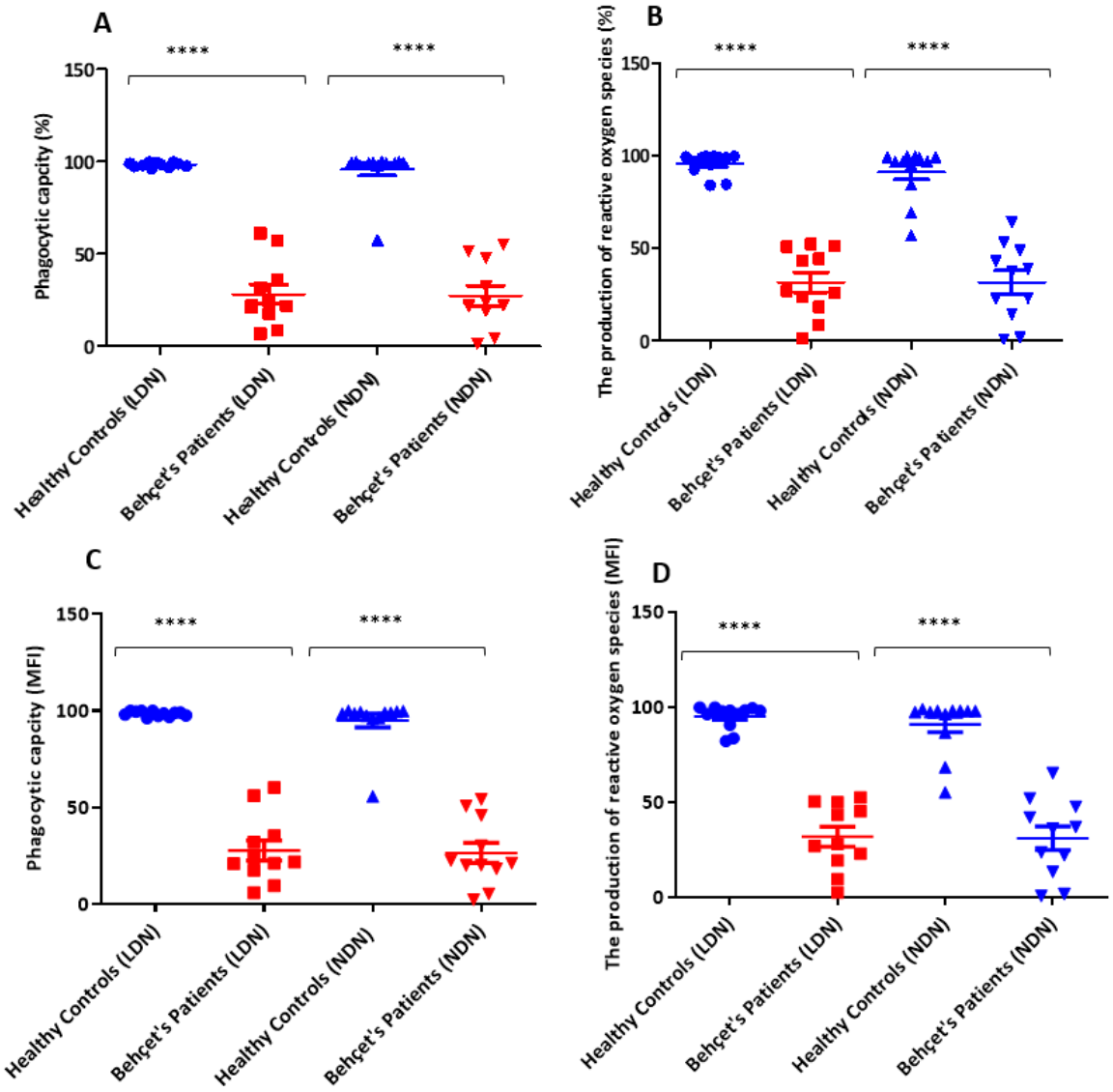


Figure 24-The percentage positive cells and mean fluorescence intensity for phagocytic activity and ROS production in LDN and NDN of BD patients and healthy individuals. (A, C) The phagocytic activity of LDN (n=11) and NDN (n=12) in BD patients in comparison to healthy controls (n=12). This was investigated by adding fluorescein isothiocyanate (FITC)-labelled *E.coli* to samples obtained from BD patients and healthy controls. (B, D) ROS production by LDN (n=11) and NDN (n=11) in BD patients and healthy controls (n=12) by stimulating the samples with non-labelled *E.coli*. ****P<0.0001.

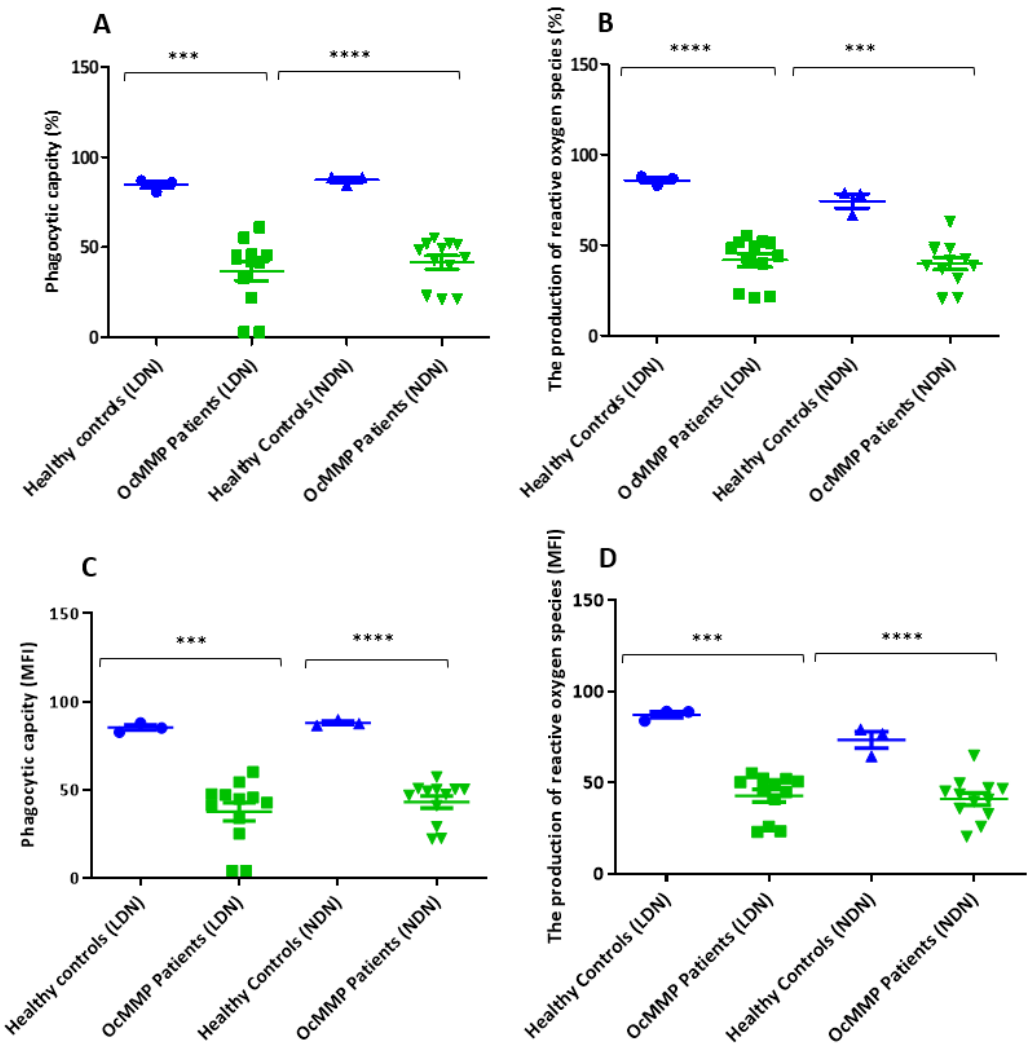


Figure 25-The percentage positive cells and mean fluorescence intensity for phagocytic activity and ROS production of LDN and NDN in OcMMP patients and healthy individuals. (A, C) The phagocytic activity of LDN (n=12) and NDN (n=12) in OcMMP patients in comparison to age matched healthy controls (n=3). This was investigated by adding fluorescein isothiocyanate (FITC)-labelled *E.coli* to samples obtained from BD patients and healthy controls. (B, D) ROS production of LDN (n=12) and NDN (n=12) in BD patients and healthy controls (n=3) by stimulating the samples with non-labelled *E.coli.* ***P <0.001, ****P<0.0001

4.3.2 The production of NETs in LDN

Figure 26A showed that cell free DNA taken from cultures of PMA stimulated LDN of patients diagnosed with BD was not only significantly higher (128 ± 1 (ng/ml) vs 49 ± 3 (ng/ml)) ($p<0.0001$) than cell free DNA content taken from unstimulated BD cell cultures, but was also significantly higher (128 ± 1 (ng/ml) vs 35 ± 2 (ng/ml)) ($p=0.0003$) in comparison to cell free DNA taken from cultures of PMA stimulated neutrophils of healthy individuals. The results showed PMA stimulated LDN of healthy controls was significantly (35 ± 2 (ng/ml) vs 29 ± 1 (ng/ml)) ($p=0.002$) higher in comparison to unstimulated LDN in healthy individuals. A significant spontaneous (49 ± 3 (ng/ml) vs 29 ± 1 (ng/ml)) NETosis was observed in unstimulated BD cell cultures in comparison to unstimulated healthy individuals ($p=0.0004$).

Figure 26B showed that cell free DNA obtained from cultures of *E.coli* stimulated LDN of BD patients was significantly lower (67 ± 1 (ng/ml) vs 82 ± 1 (ng/ml)) ($p<0.0001$) than cell free DNA content taken from *E.coli* stimulated neutrophils of healthy individuals; despite being higher (67 ± 1 (ng/ml) vs 49 ± 1 (ng/ml)) ($p=0.0002$) in comparison to unstimulated LDN of BD patients. The results also showed a significant (82 ± 1 (ng/ml) vs 55 ± 1 (ng/ml)) ($p<0.0001$) increase in the production of cell free DNA by stimulated LDN in comparison to unstimulated LDN in healthy individuals. The results showed that unstimulated LDN cell cultures produced higher amount (55 ± 1 (ng/ml) vs 49 ± 1 (ng/ml)) ($p=0.0003$) of spontaneous NET in comparison to unstimulated LDN cell cultures of BD patients (Fig.26B).

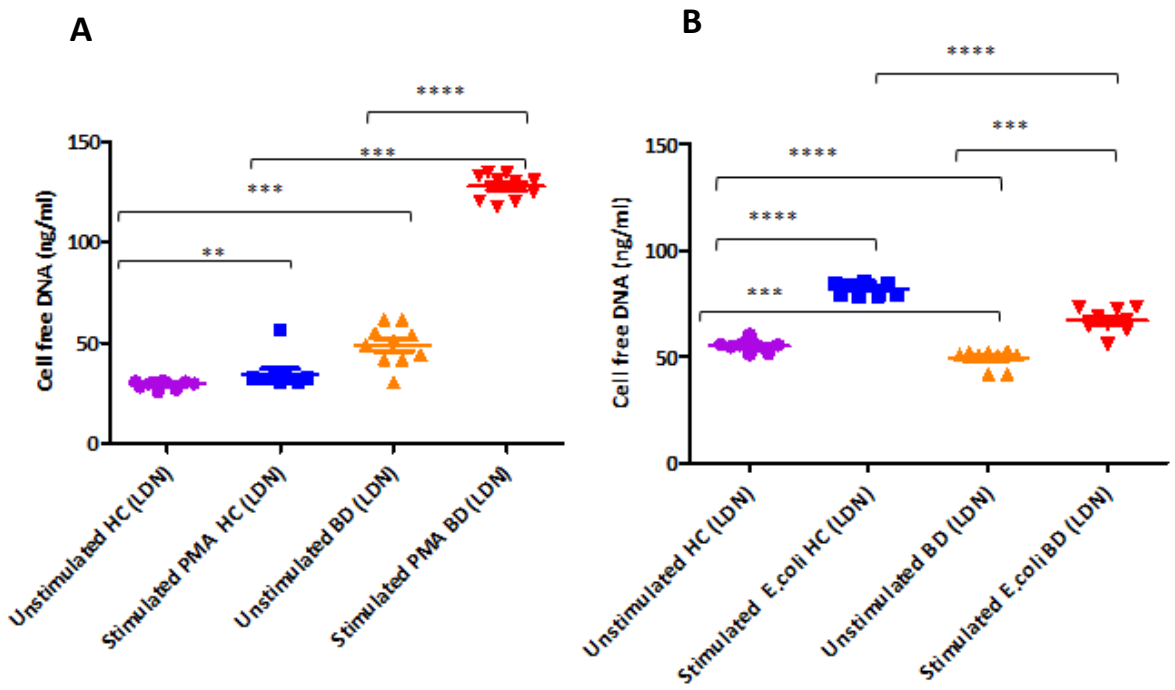


Figure 26-The production of NETs in LDN of BD patients and healthy individuals with PMA and *E. coli* stimulation. (A) The production of NETs in LDN stimulated with 25nM of PMA for 3 hours from healthy controls (n=10) and BD (n=10) patients, unstimulated healthy controls (n=10) and unstimulated BD patients (n=10). (B) The production of NETs in LDN incubated with 1-2x10^9 bacteria per ml of *E.coli* for 3 hours in healthy individuals (n=10) and BD (n=10), unstimulated healthy controls (n=10) and unstimulated BD (n=10), stimulated healthy controls (n=10) and stimulated BD patients (n=10). **P <0.01, ***P <0.001, ****P<0.0001

A similar pattern was observed in cells from patients with OcMMP showing that PMA was an effective inducer of NET formation in LDN isolated from patients with OcMMP patients and healthy individuals, but *E.coli* did not induce an increased response. Cell free DNA taken from cultures of PMA stimulated LDN of patients diagnosed with OcMMP was significantly higher (137 ± 2 (ng/ml) vs 62 ± 3 (ng/ml)) ($p<0.0001$) than cell free DNA content taken from unstimulated OcMMP cell cultures, and significantly higher (137 ± 2 (ng/ml) vs 62 ± 2 (ng/ml)) ($p=0.003$) in comparison to cell free DNA taken from cultures of PMA stimulated neutrophils of healthy individuals (Fig.27A). The results showed unstimulated OcMMP LDN cfDNA production was significantly higher (62 ± 3 (ng/ml) vs 32 ± 1 (ng/ml)) ($p=0.01$) in comparison to unstimulated LDN in healthy individuals. A significant NETosis was observed in stimulated OcMMP cell cultures in comparison to stimulated healthy individuals (137 ± 3 (ng/ml) vs 62 ± 3 (ng/ml)) ($p=0.02$).

Figure 27B shows that cell free DNA obtained from cultures of *E.coli* stimulated LDN from OcMMP patients was significantly lower (70 ± 1 (ng/ml) vs 86 ± 1 (ng/ml)) ($p=0.02$) than cell free DNA content taken from stimulated *E.coli* neutrophils of stimulated healthy individuals; despite being higher (70 ± 1 (ng/ml) vs 51 ± 1 (ng/ml)) ($p=0.002$) in comparison to unstimulated LDN of OcMMP patients. The results also showed an increase in the production of cell free DNA by stimulated LDN in comparison to unstimulated LDN in healthy individuals. Unstimulated LDN cell cultures produced high amount of spontaneous cfDNA in comparison to unstimulated LDN cell cultures of OcMMP patients (Fig.27B).

The results showed the control group (unstimulated cells) for PMA stimulated LDN of healthy individuals produced less spontaneous NETosis in comparison to the control group for *E.coli* stimulated LDN of healthy individuals. This was observed for both patient cohorts (Fig.26A, 26B, 27A, 27B).

A similar pattern was observed with the LDN showing that PMA was an effective inducer of NET formation in LDN isolated from BD and OcMMP patients and healthy individuals (Fig.26A, 27A) However, NETs in LDN a with the stimulant *E.coli* was reduced in BD and OcMMP patients in comparison to healthy controls (Fig.26B, 27B).

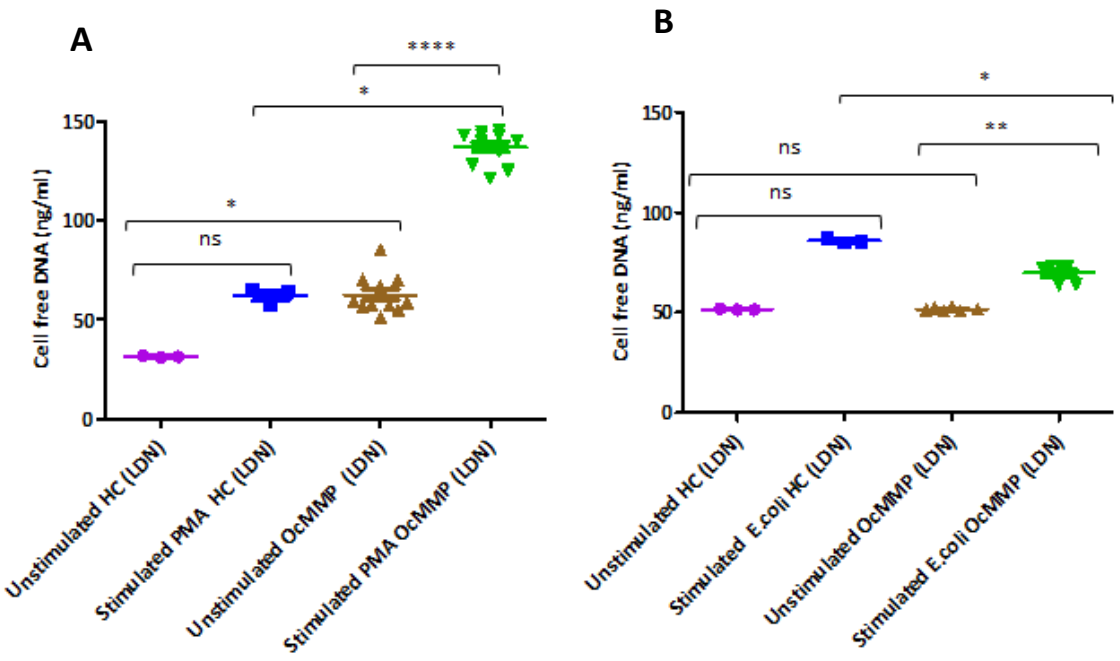
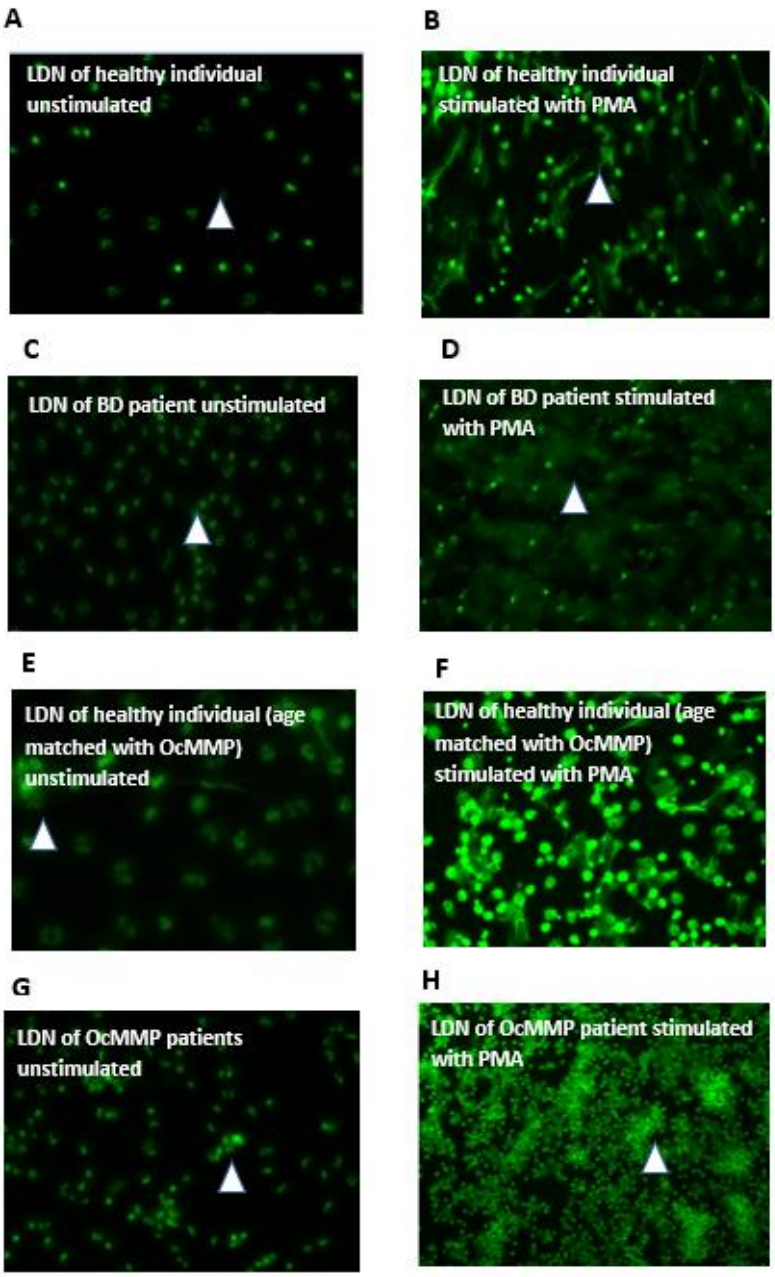


Figure 27-The production of NETs in LDN of OcMMP patients and healthy individuals with PMA and *E. coli* stimulation. (A) The production of NETs in LDN stimulated with 25nM of PMA in LDN from healthy controls (n=3) and OcMMP (n=11) patients, unstimulated healthy controls (n=3) and unstimulated OcMMP (n=11). (B) The production of NETs in NDN stimulated with 1-2x10^9 bacteria per ml of *E.coli* in LDN from healthy controls (n=3) and OcMMP (n=6) patients, unstimulated healthy controls (n=3) and unstimulated OcMMP (n=6). *P<0.05, **P <0.01, ****P<0.0001, not significant (ns)

4.3.3 The visualisation of NETs in LDN in BD and OcMMP patients

The results showed that PMA was an effective inducer of NET formation in LDN isolated from BD and OcMMP patients compared to healthy individuals (Fig..28 A-F). However, the production of NET in LDN with the stimulant *E. coli* was reduced in BD and OcMMP patients in comparison to healthy controls (Fig.28 G-L).


A
LDN of healthy individual unstimulated
B
LDN of healthy individual stimulated with PMA
C
LDN of BD patient unstimulated
D
LDN of BD patient stimulated with PMA
E
LDN of healthy individual (age matched with OcMMP) unstimulated
F
LDN of healthy individual (age matched with OcMMP) stimulated with PMA
G
LDN of OcMMP patients unstimulated
H
LDN of OcMMP patient stimulated with PMA

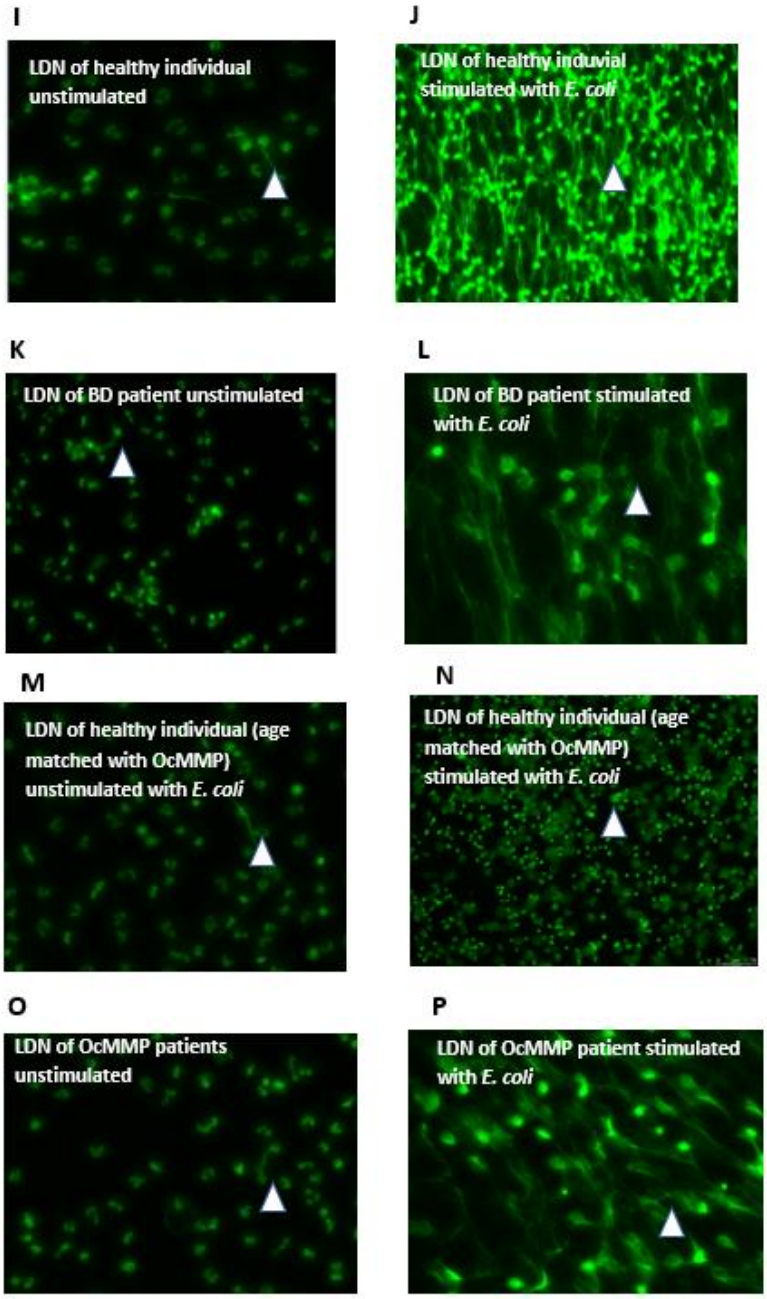


Figure 28-The production of NETs in LDN cultures of healthy individuals BD and OcMMP patients. (A-F) The isolated cells cultures were seeded into 8 well chamber slides and stimulated with PMA (25nM) and opsonised *E.coli* (1-2 x 10^9 bacteria per ml) (E-L) and stained with Sytox dye. Images were taken at x20. Arrow-representing formation of NET (strand of DNA).

4.3.4 The production of NETs in NDN

The cell free DNA taken from cultures of PMA stimulated NDN of BD was not only significantly higher (99 ± 2 (ng/ml) vs 44 ± 3 (ng/ml)) (p=0.0004) than cell free DNA content taken from unstimulated BD neutrophil cell cultures, but was also significantly higher (99 ± 2 (ng/ml) vs 55 ± 2 (ng/ml)) (p=0.0004) in comparison to cell free DNA taken from cultures of PMA stimulated neutrophils of healthy individuals (Fig.29A). The production of NETs from cultures of primed NDN of healthy individuals was also significantly (55 ± 2 (ng/ml) vs 31 ± 1(ng/ml)) (p=0.0005) enhanced (Fig.29A). An increase (44 ± 3 (ng/ml) vs 31 ± 1 (ng/ml)) in spontaneous NETosis was observed in NDN of BD patients in contrast to health individuals (Fig.29A) (p=0.0003)

However, stimulation with *E.coli* showed a significantly reduced production of NETs in stimulated (65 ± 2 (ng/ml) vs 79 ± 1 (ng/ml)) (p=0.0002) neutrophil cell cultures of BD patients in comparison to healthy controls (Fig.29B). A significant increase (65 ± 2 (ng/ml) vs 50 ± 1 (ng/ml)) (p=0.003) of stimulated *E.coli* neutrophil BD cell cultures in comparison to unstimulated was observed. A similar pattern of a significant increase (79 ± 1 (ng/ml) vs 54 ± 1 (ng/ml)) of cell free DNA in stimulated neutrophil cell cultures obtained from healthy controls compared to unstimulated (p=0.0001). Figure 29B showed significantly reduced spontaneous NETosis in in healthy individuals and BD patients (50 ± 1 (ng/ml) vs 53 ± 1 (ng/ml)) (p=0.04).

The results showed the control group (unstimulated cells) for PMA stimulated NDN of healthy individuals produced less spontaneous NETosis in comparison to the control group for *E.coli* stimulated NDN of healthy individuals. This was observed for both patient cohorts (Fig.29A, 29B, 30A, 30B).

A similar pattern was observed with the NDN showing that PMA was an effective inducer of NET formation in NDN isolated from BD and OcMMP patients and healthy individuals (Fig.29A, 30A) However, NETs in NDN a with the stimulant *E.coli* was reduced in BD and OcMMP patients in comparison to healthy controls (Fig.29B, 30B).

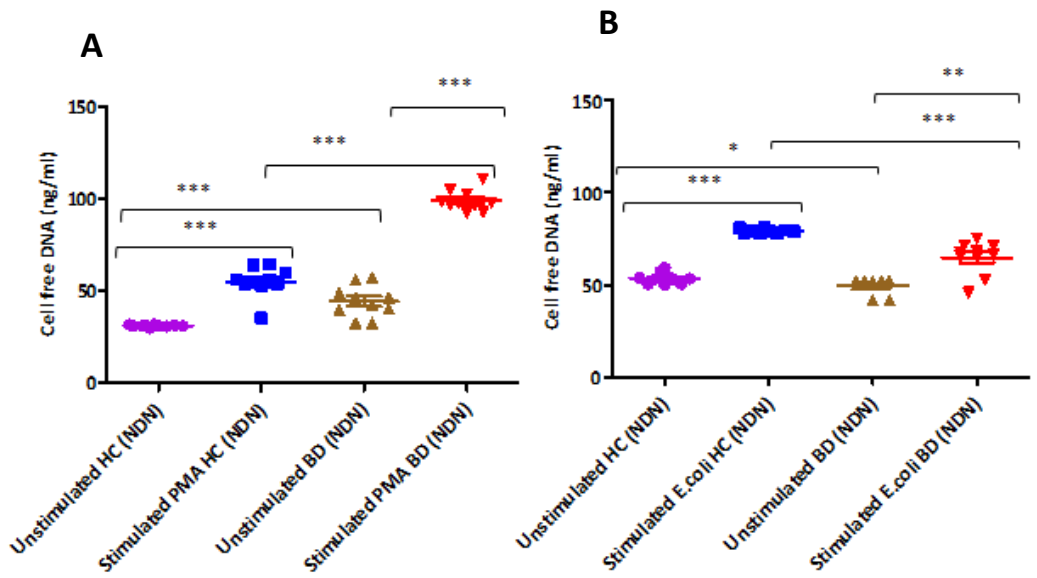


Figure 29-The production of NETs in NDN of BD patients and healthy individuals with PMA and *E.coli* stimulation. (A) The production of NETs in LDN stimulated with 25nM of PMA for 3 hours from healthy controls (n=10) and BD (n=10) patients, unstimulated healthy controls (n=10) and unstimulated BD patients (n=10). (B) The production of NETs in NDN stimulated with 1-2x10^9 bacteria per ml of *E.coli* for 3 hours of healthy individuals (n=10) and BD (n=10), unstimulated healthy controls (n=10) and unstimulated BD (n=10), stimulated healthy controls (n=10) and stimulated BD patients (n=10). *P< 0.05, **P <0.01, ***P <0.001

The cell free DNA taken from cultures of PMA stimulated NDN of OcMMP was not only significantly higher (96 ± 4 (ng/ml) vs 59 ± 5 (ng/ml)) (p=0.02) than cell free DNA content taken from unstimulated OcMMP neutrophil cell cultures, but was also significantly higher (96 ± 4 (ng/ml) vs 35 ± 1 (ng/ml)) (p=0.03) in comparison to cell free DNA taken from cultures of PMA stimulated neutrophils of healthy individuals. The production of NETs from cultures of primed NDN of healthy individuals was also enhanced (35 ± 1 (ng/ml) vs 30 ± 1(ng/ml)) (p=ns). An increase in spontaneous NETosis was observed in NDN of OcMMP patients in contrast to health individuals (59 ± 5 (ng/ml) vs 30 ± 1 (ng/ml)) (Fig.30A) (p=0.04)

A lower (70 ± 3 (ng/ml) vs 81 ± 1 (ng/ml)) (p=0.02) production of NETS from cultures of OcMMP NDN stimulated with *E.coli* was observed in comparison to stimulated NDN of healthy individuals (Fig.30B). However, an increase (70 ± 3 (ng/ml) vs 52 ± 1(ng/ml)) (p=0.01) in stimulated NDN was observed in comparison to unstimulated NDN of OcMMP patients (Fig.30B). An increase was also observed in cell free DNA of stimulated NDN of healthy individuals in contrast to unstimulated NDN. Figure 30B showed significantly enhanced spontaneous NETosis in healthy individuals and OcMMP patients (53 ± 0.3 (ng/ml) vs 51 ± 1 (ng/ml)) (p=0.03).

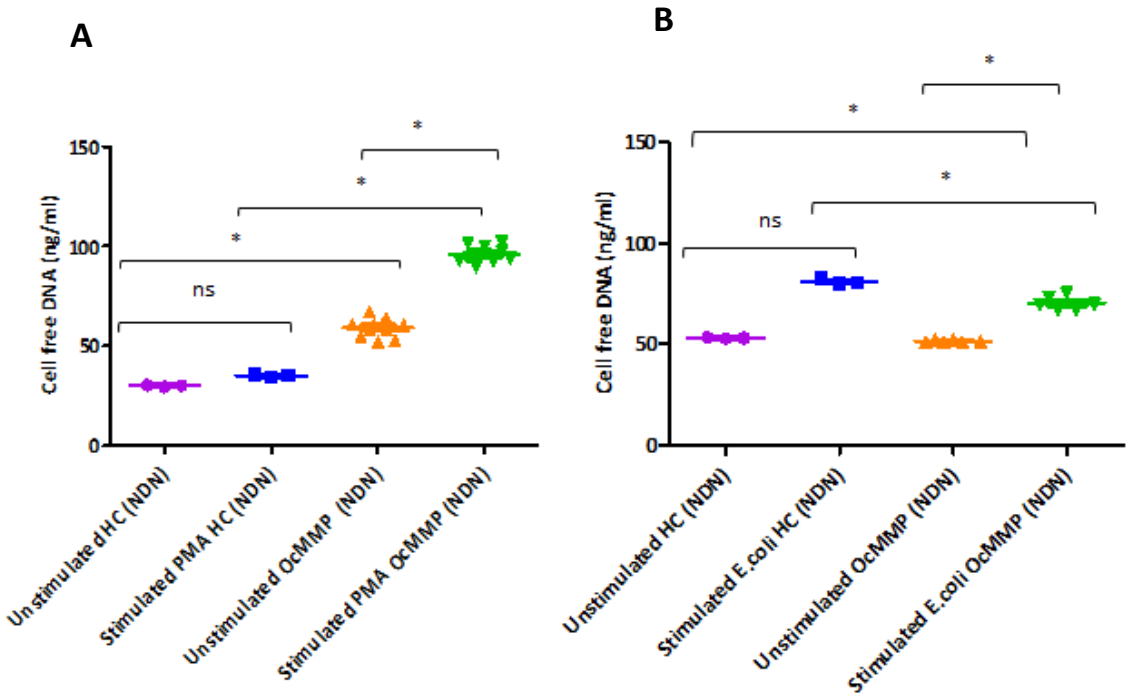


Figure 30-The production of NETs in NDN of OcMMP and healthy individuals with PMA and *E.coli* stimulation. (A) The production of NETs in NDN stimulated with 25nM of PMA for 3 hours from healthy controls (n=3) and OcMMP (n=11) patients, unstimulated healthy controls (n=3) and unstimulated OcMMP (n=11). (B) The production of NETs in NDN stimulated with 1-2x10^9 bacteria per ml of *E.coli* for 3 hours from healthy controls (n=3) and OcMMP (n=6) patients, unstimulated healthy controls (n=3) and unstimulated OcMMP (n=6). *P<0.05, not significant (ns)

To further control for our results we addressed two issues, age matching and time to preparation. It was not known which patients would consent at any given clinic and therefore it was not always possible to find a control individual who directly matched for age, and samples were stored on ice for two hours before they were processed. Therefore, we took control individuals of different ages and tested LDN and NDN function either immediately or after two hours storage (Fig.31). The results showed there was no difference in NET production whether donors were in their 20s, their 40s or their 60s. Similarly, there was no significant difference in NET production for the same sample that was used immediately or that had been stored for 2 hours on ice. This data supported our findings that differences in function were due to status of the donor rather than the methodology.

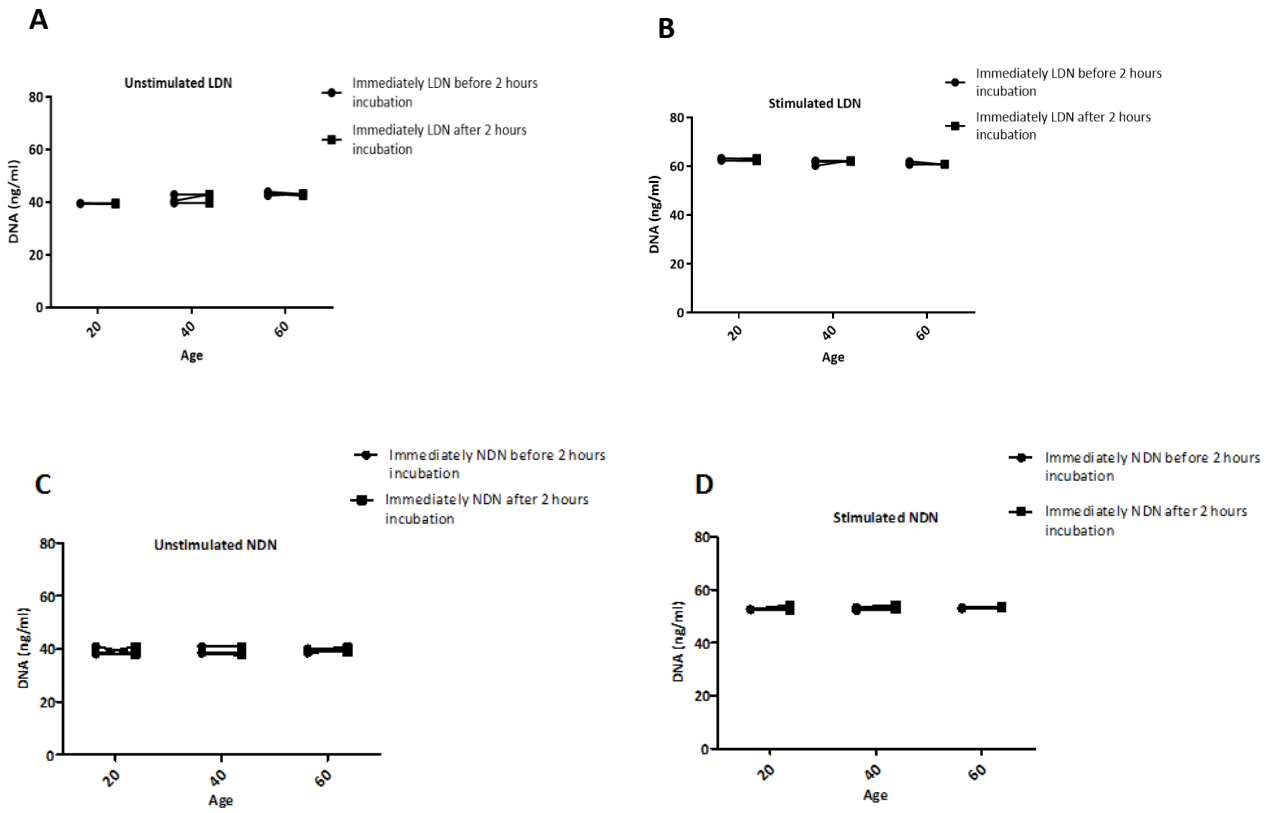
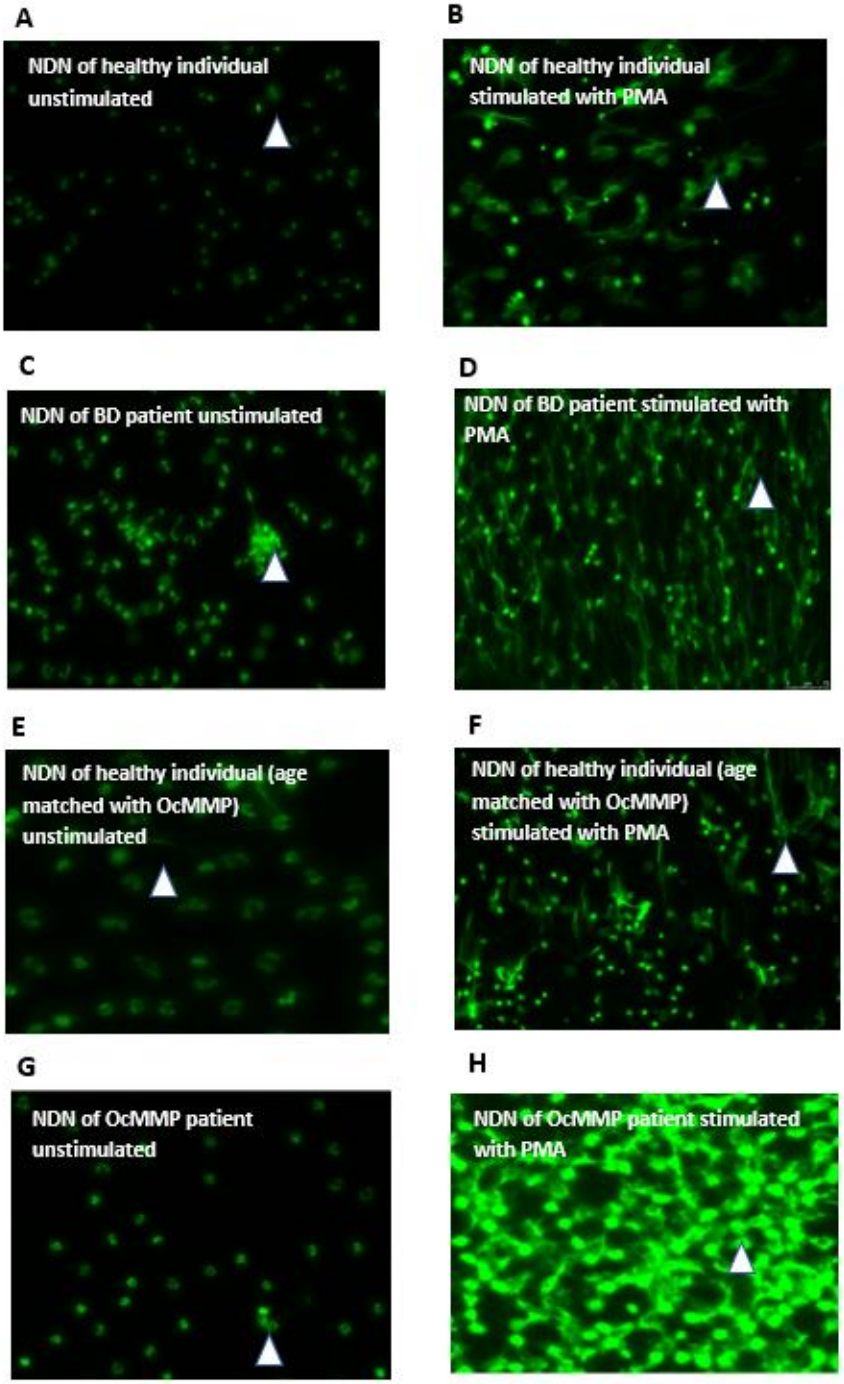


Figure 31-The production of LDN and NDN in different aged healthy individuals. Production of NETs by LDN (A, B; n=3) and NDN (C, D; n=3). from healthy individuals of different ages and used either immediately or after 2-hour incubation with PMA (25nM) of LDN and NDN cultures for from healthy individuals.

4.3.5 The visualisation of NETs in NDN in BD and OcMMP patients

A similar pattern was observed with the NDN by showing that PMA was an effective inducer of NET formation in NDN stimulated isolated from BD and OcMMP patients and healthy individuals (Fig.32). However, the production of NETs in NDN with the stimulant *E.coli* was reduced in BD and OcMMP patients in comparison to healthy controls (Fig.32E-H).


A
NDN of healthy individual unstimulated
B
NDN of healthy individual stimulated with PMA
C
NDN of BD patient unstimulated
D
NDN of BD patient stimulated with PMA
E
NDN of healthy individual (age matched with OcMMP) unstimulated
F
NDN of healthy individual (age matched with OcMMP) stimulated with PMA
G
NDN of OcMMP patient unstimulated
H
NDN of OcMMP patient stimulated with PMA

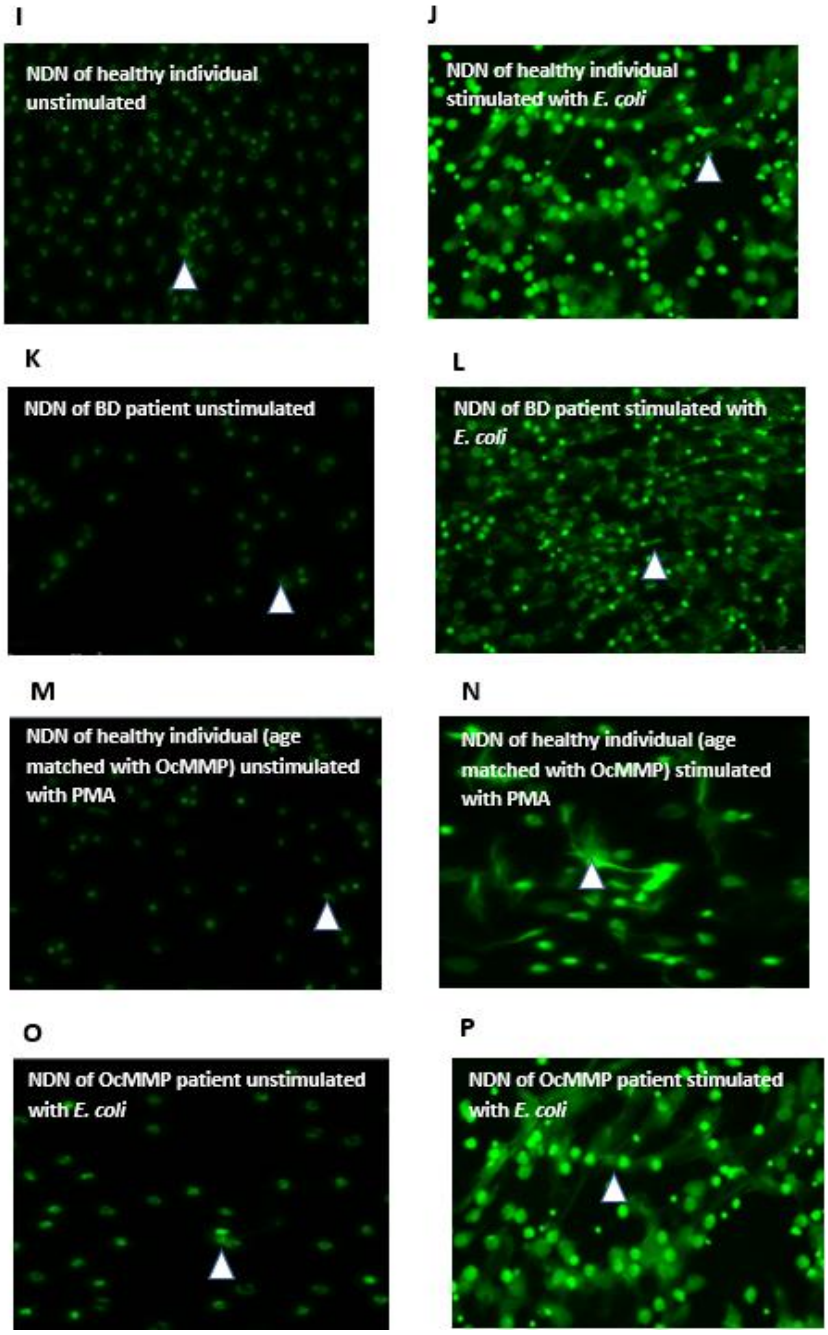


Figure 32-The production of NETs in NDN cultures BD and OcMMP patients. (A-D) The isolated cells cultures were seeded into 8 well chamber slides and were stimulated with PMA (25nM) and opsonised *E.coli* (1-2 x 10^9 bacteria per ml) (E-H) for 3 hours and stained with Sytox dye. Images were taken at X20. Arrow-representing formation of NET (strand of DNA).

4.4 Discussion

The patients with BD or and OcMMP patients. LDN show a hyper-segmented nucleus with 2 or fewer lobes in BD and OcMMP patients; in comparison to NDN which showed morphology of mature neutrophils. This may be the morphology of immature granulocytes, as LDN from patients with systemic lupus erythematosus (SLE) showed a similar immature phenotype of LDN (Carmona-Rivera *et al.,* 2013). A heterogeneous population of neutrophils LDN or Low density granulocyte (LDG) were first reported in a study by Hacbarth *et al.,* (1986) which showed the presence of LDN in the PBMC preparations obtained from adult SLE patients.

Patients with BD and OcMMP had a high LDN and low NDN count in comparison to healthy controls. These findings were comparable to studies which showed increased levels of LDN in patients with SLE (Denny *et al.,* 2010). The presence of LDN may have an important role in the persistent inflammation characteristic and immune response induced in BD and OcMMP patients.

LDN and NDN from BD and OcMMP (age matched) patients show a lower phagocytic capacity in comparison to healthy controls. The results indicate a possible deficient ability for the clearance of agents and increase predisposition of the disease. Similarly low ROS production of LDN and NDN in BD and OcMMP patients in contrast to age matched healthy controls was observed. Therefore, under maximal stimulation a reduced production of O_2 by LDN and NDN in BD and OcMMP patients may lead to a defective anti-inflammatory activity compared to healthy controls. Sagiv *et al.,* (2015) also showed a reduced production of ROS and anti-tumour properties by LDN in cancer patients.

Patients with BD had a high LDN and low NDN count in comparison to healthy controls. Both LDN and NDN subsets showed reduced ROS production and phagocytic function indicating

that difference in numbers of these subsets were not responsible for this phenotype of total neutrophils of patients with BD. Recent studies have reported reduced production of ROS and anti-tumour properties by LDN in cancer patients (Sagiv *et al.,* 2015). These findings were comparable to studies which showed increased numbers of LDN in patients with systemic lupus erythematosus (SLE) (Carmona-Rivera *et al.,* 2013). LDN from patients with systemic lupus erythematosus showed a similar immature phenotype. The presence of LDN may have an important role in the persistent inflammation characteristic and immune response induced in BD patients. Although it remains to be clarified, there is no indication that the reduced function of neutrophils from patients with BD is due the immunosuppressive drugs regimes used to control their condition. NDN of BD and OcMMP patients exhibited a reduced ROS and phagocytosis in comparison to healthy controls. This may be due to the presence of $CD16^{bright}$ $CD62L^{dim}$ population within the NDN pool (as NDN are classified as mature neutrophils therefore this subtype is most likely to be present in the NDN layer) of patients with BD and OcMMP. This phenotype is associated with reduced phagocytosis and ROS production in inflammation (Pillay *et al.,* 2012, Silvestre-Roig*, et al.,* 2016).

Enhanced production of NET was observed by the LDN in BD and OcMMP patients in comparison to NDN. This is because at baseline LDN have a greater capacity to produce NET *in vitro* without stimulation of cells with PMA, in comparison to unstimulated LDN in healthy controls and NDN as observed in this study. This may suggest that LDN might already be maximally stimulated in vivo or are immature. In other autoimmune diseases such as SLE Lood *et al*., (2016) showed that LDG displayed enhanced levels of basal mitochondrial ROS production and NET formation. Therefore, inhibition of mitochondrial ROS production reduces the enhanced basal level of NET formation. This theory can be investigated by

stimulating neutrophils with Ribonucleoprotein immune complexes (RNP ICs), inducers of NET production that require mitochondrial reactive oxygen species (ROS) for maximal NET stimulation.

Further enhanced production of NET was observed by the stimulated LDN in contrast to stimulated NDN in diseased cohorts and healthy individuals. The results suggested that LDN may be more sensitive to stimulation in comparison to NDN Carvalho *et al.,* (2015). This again supports the hypothesis of these cells being primed in diseased subjects

The results showed the control group (unstimulated cells) for PMA stimulated LDN of healthy individuals produced less spontaneous NET in comparison to the control group for *E.coli* stimulated LDN of healthy individuals. The reason for this can be that all healthy individuals stimulated with *E. coli* were from the same ethnic background (Johansson *et al.,* 2016).

CHAPTER 5

THE HETEROGENEITY WITHIN TOTAL NEUTROPHILS, LDN AND NDN IN HEALTHY INDIVIDUALS AND PATIENTS WITH BD OR OCMMP.

5. Introduction

Neutrophils are considered to be a heterogeneous population of cells. Dependent on various surface markers neutrophils can be classified into different types including low-density, activated, immature, reverse migrated and immunosuppressive (Table 17; Scapini *et al.,* 2016).

Table 17-The different types of neutrophil subsets in inflammatory disorders.

Type	*Surface Marker*
Activated Neutrophils	$CD62L^{+}$, $CD66B^{+}$ and $CD11B^{high}$
Granulocytic myeloid suppressor cells	$CD11B^{high}$, $CD14^{low}$, $CD62L^{dim}$
Low density neutrophils (LDNs)	$CD15^{+}$, $CD14^{low}$, HLA-DR^{-} and $CD33^{dim}$
Chemokine	$CXCR2^{high}$
Reverse Migrated	$CD54^{+}$
Immature cells	$CD16^{+}$

LDN have gained most attention and have been described in patients with infection, sepsis and autoimmune disorders (Table 18). LDN express CD66B (a specific neutrophil marker) and their maturation/activation phenotype is characterised by other surface markers. Analyses of LDN from different inflammatory disorders show various populations exhibiting $CD11B^{+}$ and $CD16^{+}$ mature neutrophils or a more immature phenotype of $CD11b^{low/-}$ and/or $CD16^{low/-}$cells, depending on the maturation stage (Scapini *et al.,* 2016).

Table 18-The Phenotypic and functional properties of human low-density neutrophils (LDN/G-MDSC).

Disease	*Phenotype*	*Morphology*	*Function*	*References*
HIV-Infection	CD15 ^{high} CD33 ^{high} CD11b^{+}	Activated mature cells	Not reported	Cloke *et al.,* (2012)
SLE	CD10^{+} CD11c^{low} CD15^{high} CD16^{high} CD14^{low}	Activated mature cells	Pro-inflammatory, reduced phagocytosis and enhanced spontaneous PMA NETosis	Denny *et al.,* (2010)
Sepsis	CD66b^{high} CD33^{low} CD11b^{high}	Activated mature cells with few band cells	Inhibition of T cell proliferation	Darcy *et al.,* (2014)
Severe infection	CD10^{-}	Mature de-granulated cells	A reduced chemotactic activity	Singh *et al.,* (2014)
Tuberculosis	CD15^{+} CD33^{+} and CD66b^{+}	Not Reported	Increased ROS production	Deng *et al.,* (2016)
Asthma	CD15^{high} CD16^{high} CD11b ^{high} CD66b ^{high} and CD14 ^{low} and CD33 ^{low}	Activated mature cells	Not reported	Fu *et al.,* 2014

systemic lupus erythematosus (SLE), Low density neutrophils, Granulocytic myeloid deprived suppressor cells. (G-MDSC) and Human Immunodeficiency virus (HIV)

LDN may present a pro-inflammatory phenotype in autoimmune diseases such as lupus erythematosus (SLE) and rheumatoid arthritis (RA), where they show effector functions comparable to activated neutrophils (Hacbarth *et al.,* 1986, Hong 2017). LDN may have immunosuppressive phenotype classed as granulocytic myeloid-derived suppressor cells (G-MDSCs). The G-MDSCs are also found within the PBMC layer of the density gradient in patients with different inflammatory conditions (Garley *et al.,* 2018). The immunosuppressive phenotype of LDN is described as $CD66b^{+}$ $CD15^{+}$ $CD14^{-/dim}$ $CD33^{dim}$ $HLA\text{-}DR^{-}$ and/or reduced/greater levels CD11b, CD16, CD124/IL-4R (maturation markers). The immunosuppressive LDN also express a reduced/ enhanced levels of activation markers (CD62L, CD54/ICAM-1, CD63, and CD274/PD-L1) or chemokine receptors (e.g. CXCR2, CXCR4) (Scapini *et al.,* 2016). LDN have been described as bone marrow-derived immature PMN classified as $CD10^{low/-}CD16^{low}$ cells, released during severe systemic infection (Manz *et al.,* 2014). However, precise LDN characterisation and differentiation remains challenging due to the major differences found between nuclear morphology and classification of cells, and the relevance of phenotype to function.

Denny *et al.,* (2010) demonstrated that proinflammatory LDN can be isolated from PBMC by negative selection using LDG isolation cocktail, with increased production of proinflammatory cytokines (TNF-α, IL-17, IFNγ, and IFNα) and NET, but decreased phagocytosis. These LDG induced endothelial cell destruction and reconstruction processes in the immune response (Denny *et al.,* 2010). G-MDSC are categorised as an immunosuppressive sub-group of LDN, inhibiting different T cell responses via production of arginase 1 (Arg1) and reactive oxygen species in patients with cancer (Dumitru *et al.,* 2012). Immunosuppressive mechanisms

associated with LDN include programmed cell death ligand 1 dependent interactions and induction of $CD4^+$ $FoxP3^+$ T regulatory cells (Scapini *et al.,* 2016).

Normal density neutrophils can be identified as $CD15^+$ population, but may switch to LDN and a gain of immunosuppressive properties (Sagiv *et al.,* 2015). NDN can also exhibit an immunosuppressive phenotype ($CD66b^+$ $CD15^+$ $CD14^{-/dim}$ $CD33^{dim}$ HLA-DR–) similar to immunosuppressive LDN/G-MDSCs population, (Scapini *et al.,* 2016). In comparison to LDN, NDN also show a comparable type I interferon transcriptional signature which promotes a transformed migration towards CCL3 and CXCL8 and a phagocytic activity during an infection (Rocha *et al.,* 2015). Interestingly, incubation of NDN from healthy controls with *M. tuberculosis* leads to a generation of LDN *in vitro* (Su *et al.,* 2019). Immunosuppressive NDN from patients with HIV were observed to have elevated levels of PD-L1 and inhibition of T cell function was inhibited via the production of reactive oxygen species and PD-L1/PD-1 interaction similar to LDN. (Bowers *et al.,* 2014). These data suggest plasticity in the neutrophil population *in vivo*, dependent on the microenvironment rather than polarisation of specific subset (Faurschou *et al.,* 2003; Sagiv *et al.,* 2015; P. Kubes personal communication). Given all the evidence described above regarding neutrophil heterogeneity and phenotype no study to date have investigated neutrophil phenotype and heterogeneity within the total neutrophil population, LDN and NDN of BD and OcMMP patients in vitro. In this chapter neutrophil phenotype and heterogeneity within total neutrophil population, LDN, NDN of BD and OcMMP patients. This was investigated by evaluating surface expression by performing flow cytometry as outlined in chapter 2.

5.1. Results

5.1.1. The identification of the mean % expression surface markers in total neutrophils in BD patients in comparison to healthy controls

Neutrophils from BD patients had a significantly higher expression of activation markers CD11B (91 ± 9 (%) vs 55± 9 (%)) (p=0.04) (Fig.31E), CD66B (Fig.33F) (99 ± 0.3 (%) vs 6 ± 1 (%)) (p=0.02) CD62L (Fig. 33G) (83 ± 1 (%) vs 36 ± 4 (%)) (p=0.02), reverse migrated marker CD54 (Fig.33J) (50 ± 7 (%) vs 0.01 ± 0.02(%)) (p=0.003) and CD33 (Fig.33H) (38 ± 2 (%) vs 0.02 ± 0.4 (%)) (p=0.01) compared to cells with healthy controls. CD15 (Fig 33A) (85 ± 7 (%) vs 75 ± 16 (%)) (p=ns) and CD16 (Fig. 33B) (75 ± 18 (%) vs 92 ± 1 (%)) (p=ns) expression was comparable and very highly expressed on cells from both cohorts and the expression of chemokine receptor CXCR2 (Fig.33I) (18 ± 2 (%) vs 13 ± 2 (%)) (p=ns) was low, while CD14 (Fig.33C) (0.5 ± 0.02 (%) vs 0.4± 0.06 (%)) (p=ns) and HLA-DR (Fig.33D) (0.9± 0.4 (%) vs 2.5 ± 0.8 (%)) (p=ns) expression was very low.

5.1.2. Surface marker expression on neutrophils from patients with OcMMP compared to healthy controls

The expression of the activated markers CD11B (Fig.34E) (98 ± 0.4 (%) vs 48 ± 6(%)) (p=0.01), CD62L (Fig.34G) (94 ± 1 (%) vs 47 ± 0.7 (%)) (p=0.003) and CD66B (Fig.32F) (67 ± 9 (%) vs 5.8 ± 0.2 (%)) (p=0.03) reverse migrated marker CD54 (Fig.34J) (70 ± 9.3 (%) vs 0.1 ± 0.02 (%)) (p=0.02) and CD33 (Fig.34H) (56 ± 16 (%) vs 0.04 ± 0.01 (%)) (p=0.03) was significantly higher on neutrophils from patients with OcMMP in comparison to healthy controls. There was no significant difference between expression of CD15 (Fig.34A) (99 ± 0.3 (%) vs 95 ± 1.6 (%)) (p=ns) or CD16 (Fig.32B) (97 ± 0.5 (%) vs 93 ± 1.3 (%)) (p=ns) which was very high on cells from both cohorts. The expression of HLA-DR^{+} (Fig.34D) (1.1 ± 0.5 (%) vs 0.03 ± 0.01 (%)) (p=0.03)

or CD14 (Fig.34C) (0.7± 0.3 (%) vs 0.4 ± 0.01 (%)) (p=ns) was very low on cells from OcMMP patients in comparison to healthy controls. The expression of chemokine receptor CXCR2 (Fig.34I) (21.1 ± 1.0 (%) vs 18 ± 1.4 (%)) (p=ns) was high on cells from OcMMP patients (Fig.34I).

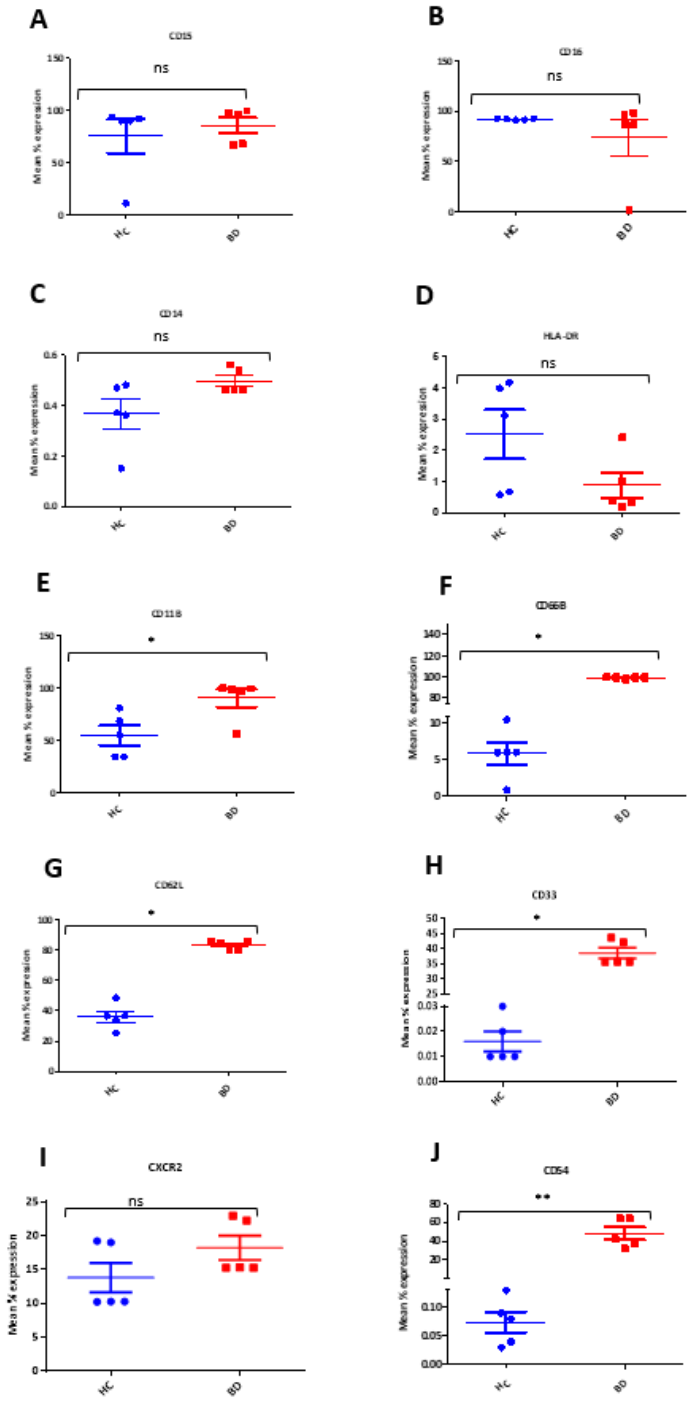


Figure 33-The percentage mean expression of surface markers of isolated total neutrophils of BD (n=5) patients and healthy controls (n=5). The surface marker expression of CD15 (A), CD16 (B), CD14 (C), HLA-DR (D), CD11B (E), CD66B (F), CD62L (G), CD33 (H), CXCR2 (I) and CD64 (J) was investigated by antibodies, isotype controls and flow cytometry. *P< 0.05, **P <0.01, not significant (ns)

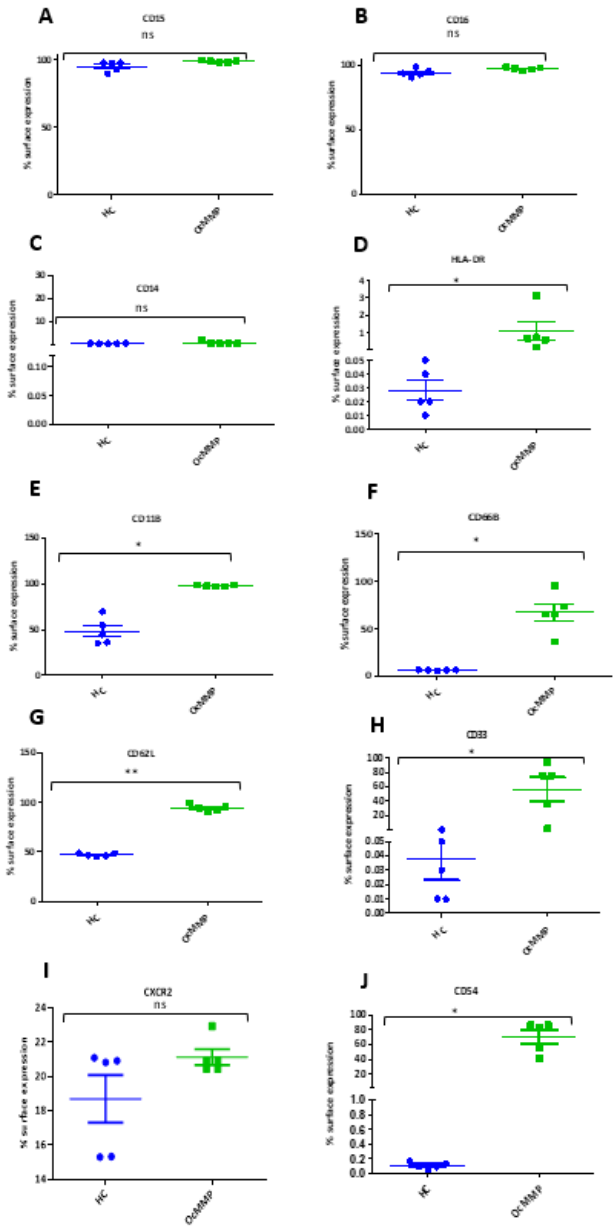


Figure 34-The percentage mean expression of surface markers of isolated total neutrophils of OcMMP patients (n=5) and healthy controls (n=5). The surface marker expression of CD15 (A), CD16 (B), CD14 (C), HLA-DR (D), CD11B (E), CD66B (F), CD62L (G), CD33 (H), CXCR2 (I) and CD64 (J) was investigated by antibodies, isotype controls and flow cytometry. *P< 0.05, **P <0.01, not significant (ns)

5.1.3. Heterogeneity within the LDN and NDN population

LDN and NDN populations in OcMMP patients showed a similar pattern of surface expression as total neutrophils. CD15 was highly expressed and CD14 was very low on neutrophil subsets from both cohorts (Fig.35 (C and D), 36 (C and D)). Expression of CD66B and CD33 was higher in both LDN and NDN populations from patients with BD (n=10) or OcMMP (n=10) in comparison to healthy volunteers (young (n=10) and age-matched (n=5) (Table 19,20 and Fig.37,38. Interestingly expression of HLD-DR was significantly higher on NDN (27 ± 9 vs 0.8 ± 0.03) (Fig.38B) ($p=0.0003$) in population of OcMMP compared to age-matched cataract controls (n=5).

Table 19-The mean (%) expression of surface markers on LDN. The mean (%) expression of surface markers on LDN of healthy individuals, BD and OcMMP patients.

Surface expression	*HC*	*BD*	*P value*	*HC (Aged matched with OcMMP)*	*OcMMP*	*P value*
	% mean expression					
CD15	95.15	92.20	0.90	94.38	93.94	0.85
CD14	0.46	0.55	0.10	0.32	0.50	0.11
HLA-DR	1.23	10.08	0.80	3.3	3.45	0.70
CD66B	15.41	49.19	0.004	0.75	56.84	0.002
CD33	3.13	38.57	0.0006	1.89	41.20	0.004

Healthy controls (HC), Low density neutrophils (LDN), Behçet's disease (BD) and Ocular mucous membrane pemphigoid (OcMMP).

Table 20-The mean (%) expression of surface markers on NDN. The mean (%) expression of surface markers on NDN of healthy individuals, BD and OcMMP patients.

Surface expression	*HC*	*BD*	*P value*	*HC (Aged matched with OcMMP)*	*OcMMP*	*P value*
	% mean expression					
CD15	95.49	95.10	0.07	94.38	93.94	0.07
CD14	0.46	0.55	0.1000	0.31	0.49	0.09
HLA-DR	6.04	6.32	0.08	0.75	27.33	0.1000
CD66B	3.7	48.13	0.003	0.17	19.31	0.002
CD33	2.57	38.57	0.0005	0.90	24.56	0.005

Healthy controls (HC), Low density neutrophils (LDN), Behçet's disease (BD) and Ocular mucous membrane pemphigoid (OcMMP).

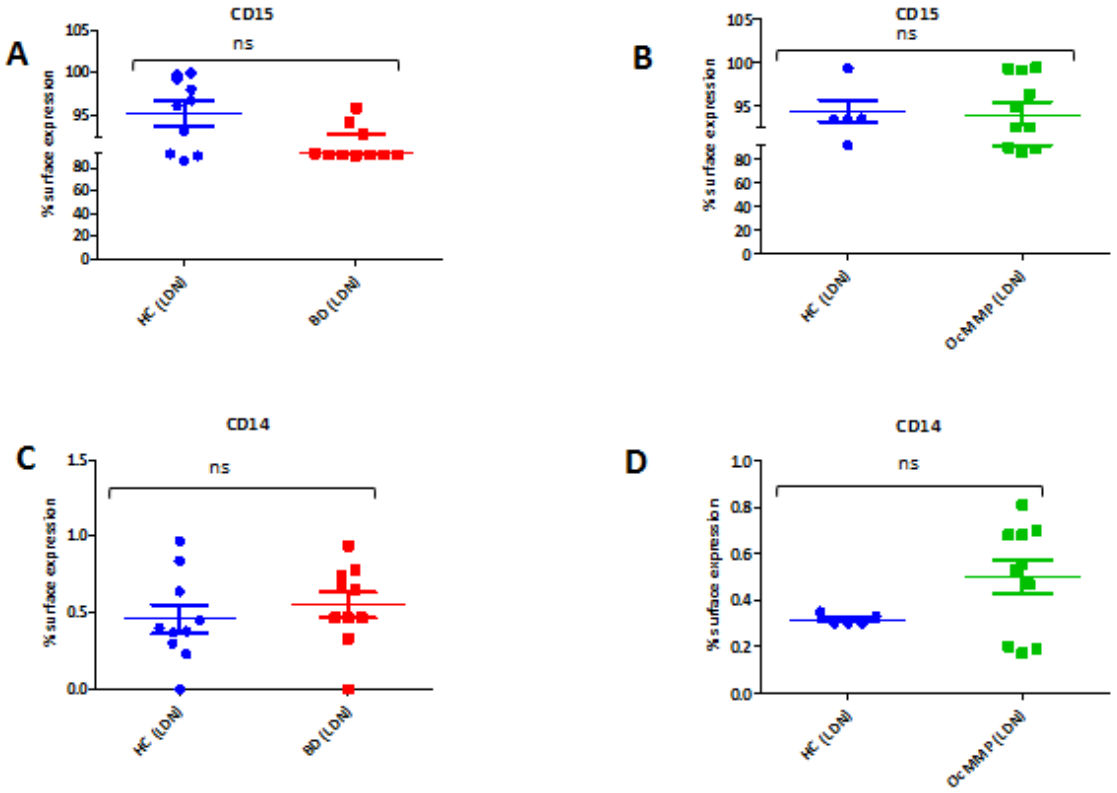


Figure 35-The expression of CD15 and CD14 positive neutrophils. The expression of CD15 positive neutrophils (A, B) and CD14 positive (C,D) within the LDN population in BD (n=10), OcMMP (n=10) patients and healthy controls (n=10, n=5).The expression was investigated using antibodies, isotype controls and flow cytometry. Not significant (ns)

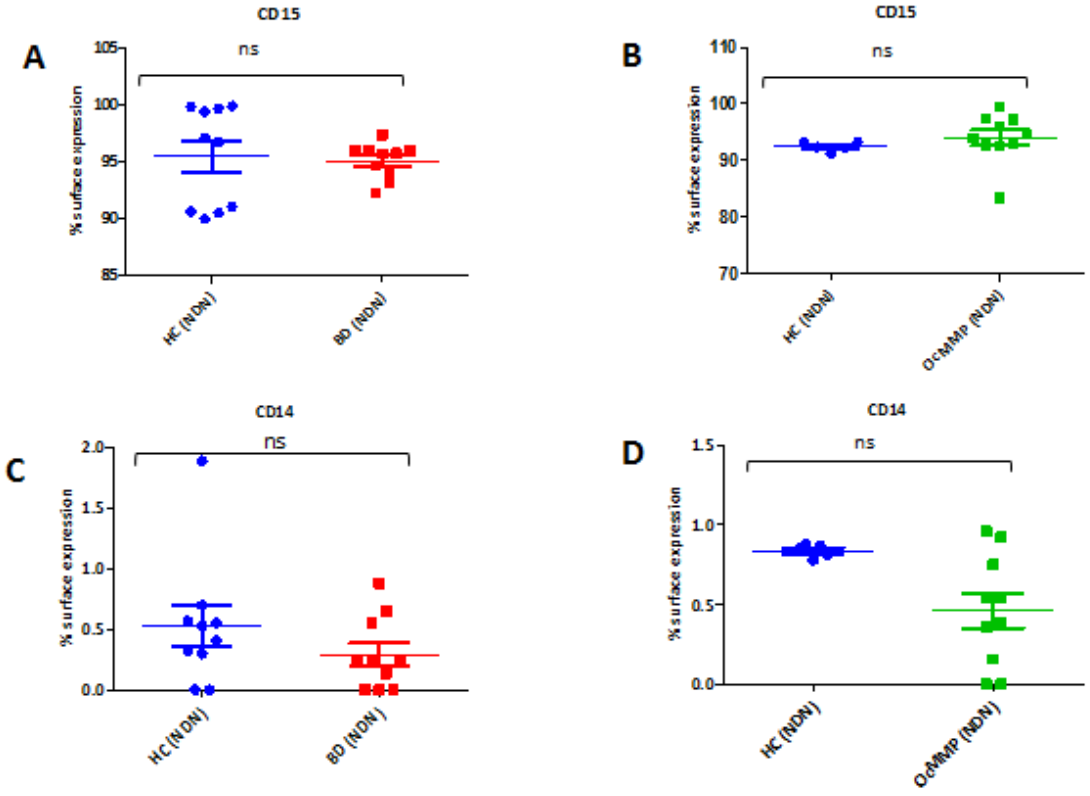


Figure 36-The expression of CD15 and CD14 positive neutrophils. The expression of CD15 positive neutrophils (A,B) and CD14 positive (C,D) within the NDN population in BD (n=10), OcMMP (n=10) patients and healthy controls (n=10, n=5). The expression was investigated using antibodies, isotype control and flow cytometry. Not significant (ns)

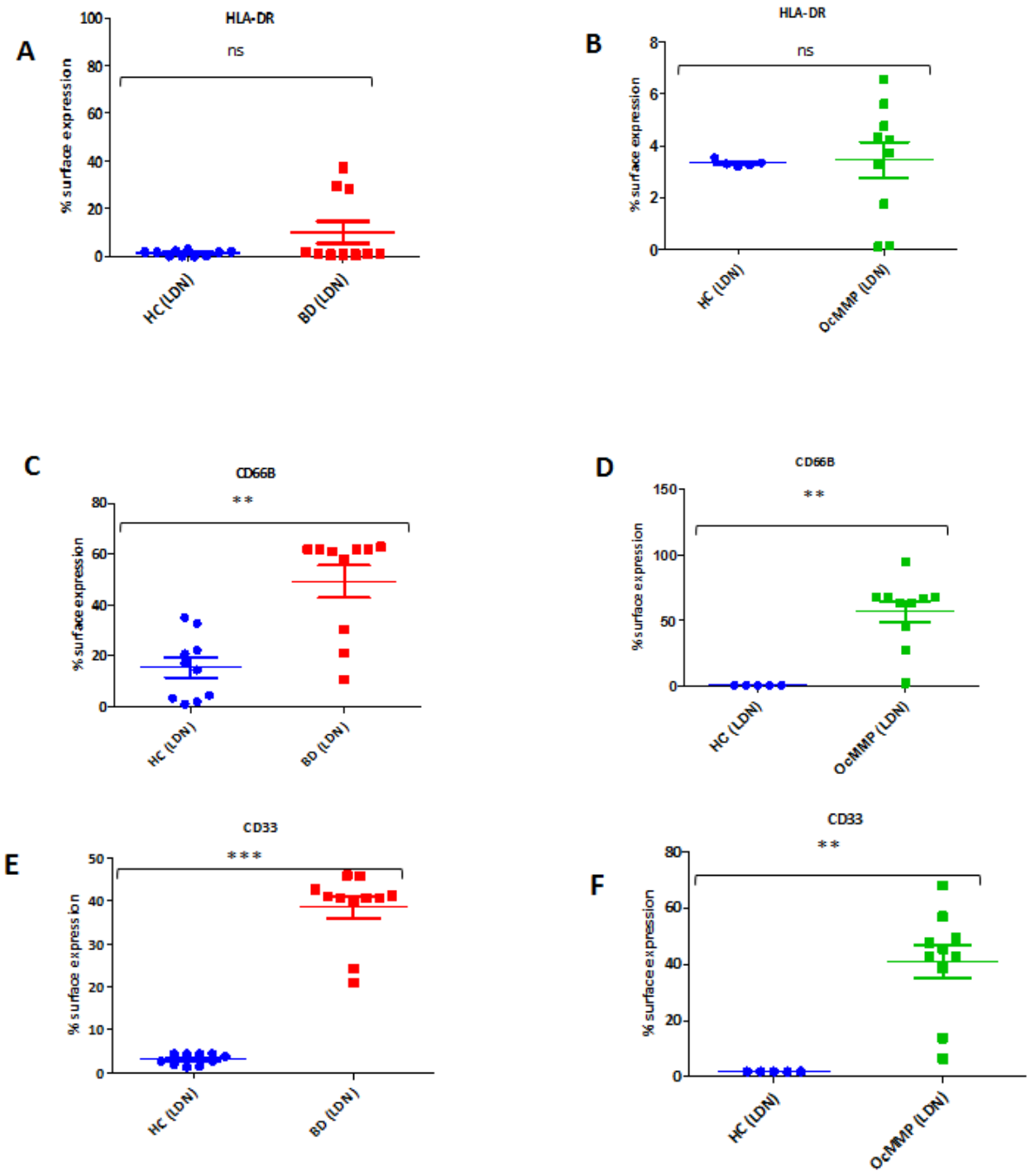


Figure 37-The expression of surface markers within the LDN population. The expression of different surface markers within in the LDN population in BD (n=10), OcMMP (n=10) patients and healthy controls (n=10, n=5). The expression was investigated using testing antibodies, isotype controls and flow cytometry. **P <0.01, ***P <0.001, not significant (ns)

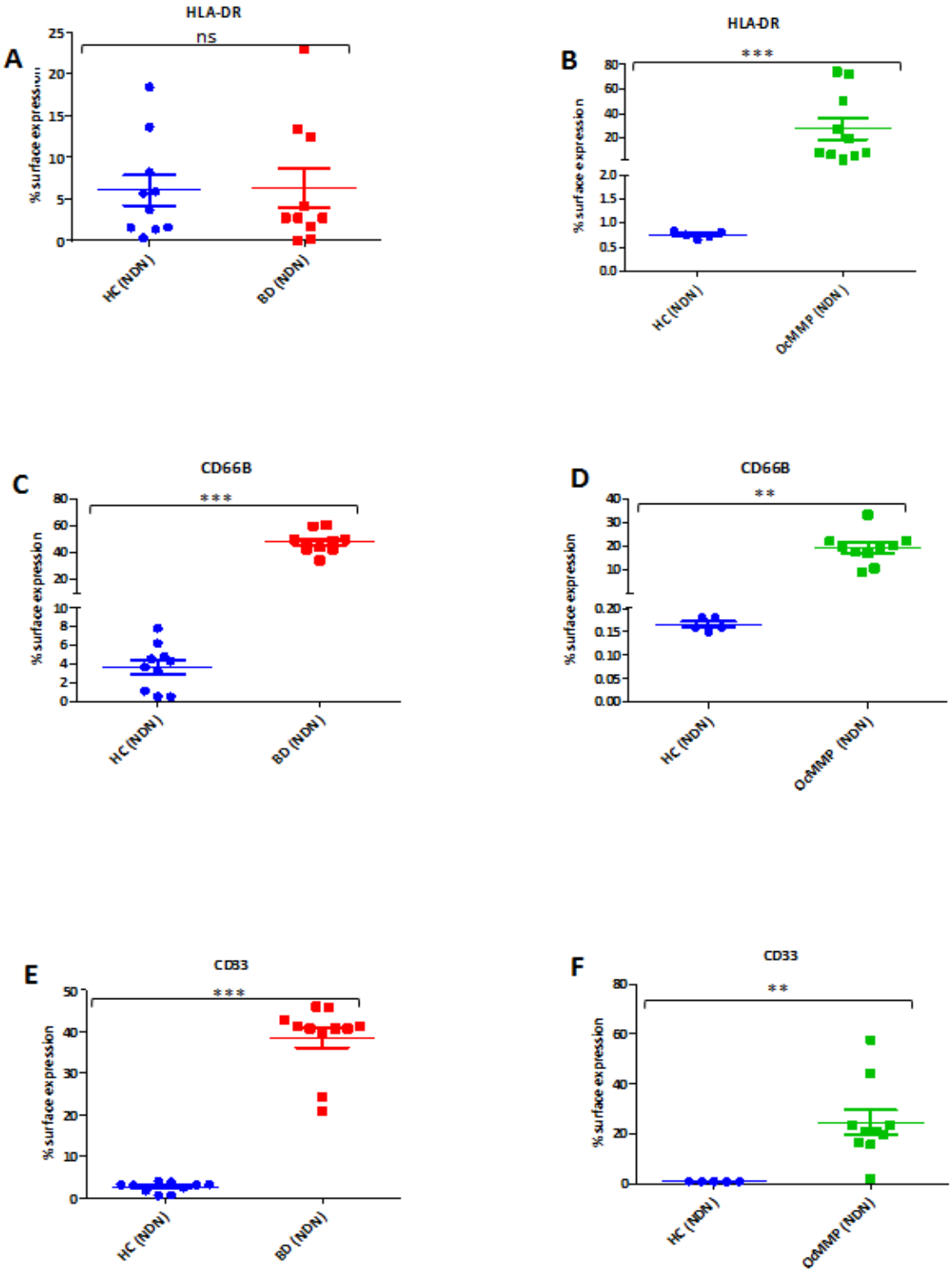


Figure 38-The expression of surface markers within the NDN population. The expression of different surface markers within in the LDN population in BD (n=10), OcMMP (n=10) patientsand healthy controls (n=10, n=5). The expression was investigated using testing antibodies, isotype controls and flow cytometry. **P <0.01, ***P <0.001, not significant (ns)

5.3. Multiparameter expression analysis of surface markers on neutrophil subsets from patients with BD or OcMMP

Multiparameter analysis of various surface markers was performed on total neutrophils from patients with BD or OcMMP. A higher expression of the neutrophil subset with a $CD33^{+}/CD62L^{dim}/CD14^{low}$ phenotype (LDN/activated) was also observed in OcMMP patients in comparison to BD (even though an expression was observed in BD in comparison to healthy controls) and healthy controls (Table 19). The neutrophil phenotype $CD62L^{+}/CXCR2^{bright}/CD14^{-}$ was more highly represented (mean %) in OcMMP in comparison to patients with BD and was not present in either control population (Table 6). Similarly, a $CD62L^{+}/CXCR2^{bright}/CD14$ population was only found in patients with OcMMP. By comparison, $CD62L^{+}/CD14^{high}/CD33^{dim}$ cells were more highly presented on neutrophils from patients with BD. A $CD11B^{+}/CD16^{bright}/CD66B^{bright}$ and $CD15^{+}/HLA\ DR^{-}/CD54^{bright}$ subsets highly represented in cells from both patient cohorts, in comparison to healthy controls. The neutrophil subset of was highly observed in BD in comparison to OcMMP. Overall, although results of single staining of surface markers was comparable on cells from both patient groups multiparameter analysis showed significant differences which may be relevant to each condition.

Table 21-The mean % expression of multiple neutrophil subsets expressed by neutrophils of BD, OcMMP and healthy controls.

	BD (n=8)	*Healthy controls (n=8)*	*P value*	*OcMMP (n=8)*	*Age matched healthy controls (n=8)*	*P value*
			Mean (%) expression			
CD11B^{+}/CD16^{bright}/CD66b^{bright}	97.37	1.69	0.0003	97.17	0.95	0.0002
CD62L^{+}/CD14^{high}/CD33^{dim}	88.37	0.92	0.0001	28.63	0.95	0.003
CD33^{+}/CD62L^{dim}/CD14^{low}	1.94	0	0.003	35.3	0	0.004
CD62L^{+}/CXCR2^{bright}/CD14^{-}	0	0	n/a	92.07	0	0.0001
CD15^{+}/HLA-DR^{-}/CD54^{bright}	95.21	51.78	0.004	91.42	51.34	0.004

Behçet's disease (BD) and Ocular mucous membrane pemphigoid (OcMMP).

5.4. The characterisation of granulocytic myeloid suppressor cells (G-MDSCs) in LDN and NDN population

To characterise the G-MDSCs (gated on $CD66B^{+}$) in LDN and NDN multiparameter staining was used ($CD66B^{+}CD33^{dim}HLA\text{-}DR^{-}$). The mean percentage expression of the activated phenotype was significantly higher in LDN (96.0 ± 0.8 (%) vs 3.0 ± 1.3 (%)) ($p<0.0001$), (97.65 ± 1 (%) vs 1.3 ± 0.3 (%)) ($p<0.0001$) and NDN (95.9 ± 1.0 (%) vs 1.3 ± 0.6 (%)) ($p<0.0001$), (84.6 ± 3.1 (%) vs 0.7 ± 0.2 (%)) ($p<0.0001$) populations from patients with BD (n=10) and OcMMP (n=10) in comparison to young (n=10) and elderly healthy controls (n=5) (Fig.39).

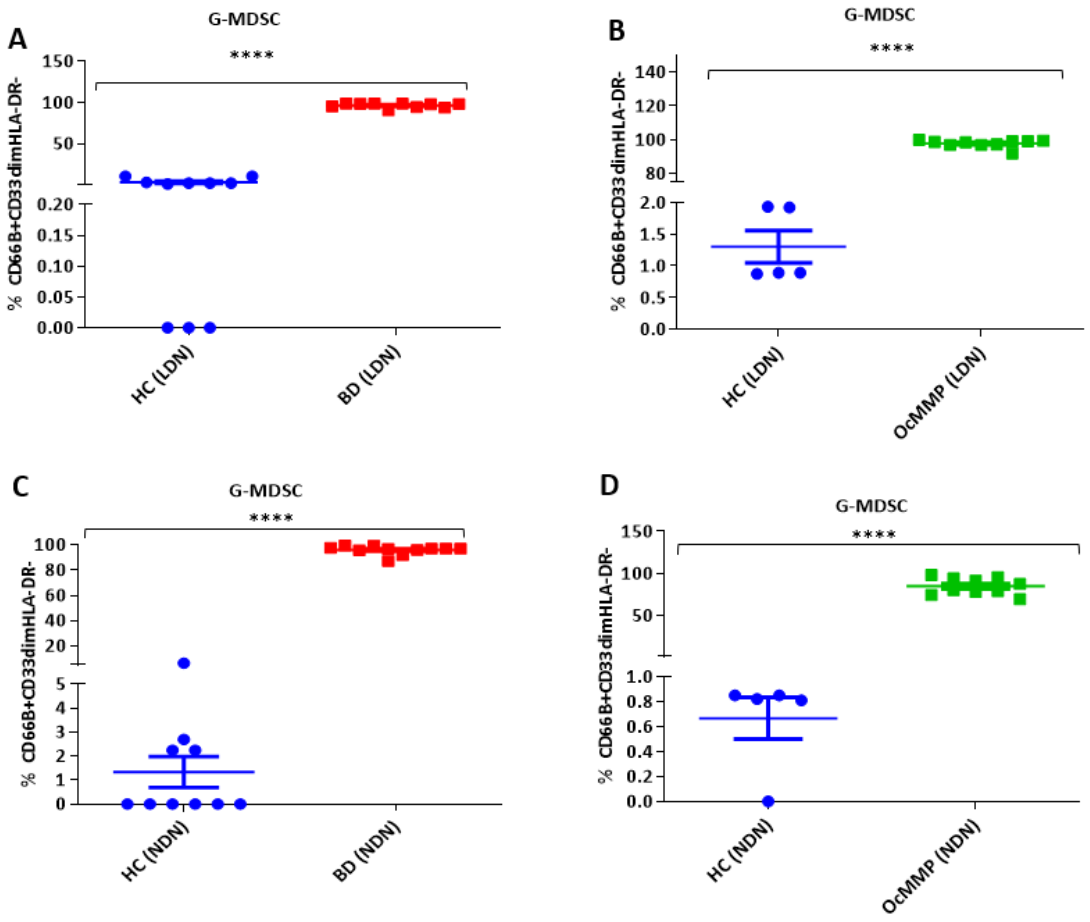


Figure 39-The expression of granulocytic myeloid suppressor cells. The mean percentage expression of $CD66B^{+}CD33^{dim}HLA\text{-}DR^{-}$ (G-MDSC) within the LDN and NDN population of BD (n=10), OcMMP (n=10) and healthy controls (n=10, n=5). The expression was investigated by antibodies, isotype controls and flow cytometry. ****P <0.001

5.5. Discussion

Single surface expression of markers of neutrophil subtype and possible function showed a similar pattern on cells from patients with either BD or OcMMP whether total cells, LDN or NDN. High expression of CD15 and low expression of CD14 confirmed our purification steps for neutrophil populations.

The surface expression of $CD66B^{+}/^{bright}$ is increased due to the mobilisation of various granules during cellular activation activity as CD66B is present in the intracellular membrane of many subsets of cytoplasmic granules. The surface expression of CD66B population indicates an activated migrated maturation state (Watt *et al.,* 2005). CD66B can contribute towards vasculitis as it facilitates fast and effective cell adhesion to endothelium (Schmidt *et al.,* 2012). Therefore, vascular damage associated with BD and OcMMP patients may not only be related to the functional role of LDN and NDN but can be related to upregulation of CD66B.

Likewise, CD11b is increased on the cell surface from granules during activation suggesting that CD11b can be presented in a resting state in the cell, but the expression of CD11B is highly increased during neutrophil activation as in patients with cutaneous T-cell lymphoma (Goddard *et al.,* 2005)

High expression of $CD62L^{+}$ cells were observed in cells from patients in comparison to healthy controls. CD62L is an activation marker of neutrophils but functionally is involved in neutrophil migration as is the chemokine receptor CXCR2 which was similarly raised on cells from patients. These results suggest that neutrophil activation occurs via CXCR2, receptors and enter into tissue if required (Kolaczkowska and Kubes 2013).

A higher expression CD54 on neutrophils from patients with OcMMP and BD in comparison to healthy controls was observed. CD54 expressing neutrophils are correlated with reverse migration (Buckley *et al.,* 2006; Kruger *et al.,* 2015). These results suggest that neutrophils from patients with BD and OcMMP may have entered one tissue before leaving and re-entering the blood. Whether these cells are homing to the bone marrow for destruction or potentially entering another tissue is not clear at this time. Interestingly, the results showed a higher expression of $CD54^{+}$ on cells from patients with OcMMP in comparison to BD, but the relevance of this is not clear.

Multiparameter staining identified neutrophils from patients showed an activated maturation state compared to healthy controls, $CD11B^{+}/CD16^{bright}/CD66b^{bright}$ and $CD33^{+}/CD62L^{dim}/CD14^{low}$. The expression of $CD33^{+}/CD62L^{dim}/CD14^{low}$ was high in neutrophils from patients with OcMMP in comparison to cells from patients with BD. It has been suggested this activated phenotype may define both immature and mature cells highlighting a potential difference between the patient cohorts.

The expression of immunosuppressive phenotypes was highly expressed in BD and OcMMP in comparison to healthy controls ($CD62L^{+}/CD14^{high}/ CD33^{dim}$) even though, the expression of this subset was high in BD in comparison to OcMMP. The expression of $CD62L^{+}/CXCR2^{bright}/CD14^{-}$ was high in OcMMP in comparison to BD and healthy controls, suggesting an immunosuppressive phenotype in both disease states. This may contribute towards the altered phagocytic capacity of neutrophils in patients diagnosed with BD and OcMMP in comparison to healthy controls, as an impaired phagocytic capacity is associated with immunosuppressive neutrophils (Carmona-Rivera and Kaplan 2013).

A higher number of G-MDSC CD66b^{+}CD33^{dim}HLA-DR^{-} (Marini *et al.,* 2016) were observed in BD and OcMMP patients in comparison to age matched healthy controls within the LDN and NDN population. The reduced phagocytic function and ROS production by the LDN and NDN may be associated with an immunosuppressive phenotype. Although it remains to be clarified, there is no indication that the reduced function of neutrophils from patients with BD or OcMMP is due the immunosuppressive drugs regimes used to control their condition.

Neutrophil heterogeneity has been described in several other conditions. In patients with lung cancer LDN numbers were raised in patients with advanced disease. Specifically, CD66b^{+}CD10^{low}CXCR4^{+}PDL1^{int} were found exclusively in patients with advanced disease (Shaul *et al.,*2020). Patients with pyogenic arthritis, gangrenosum and acne (PAPA), an autoinflammatory condition showed increased circulating LDG (CD10$^{=/-}$CD14^{low}CD15^{+}) compared to control cells. LDG from patients with PAPA had increased ability to spontaneously form NET compared to NDN or healthy control cells. Serum from patients with PAPA induced NET formation due to IL-1β but a reduced ability to degrade NET, a response that was reversed by addition of DNase 1. Therefore a combination of increased production and reduced degradation of NET leads to an increased circulating NET which may be relevant to the pathogenesis of PAPA (Mistry *et al.,* 2018) Circulating NET and LDG were raised in patients with idiopathic inflammatory myopathies and correlated with muscle damage and decreased viability of myotubes (Seto *et al.,* 2020). In patients with active LDN were increased compared to healthy controls with higher expression of CD66b, CD33, CD15 and CD16 compared to NDN from the same patient. Transcriptomic analysis showed increased expression of *CCL5, CCR5, CD4, IL10, LYZ* and *STAT4* and downregulation of *CXCL8, IFNAR1, STAT1, TICAM1* and *TNF* in LDN versus NDN. LDN form patients with active TB did not

phagocytose live *Mycobacterium tuberculosis*, produce ROS or NET, rather showed an immunosuppressive phenotype, mainly mediated by IL-10 (La Manna *et al.,* 2019) LDG from patients with ANCA vasculitis are raised compared to controls, heterogeneous (mature and immature populations), and respond weakly to anti-myeloperoxidase antibodies (Ui Mhaonaigh *et al.,* 2019) These data support a heterogenous population of neutrophils, driven by disease processes, that relate to function.

In conclusion, total neutrophils, LDN and NDN show surface marker expression associated with increased activation, trafficking and potential immunosuppressive phenotypes. A direct correlation with function is difficult in neutrophils as unlike lymphocytes or monocytes sorting by flow cytometry is extremely difficult. As such, these data presented implies function. That said, an activated, trafficking phenotype would be compatible with the known pathogenesis of BD and OcMMP, and a potential role for neutrophil mediated immunosuppression an interesting possibility.

CHAPTER 6

GENERAL DISCUSSION

6. General discussion

In this thesis two multisystem ocular inflammatory disorders were explored, Behçet's disease and Ocular Mucous Membrane Pemphigoid. Behçet's disease is an auto-inflammatory disorder defined by recurrent oral aphthae, genital ulcers, ocular inflammation, and vasculitis and skin lesions. Patients diagnosed with BD (50-90%) show intraocular inflammation (Mendes *et al.,* 2009). Posterior segment inflammation involves occlusion of retinal veins that is recurrent and may occur at any location from the central retinal vein to the miniscule, small branches (Cunningham *et al.,* 2017). Currently, there is no specific diagnostic tool or serum biomarker to identify and quantify the severity of BD (Mendes *et al,* 2009).

Mucous Membrane Pemphigoid (MMP) also known as Ocular Mucous Membrane Pemphigoid (OcMMP) is a chronic ocular, multisystem autoimmune immunobullous disorder characterised by blistering lesions that primarily affect orificial mucous membranes of the mouth, eyes, digestive tract and genitalia with average age of onset in the seventh decade (60 to 70 years of age), but can occur at any age. When ocular mucosa is affected, the disease is characterised by conjunctival inflammation and erosions leading to subepithelial scarring and adhesions between the eyelids and the bulbar conjunctiva. Women seem to be more often affected than men by a factor of 1.5 to 1 and in the UK the prevalence rate of MMP in adults is 0.8 per 100,000 (Williams *et al.,* 2013). The aetiology of MMP remains unclear.

Although, the initiating cause of BD and OcMMP remains undefined, it is clear that neutrophils have a central role in BD and OcMMP pathogenesis and progression. Neutrophils have been described as active in patients with BD and colchicine, a neutrophil inhibitor has been successfully used to treat patients with BD (Eksioglu-Demiralp *et al.,* 2001; Saleh *et al.,*

2014). However, other studies have showed that patients diagnosed with BD display significantly higher ROS production in comparison to healthy controls in response to monosodium urate crystals (Gogus, *et al.,* 2005). The differences identified may be due to the heterogeneity of the patients, treatment provided, or due to the stimulus used.

In patients with OcMMP, a persistent neutrophil infiltrate in the conjunctival mucosa is observed in independent of observable levels of inflammation and is associated with progression of the condition (Williams *et al.,* 2016). This is supported by elevated levels of neutrophil collagenase, matrix metalloproteinase-8 and 9 and myeloperoxidase in patients with OcMMP compared with controls (Arafat *et al.,* 2014).

The results in this thesis showed a reduced phagocytic capacity and ROS production in total neutrophils in patients diagnosed with either BD or OcMMP in comparison to age matched healthy controls. The deficits were not due to age, therapeutics or ethnicity. Consequently, these findings are disease dependent due possibly to persistent stimulation by cytokines and a constant inflammatory response ultimately leading to a form of exhaustion. This suggests a collective denominator in maintaining chronic inflammation in BD and OcMMP. Johansson *et al.,* showed a reduced phagocytic capacity and ROS production in ANCA associated vasculitides patients, supporting our hypothesis (Johansson *et al.,* 2016) Patients with SLE exhibited reduced ROS production compared to healthy controls although this was related to specific polymorphisms in many patients (Urbonaviciute *et al.,* 2019; Olsson 2017).

The persistent neutrophil infiltrate in the OcMMP conjunctiva has been identified by surface markers, and no studies to date, have examined neutrophil function. As these data presented in this thesis show OcMMP peripheral neutrophils have less phagocytic capacity and ROS production, it is possible these neutrophils infiltrate ocular conjunctival tissue and persist in

clinically quiescent eyes. Evidence suggests that these neutrophils are capable of cytokine production and their persistence is associated with progressive conjunctival fibrosis (Williams *et al.,* 2013; Williams 2016). Classically, neutrophils are thought to have a short life span mediated by apoptosis and changes in apoptotic parameters, can lead to prolonged inflammatory responses (Wright *et al.,* 2010). The communication with other inflammatory cells in any inflamed site is a very complex procedure however, there is current evidence for dysregulation causing ocular surface pemphigoid and analysis of immune cells may lead to targeted treatment options (Bose 2019).

It is vital to fully understand the role of ROS. Neutrophils from patients with BD are more effective at transforming fibrinogen, a response involved in clot formation and thrombosis (Emmi *et al.,* 2019; Elloumi, *et al.,* 2017). Several studies have suggested that many different cell types are involved in the production of IL-17, an inducer of neutrophil production, in BD patients suggesting a role in the proliferation of the inflammation in BD (Emmi *et al.,* 2016; Sonmez *et al.,* 2018).

This thesis has shown an increase in NET production in total neutrophils (LDN and NDN) with PMA in patients with both BD and OcMMP in comparison to healthy controls. The converse is seen with *E.coli* (Chapter 3 and 4). The production of NET during NETosis is important in the development and protection of many autoimmune diseases that include chronic inflammatory disorders which eventually can lead to organ damage (Pruchniak *et al.,* 2015). It is acknowledged that production of NET is considered to form part of the host defence during Bechet's syndrome. In healthy individuals, phagocytosis and ROS production contributes towards killing of bacteria. This function is reduced in BD and OcMMP that could lead to inadequate eradication of microbes driving inflammation in BD and OcMMP. The

depletion of local tissue ROS levels thereby perpetuates disease. Safi *et al.,* (2018) suggested that ROS is vital for NET generation. However, PMA is a potent stimulator, and PMA induced NET formation is highly ROS dependent and results in cell death. The results showed a low ROS production in BD and OcMMP patients therefore, identifying whether the cf-DNA released by neutrophils, LDN and NDN of OcMMP and BD patients is due to ROS, apoptosis or necrosis will need further investigation. Similarly, challenging neutrophils with more physiological stimuli is required. Boeltz *et al.,* (2019) states that the use of other additional and more physiologically appropriate stimuli is highly encouraged with NET assays.

A study by Perazzio *et al.,* (2017) demonstrated that patients with active and inactive BD encountered greater levels of CD40L in their plasma, which as a result induces ROS and NET production in neutrophils. As a result, an increased expression of Mac-1 is observed in neutrophils which can also contribute towards vasculitis in BD patients. This supports the findings by Hashiba *et al.,* (2015) which also observed that plasma cfDNA was increased as a result of *in vivo* NET formation in sepsis patients. However, it was shown that *ex vivo* generation of NET is down regulated in neutrophils isolated from sepsis patients. This study contradicts the findings in this thesis regarding NET production by neutrophils, LDN and NDN upon stimulation with PMA, but supports the results obtained by NET production by neutrophils, LDN and NDN upon stimulation with *E.coli* in this study.

One possibility can be due to a greater presence of immature neutrophils (within the LDN population) in BD and OcMMP patients which may result in reduced bacterial killing by phagocytosis and production of reactive oxygen species. In this study opsonised *E.coli* was used to assess the production of ROS and NET in patients with BD or OcMMP and both functional aspects demonstrated a reduced capacity compared to healthy controls. As

previously discussed, ROS is vital for NET production. This may provide an explanation to reduced NET production in BD and OcMMP with *E.coli,* as mature neutrophils of patients are be depleted due fighting against the infection or autoimmune response and immature neutrophils that substitute may have a reduced aptitude to respond to microbes. A study by McDonald *et al.,* (2012) showed that NET were stably generated within the vasculature, especially in the liver sinusoid in septic mice due to presence of immature neutrophils.

It is highly possible that continued inflammatory elevation or stimulation in BD and OcMMP may lead to receptor downregulation and result in impaired signal transduction which may influence these cells to become less responsive to stimuli. This was confirmed by Hashiba *et al.,* (2015) which showed high plasma levels of inflammatory cytokines in sepsis patients and reduced NET production. In order to gain insight of the actual NET formation *in vivo*, inducers such as bacteria should be considered as used in this study.

Middle Eastern patients diagnosed with BD release significantly more NET in comparison to healthy matched controls. This was associated with higher levels of PAD4 expression. Neutrophil extracellular traps are considered to contribute towards vasculitis and thrombosis in BD. NET were recognised in areas of vasculitis in biopsy specimens of BD patients (Safi *et al.,* 2018). Le Joncour *et al.,* showed that NET from patients with BD exhibited increased production of cfDNA and MPO-DNA complexes in patients with active BD compared to inactive patients and healthy controls, and in patients with vasculitis compared to those without. In addition, plasma from patients with BD generated higher thrombin formation in correlation with cfDNA and MPO-DNA levels (Le Joncour *et al.,* 2018).

A recent study described NET production by saliva. NETosis was independent of elastase or NADPH from saliva samples, and resulting NET had a greater capacity to kill bacteria and were

more resistant to DNAase. In comparison, saliva obtained from patients with aphthous ulcers or BD did not induce NET production due different reasons. Sialyl Lewis X, which drives NET production was lost from salivary mucins in patients with aphthous ulcers. However, this was not observed in BD patients and the inhibitory mechanism in these individuals remains unknown stimulus (Mohanty 2015). A possible explanation to this particular response is that these responses may have been influenced by the change in the oral microbiome which is different in patients with aphthous ulcers (Seoudi *et al.,* 2015). Therefore, factors of other microbiomes at sites of mucosal inflammation in patients with BD that may well influence the immune response observed. A second possibility is that the oral cavity houses a "resident" neutrophil population in the gingival sulcus supported by monocytes derived from a local haematopioetic stem cell niche under homeostatic conditions. Mechanisms to prevent NETosis in these neutrophils may protect the environment, but why such processes would differ between disease states, and how this relates to blood borne cells is not clear (J Konkel University of Manchester personal communication)

The results from the current study with regards to functional aspects suggested that even though the pathology or pathogenesis of BD remains unknown, constant mucosal ulceration leads a dysfunctional inflammatory response, subsequent endothelial activation and vasculitis. Grieshaber-Bouyer *et al.,* (2019) suggested that although inflammation is treated in patients with BD, NET production by neutrophils is a distinct and novel therapeutic target in patients with BD or OCMMP.

6.1.1 Low density (LDN) and Normal density neutrophils (NDN)

A second possible explanation for the results described for total neutrophils is that there are different subsets of these cells in patients compared to controls, and that these cells have different functional responses. To address this, we investigated LDN and NDN from patients with BD or OcMMP. The results showed a high LDN, low NDN count in patients compared to healthy volunteers. To determine whether the increase in LDN explained the functional differences seen in total neutrophils, LDN and NDN from patients with BD or OcMMP were assessed for ROS production and phagocytic activity. Both subsets from patients with both diseases demonstrated a reduced phagocytic capacity and ROS production, but an increase in NET production with PMA was observed in comparison to healthy controls.

This is the first time neutrophil subsets have been investigated in these diseases, but the results were consistent with studies on solid cancer, human immunodeficiency virus (HIV)-1 infection, hematologic malignancies and sepsis (Scapini *et al.,* 2016; Bowers *et al.,* 2014; Darcy *et al.,* 2014; Janols *et al.,* 2014). There was a direct correlation between tumour progression and the proportion of LDN which became the main sub-population in the circulation of cancer patients. In a mouse model, following zymosan injection adoptive transfer of LDN into inflamed peritoneum reduced inflammation and correlated with resolution of inflammation. Moreover, evidence suggested that LDN could convert into NDN. These data described different subsets of LDN some with immunosuppressive function and with the ability to convert (Sagiv *et al.,* 2015). In relation to BD or OcMMP increased numbers of LDN can be associated with inflammation. If an inflammatory response were acute this would aid resolution. However, chronic LDN could contribute to disease by inhibiting resolution at mucosal surfaces and perpetuating other manifestations of disease.

The presence of LDN in autoimmune or rheumatic diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis and acute rheumatic fever was originally reported in 1986 (Hacbarth *et al.,* 1986). Currently LDN isolated from patients diagnosed with SLE, psoriasis, chronic granulomatous disease (CGD) and ANCA-associated vasculitis, have been associated with pro-inflammatory function. Such LDN are classified as low density granulocytes (LDG) (Midgley *et al.,* 2016; Denny 2010; Lood *et al.,* 2016). A second subset of LDN is defined as granulocytic-myeloid derived suppressor cells (G-MDSCs) correlating to the immunosuppressive LDN (Sagiv *et al.,* 2015). A distinct subset of neutrophils (LDG) were found in patients with SLE patients showed an enhanced capacity to form NET, with increased expression of many different neutrophil proteins and enzymes involved in NET formation and in the initiation of autoimmunity. NET contained ds-DNA which can act as an autoantigen and has a key role in SLE pathogenesis. Furthermore, LDG increased EC cytotoxicity through NET formation and induced IFN-α synthesis by pDCs. Neutrophils from blood and skin of SLE patients produced IL-17 as part of NET production which can cause tissue damage and immune dysregulation in SLE patients (Villanueva *et al.,* 2011).

One of the main manifestations of BD is vasculitis and the link between the production of NET and vasculitis has been suggested (Safi *et al.,* 2018). In ANCA-associated vasculitis NET formation was ANCA independent, with increased NETosis during the active disease in comparison the remission phase. It was concluded that excess NET formation (ex vivo) correlated with active clinical disease and could be classified as a biomarker of autoimmunity rather than infection. Genetic analysis showed that LDG were the likely source of the neutrophil gene expression in these patients, with LDG playing a vital role in the pathogenesis

of ANCA by promoting direct toxicity to endothelial cells by NET production (KraaIj *et al.,* 2018).

The results in the current study showed increased levels of G-MDSC in LDN isolated from BD and OcMMP patients in comparison to healthy controls. The main functions of G-MDSC are to suppress and inhibit T cell responses such as interferon-γ production and proliferation. In patients with sepsis G-MDSCs inhibited T cell functions via T cell CD3γ -chain expression and T cell function via L-arginine metabolism contributing towards T cell dysfunction in sepsis (Darcy *et al.,* 2014). A study by Favaloro *et al.,* showed a significant increase in G-MDSCs and regulatory T cells in patients with progressive multiple myeloma (MM). After administration of granulocyte colony stimulating factor to induce stem cell mobilization there was an increase in G-DMSC in the peripheral stem cell collection (Favaloro *et al.,* 2014). Finally, immunosuppressive G-MDSCs isolated from cancer patients showed a lower density and different genetic profile in comparison to NDN from the same cancer patients, suggesting a potential link between LDN and G-MDSCs but this remains unclear (Condamine *et al.,* 2016).

As stated an increase in the population of $CD45^{INT}CD11b^{+}CD16^{+}CD14$ in the conjunctiva of patients with ocular MMP and CD11b can be marker for G-MSDCs (Williams *et al.,* 2016; Elliott *et al.,* 2017). Therefore, a reduced phagocytic capacity and enhanced expression of CD11b could represent a possibility that G-MSDCs are expressed in a closed eye environment and in patients with ocular SJS-TEN. However, how this would correlate with progression to inflammation needs to be investigated.

In the current study the function of LDN in terms of phagocytosis, ROS and NET production is influenced by the local microenvironment and pathology associated with BD and OcMMP. Recent studies examined the phenotype of tear neutrophils between early eye lid closure and

following after a full night of sleep. The neutrophils markers CD16, CD11B, CD14 and CD15 were downregulated in cells following sleep and cells showed a reduced phagocytic capacity, suggesting that the closed eye is more a inflammatory environment and therefore, primary degranulation and release of elastase leads to a reduced phagocytic function. Furthermore, neutrophils control and regulate the recruitment of T cells to the ocular surface (Postnikoff *et al.,* 2019). Regulation of T cell proliferation inhibition by neutrophils is highly dependent upon Mac-1 integrin and production of ROS between PMN and T cell (Pillay *et al.,* 2012).

In summary, it can be suggested that mature neutrophils in NDN fraction in this study are initiated from fully mature cells in the bone marrow. However, under certain pathological conditions, such as BD or OcMMP, the progenitor neutrophils leave the bone marrow early, migrate into the blood and displayed a multiple phenotype in the LDN faction. Alternatively, it is possible that suppressive neutrophils were initiated from NDN and appeared in the LDN fraction whereas immature neutrophils completed their maturation process in the peripheral circulation allowing an additional source of mature neutrophils.

6.1.1. Phenotyping total neutrophils

The results obtained in this study demonstrated that neutrophils from patients with BD and OcMMP patients showed activated and immunosuppressive subpopulations. An activated phenotype of neutrophils can mediate the involvement of inflammation of blood vessels in BD. Activated neutrophils induce tissue injury by perivascular penetration in lesions in BD patients (Becatti *et al.,* 2016). Our results show an immunosuppressive phenotype in total neutrophils in BD and OcMMP patients in comparison to healthy individuals. This suggests that the total neutrophil population contains low density neutrophils (LDN) based upon surface expression ($CD15^{+}$/HLA-DR^{-}/$CD54^{bright}$) and a subset of LDN display

immunosuppressive functions. The immunosuppressive function of neutrophils requires clarification as many studies were based on stimulation of PBMC with antiCD3/CD28 beads. In such experiments, neutrophils remove the antibodies from the beads lead to reduced activation rather than suppression. Future studies should utilise mixed lymphocyte reactions or plate bound stimulating antibodies (Negorev *et al., 2018*).

A subset of neutrophils from patients with BD or OcMMP show high expression of CD54, a phenotype associated with reverse transendothelial migration of neutrophils. Mathias *et al.,* described this phenomena used intravital imaging in zebrafish which showed that neutrophils migrate away from a wound back into the vasculature (Mathias *et al.,* 2006). A high CD54 expression was observed in on cells from patients with systemic inflammation where many neutrophils undergo reverse migration (de Oliveira *et al.,* 2016). Studies on mice and humans with acute pancreatitis showed that neutrophils with a reverse migration phenotype were found in the peripheral blood and lungs and associated with severe lung injury (Wu *et al.,* 2016; Peiseler *et al.,* 2019). Reverse migration was demonstrated in a model of focal hepatic sterile injury where neutrophils performed vital repair function before returning back to the bone marrow, and eventually dying via apoptosis (Wang *et al.,* 2017). It has been suggested that reverse migration not only contributes towards the resolution of inflammation but can cause additional inflammation in severe injury (Peiseler *et al.,* 2019). The molecular mechanisms of reverse transmigration are unknown, however, a study by Powell et al., that showed CXCR2 signalling stimulates reverse migration of neutrophils in sterile injury in zebra fish (in vivo) and in human neutrophils (Powell *et al.,* 2017). Therefore, a high CD54 expression in total neutrophils of BD or OcMMP patients may implicate retrograde chemotaxis as a novel

procedure that controls the both the maintenance of activity and resolution of the inflammatory response in these patients depending on circumstances yet to be defined..

An alternative to analysis of surface markers to define subsets is transcriptome analysis. A recent study described LDG as having an immature morphology and an altered transcriptome by expressing more than 5000 genes at significantly different levels in comparison to neutrophils from patients with RA. LDG showed a reduced phagocytic capacity, TNF signalling, ROS and NET production that may contribute to the pathogenesis of RA (Wright *et al.,* 2017). This study suggests that LDN are a heterogeneous population and within the LDN the LDG have a distinct function with an immature morphology. Transcriptomic, epigenetic and functional analysis on LDG in SLE patients showed intermediate-mature ($CD10^{+}$) and immature ($CD10^{-}$) LDG populations. High NET production was observed by $CD10^{+}$ SLE LDG in comparison to $CD10^{-}$ LDG. In addition, $CD10^{+}$ showed increased expression of genes involved in phagocytosis compared to $CD10^{-}$ LDG (Mistry *et al.,* 2019). LDG were not investigated in the current study but it is probable that LDG may exist in the LDN fraction obtained from BD and OcMMP patients. This supports the idea that maturation status and heterogeneity of BD and OcMMP neutrophils subsets affects their ability to perform the important functions such as phagocytosis , ROS production and NET formation

6.1.2. The relevance of results in Behçet's Disease

Genetic studies have implicated several mutations in immune mediating with Behçet's disease. *IL10* variants associated with reduced production of IL-10 have been described leading to a proposed reduced anti-inflammatory control in the pathogenesis of BD (Wallace *et al.,* 2007; Remmers *et al.,* 2010). Functional analysis indicates that the same BD related *IL10* variants cause a reduced expression of anti-inflammatory cytokines and therefore, most

likely in concert with commensal microorganisms, result in an inflammatory state (Nahoum *et al.,* 2006). Functional variants in the *TNF* gene are associated with the severity of ocular involvement particularly retinal occlusion in patients with BD (Verity *et al.,* 1999). In support of these findings anti-TNF therapy is effective in severe and refractory BD symptoms. A variant gene encoding the TIR domain-containing adaptor protein (TIRAP 180Leu) linked to a reduced response to TLR signalling and protection against infectious disease, was associated with BD (Durrani *et al.,* 2011). Finally, a variant in the gene encoding protein tyrosine phosphatase non receptor type 22 (PTPN22 620W) was inversely correlated with patients with BD. This SNP has been associated with several autoimmune diseases such as rheumatoid arthritis and type 1 diabetes and is associated with a gain of function in neutrophils. Following fMLP stimulation, neutrophils heterozygous and homozygous for R620W released significantly more Ca(2+) and fMLP plus TNF stimulation provoked more ROS production in patients with RA (Baranathan *et al.,* 2007; Bayley *et al.,* 2015). Current analysis suggest challenge at a mucosal site leads to a failure of resolution, reduced IL-10 and TIRAP activity combined with increased TNF production and PTPN22 gain of function acting on endothelium and neutrophils will lead to persistent ulceration and vascular activation, breakdown at immune privileged sites and vasculitis (Fig.40).

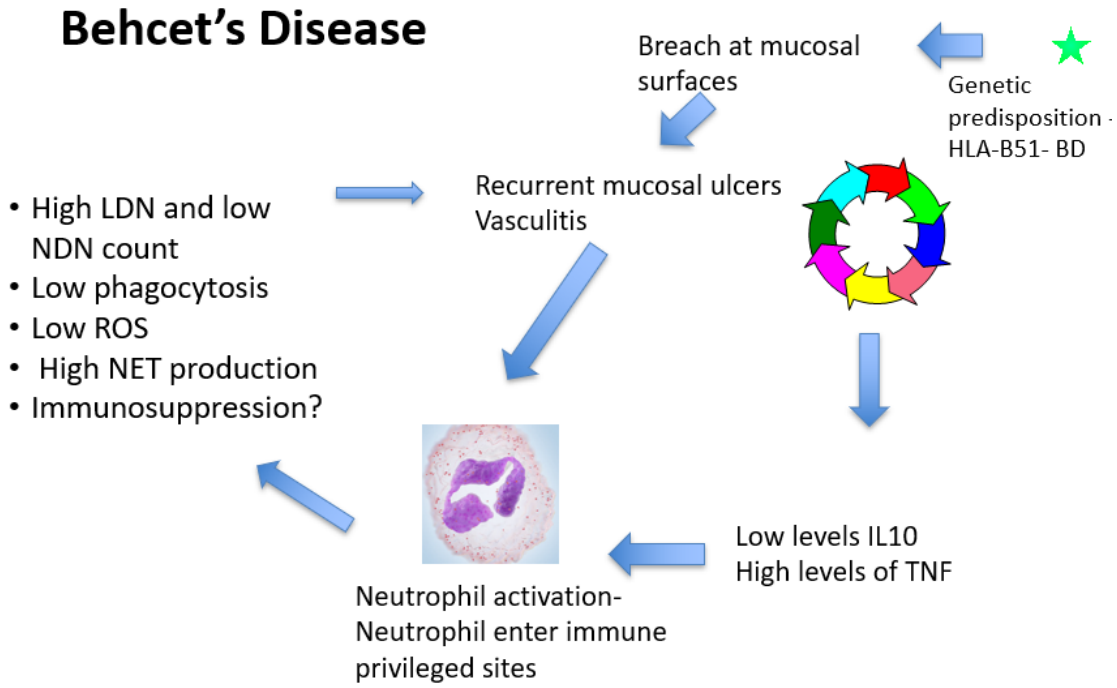


Figure 40-The representation of neutrophil function in BD. The genetic predisposition of HLA-B51 in BD patients affects the mucosal surfaces which as a result leads to recurrent of mucosal ulcerations and vasculitis. This process leads to low levels of IL-10 and high production TNF resulting constant neutrophil activation, and neutrophils enter the immune privileged sites. This study showed high LDN and low NDN count, low phagocytosis, low ROS production and high NET production leading to immunosuppression and recurrent mucosal ulcerations and vasculitis in BD patients.

6.1.3. The relevance of results in OcMMP

The development of dry eye disease is a result of inflammation and hyperactivity of neutrophils leads to inflammation and especially tissue damage during inflammation (Mortaz *et al.,* 2018;Wei *et al.,* 2014). In OcMMP the genetic predisposition of HLA-DQB1*0301 causes a breach at basement membrane and production of cytokine and autoantibodies which in response causes autoimmunity inflammation and recruitment of neutrophils in the conjunctiva. High LDN and low NDN count low phagocytosis , low ROS production, high NET production, enhanced expression of activated markers, immunosuppressive markers reverse transmigrated markers, high production of G-MDSCs within the LDN population in the OcMMP patients in this study. As a result, leads to autoimmunity and auto-inflammation in OcMMP patients (Fig..41).

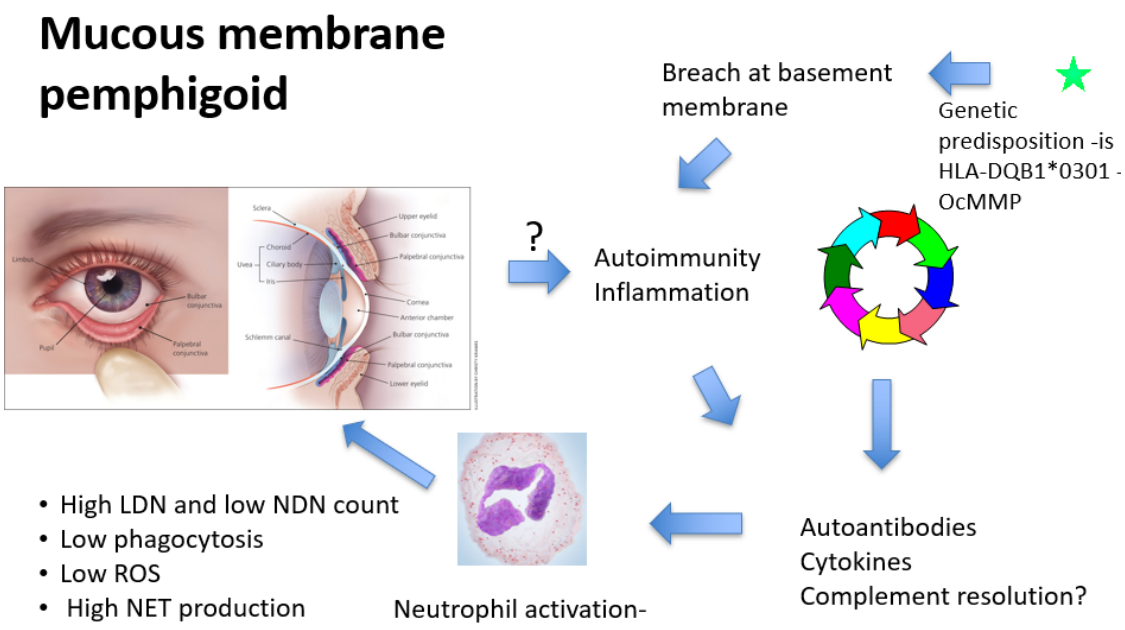


Figure 41-The representation of neutrophil function in OcMMP. The genetic predisposition of HLA-DQB*0301 affects the basement membrane leading to autoimmunity and inflammation. In response this causes autoantibody and cytokine production leading to complement resolution. Furthermore, this leads to neutrophil activation which causes high LDN and low NDN count, low phagocytosis, low ROS production and high NET production in OcMMP patients. These function further cause autoimmunity and inflammation in OcMMP patients.

6.1.4. Limitations

There are several different limitations to this study as follows;

1) Different analysis was not performed on all of the patients or healthy volunteers. This was due to the requirement to use neutrophils within few hours of obtaining blood from patients dependent on those attending each clinic and healthy individuals.
2) Patients diagnosed with OcMMP or BD were on different treatment and BD patients encountered different manifestations of the condition. Therefore, this should be addressed in future studies.
3) LDN are not a homogenous population as discovered and discussed in this thesis therefore, further investigation in terms of diversity is needed. Relation between different subsets and function, phagocytosis, ROS production, NETosis and immunosuppression needs to be investigated.
4) PMA is not a physiological stimulus and may not reflect the complexity of response in neutrophil subsets.
5) NET production was measured as cell free DNA and Sytox green. However, multiparameter staining of NET should be undertaken (Nauseef and Kubes 2016).

6.1.5 Future work

The future considerations (amendments) for this study as follows;

1) To investigate functional analysis and expression of surface markers on samples from the same patient with either BD or OcMMP and healthy individuals. This could involve

transcriptomic analysis but ideally the ability to sort neutrophil subsets based on surface markers to compare function response.

2) To address different manifestations of disease in patients with BD and to study treatment even though, the data was tightly clustered.
3) To investigate heterogeneity and function of neutrophils in tissues to compare with blood derived cell. In particular tear film and conjunctival impression cytology in OcMMP patients. This aspect of the research would be greatly advanced by the ability to sort neutrophil subpopulations
4) To fully define NET derived from different neutrophil subsets by immunohistochemistry and time lapse photography.
5) Perform longitudinal studies to investigate neutrophil phenotype and function in BD and OcMMP patients.

6.1.6 Conclusions

In summary, the data presented in this thesis indicates a dysfunctional neutrophil phenotype and aberrant function in chronic ocular inflammatory disorders. It highlights, for the first time, a possible contribution of neutrophil subsets in BD and OcMMP patients as drivers of disease. Understanding the effects of the functional outcomes of neutrophils discussed in the thesis in each disease to determine whether neutrophils are friend or foe should define future research in these and other conditions (Peiseler *et al.,* 2019).

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