ALPHA DEFENSIN EXPRESSION IN WHITE BLOOD CELLS

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ABSTRACT

Copy number variation (CNV) accounts for a large proportion of genetic variation. The *DEFA1A3* CNV has variable numbers of copies of the *DEFA1* and *DEFA3* genes that encode alpha defensins 1-3, antimicrobial peptides abundant in human neutrophils. Association studies have found associations between this locus and autoimmune diseases such as IgA nephropathy and periodontitis, though the disease mechanisms by which this association occurs are not yet fully understood. Previous investigations into the locus have found inconsistent results for expression of alpha defensin in subsets of white blood cells other than neutrophils. The objective of this project was to investigate the expression of alpha defensins in white blood cells and to determine if expression was variable based on genotype.

This project shows that expression of alpha defensin RNA is present in mononuclear cells as well as granulocytes but is variable independent of copy number or flanking SNP genotypes. No detectable expression of alpha defensin protein was found by immunocytochemistry in cells other than neutrophils. This project shows evidence of alpha defensin in mononuclear cells, highlighting new avenues in which to investigate when researching disease mechanisms of IgAN and periodontitis.

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ABBREVIATIONS

Ag	Antigen
BC	Buffy coat
BCR	B cell receptor
Bp	Base pair
C1	Class 1 haplotype
C2	Class 2 Haplotype
CAMs	Cell adhesion molecules
CGH	Comparative genome hybridisation
CLP	Common lymphoid progenitor cells
CN	Copy number
CNVs	Copy number variant
E1	Exchange 1 haplotype
E2	Exchange 2 Haplotype
FACS	Fluorescence-activated cell sorting
FISH	Fluorescence in situ hybridisation
Gd-IgA1	Galactose deficient IgA1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-CSF	Granulocyte colony-stimulating factor
GRN	Granulocytes
GWAS	Genome wide association study

HNP	Human neutrophil peptide
HRP	Hydroperoxidase
IgAN	IgA nephropathy
ICC	Immunocytochemistry
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MN	Mononuclear cells
NETs	Neutrophil extracellular traps
NK	Natural killer
PAMPs	Pathogen associated molecular pattern
pBCR	Pre-B cell receptor
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PMNs	Polymorphonuclear leukocytes or granulocytes
Pro-B cells	Progenitor B cells
ProHNP	Pro human neutrophil peptide
qPCR	Qualitive PCR
RBC	Red blood cells

REF	Reference Haplotype
ROS	Reactive oxygen species
SNPs	Single nucleotide polymorphisms
TCR	T cell receptor
WBC	White blood cell

CHAPTER 1: INTRODUCTION

1.1: Blood cells

Blood cells are cells of haematopoietic lineage that specialise in a number of functions including, and not limited to, the carrying of oxygen and nutrients to cells, protection against infection and disease, and the prevention of blood loss. Blood cells are characterised into three subtypes; erythrocytes, thrombocytes and leukocytes.

1.1.1: Erythrocytes

Erythrocytes, also known as red blood cells (RBCs), have a primary role in the delivering of oxygen to living cells and the removal of waste product. They are specialised cells that lack nuclei and most organelles in order to increase levels of haemoglobin and, subsequently, the efficiency of oxygen transport. RBCs make up the majority of blood volume (between 40 and 45%) and circulate in the blood for 100-120 days before they are removed by macrophages.

1.1.2: Thrombocytes

Thrombocytes, or platelets, are specialised in the prevention of blood loss through clotting. This also aids in the innate immune response, through confinement of infections. Much like RBCs, platelets also lack nuclei, as they are derived from cytoplasmic fragments of the megakaryocyte, although evidence of platelet RNA has emerged over the years (Rowley 2012).

1.2: White blood cells

1.2.1: The immune system

The human immune system is made up of white blood cells (WBCs) or leukocytes that have several specialised functions that protect the body against infectious disease. The immune response is characterised into two responses based on differences in speed and specificity; the innate (Koenderman *et al.*. 2014) and adaptive responses (Medzhitov and Janeway Jr 1997). The innate immune response encompasses the use of rapid response WBCs, such as phagocytes, that are specialised to respond immediately but non-specifically to infection. The cells involved in the adaptive immune response develop into prolonged and specialised immune cells that act against specific pathogens to prevent recurrent infections.

While WBCs are not abundant in total blood cell count (making up less than 1% of total blood cell count) each WBC has a specialised function. They are distinctive from other blood cells, erythrocytes and platelets, due to the presence of a nucleus (Schmid-Schonbein *et al.*. 1980). It is the combined interaction between the cells that ensures a rapid response to pathogens to prevent and protect against disease. All WBC originate in the bone marrow and are made from hematopoietic stem cells in a process known as haematopoiesis (Birbrair and Frenette, 2016). WBCs are separated into two subgroups based on their different structures; agranulocytes (or peripheral blood mononuclear cells) and granulocytes.

Peripheral blood mononuclear cells (PBMC), characterised by their distinctive round nucleus, consist of lymphocytes (T cells, B cells and NK cells) which make up to 90% of the total PBMC population, and monocytes which make up the remaining 10%. Lymphocytes function in an orchestrated attack against pathogens, often interacting with one another to initiate a response. Monocytes, alternatively, can differentiate into the phagocytic macrophage, or into the less common dendritic cell, which are able to induce a response in the inactive T cell.

The second subtype of WBC are granulocytes, distinct in the presence of cytoplasmic granules and lobular nuclei. Granulocytic cells include eosinophils, basophils and neutrophils, the last of which is the most abundant. They all, however, share a very similar function of phagocytosis, a process that leads to the engulfing and digestion of pathogens and toxic materials to prevent infection and disease. Granulocytes are often active in the initial response against pathogens, engulfing any cell deemed to be foreign. The combined interaction of both subtypes of WBCs can protect the host from infection of pathogens, ranging from bacterial infections to parasitic infections.

1.2.2: Mononuclear cells

1.2.2.1: Monocytes

10% of PBMCs are made up of monocytes, distinct in their bean shaped nuclei. Like other WBCs, monocytes are produced in the bone marrow from haematopoietic stem cells. Monoblast precursors differentiate into monocytes through the action of transcription factors and cytokines such as GM-CSF and IL-3 (Terry and Miller 2014). Once synthesised, monocytes leave the bone marrow. There are three types of monocytes present in human blood; classical, non-classical and intermediate, distinct in the levels of expression of CD14 and CD16 receptors on the cell surface. Roughly 40% of the body's monocytes are stored in the spleen (Swirski *et al.*. 2009) with the remaining 60% circulating through the bloodstream. Classical monocytes (also known as $CD14^{++}CD16^{-}$ monocytes), characterised by high expression of CD14, typically circulate the bloodstream for 1 day, while intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocytes circulate for up to 7 days (Patel at al 2017). Circulating monocytes patrol along healthy tissue (Auffray at al 2007) until they migrate to infected tissue where they differentiate into macrophages and dendritic cells.

Monocytes have three main functions; phagocytosis, antigen presentation and production of cytokines TNF-α and IL-1α (Danis *et al.*. 1995). Phagocytosis via monocytes is done using opsonising proteins such as antibodies and complement factors (Helmy *et al.*. 2006). Though monocytes are not categorised as granulocytic cells, they harbour some

similarities. Azurophil granules are present in the Golgi of a developing monocyte which fuse with the phagosome during phagocytosis (Nichols *et al..* 1971). Unlike other phagocytes, namely mature neutrophils, monocytes can synthesise granule proteins even after leaving the bone marrow. Expression analysis of granule proteins such as alpha defensins have remained inconclusive and contradictory. However, monocytes are known to interact with alpha defensins directly, whereby alpha defensins induce cytokine release in monocytes (Chaly *et al..* 2000)

Monocytes can differentiate into phagocytic macrophages and antigen presenting dendritic cells (Yang *et al..* 2014). Once monocytes differentiate into macrophages through the process of leukocyte extravasation, they are considered ‘professional phagocytes’ based on the efficiency in which they induce phagocytosis (Rabinovitch 1995). Macrophages are critical cells in chronic inflammation due to their specialised ability to remove dying or dead cells, including neutrophils (Savill *et al..* 1989) to prevent prolonged inflammation and tissue damage. Once pathogens are phagocytosed, macrophages present the protein on the cell surface in complex with MHC class II molecules which bind to and are recognised by T helper cells (Murray and Wynn 2011).

Monocytes also differentiate into antigen presenting dendritic cells (O’Neill and Bhardwaj, 2005) that are present in tissue that have direct contact with pathogens and the external environment. These specialised cells include proliferating keratinocytes (Banchereau and Steinman 1998) and Langerhans cells in the epidermis of the skin. Once activated

through activation of PAMPs and cytokines, dendritic cells migrate to the lymph nodes and interact with T cells and B cells in the adaptive immune response. Monocytes interact directly with the other cells of the immune system and it is changes in the function of monocytes that have potential knock on effects on the function of other WBCs.

1.2.2.2: Lymphocytes

Lymphocytes make up 90% of mononuclear cells in human blood. They are characterised into three cell subtypes; T cells, B cells and Natural Killer (NK) cells. Lymphoid cells have distinct roles in the adaptive immune response that leads to prolonged immunity against specific pathogens in order to prevent recurring disease.

1.2.2.2.1: T cells

T cells are involved in the cell-mediated (or adaptive) immune response. They are distinct from other lymphocytes (B cells and NK cells) due to the presence of T cell receptors on the cell surface that bind to antigens presented on MHC molecules (Corr *et al.*. 1994). T cell lymphocytes are produced in the bone marrow from hematopoietic stem cells known as lymphoid progenitor cells. These stem cells undergo expansion through cell division, leading to the production of immature thymocytes that do not express CD4 or CD8 which then migrate to the thymus (Sitnicka 2009). In the thymus these double negative ($CD4^{-}CD8$) cells undergo selection which leads to the production of single positive T

cells that express either CD4 or CD8 glycoproteins on their cell surface (Germain 2002). The selection process of T cells involves positive selection, whereby T cells able to interact with MHC are selected for, and negative selection, where T cells that bind to self MHC peptides are removed (Klein *et al.*. 2014), thereby preventing the production of autoimmune cells against the self. Roughly 98% of T cells that develop during the selection process are removed and the remaining 2% positively selected T cells then migrate to the periphery.

Peripheral T cells are made up of naïve T cells, memory T cells and regulatory T cells. Naïve T cells interact with novel antigens presented by dendritic cells (Sallusto and Lanzavecchia 2002). This interaction leads to the production of IL-2 and the proliferation and differentiation of naïve T cells into Th1 or Th2 (T helper) effector cells (O'Garra and Murphy,1996), short lived cells that promote pathogen clearance. Most T cells are classified as either CD4+ (T helper) or CD8+ (cytotoxic) T cells. Activation of CD4+ T lymphocytes occurs through the binding of the T cell receptor (TCR) on the T cell with MHC-II peptides on antigen presenting cells, alongside binding of co-stimulatory molecules such as CD28 (Beyersdorf *et al.*. 2015) or ICOS on the T cell with co-stimulatory molecules on an antigen presenting cell. The combined binding signals the engagements of the PI3K signalling pathway involved in the development and differential of T and B cells (Juntilla and Koretzky 2008). Helper T cells are critical in the adaptive immune response by stimulating B cells to secrete antibodies (Parker 1993), signalling phagocytosis of microbes by macrophages (Underhill *et al.*. 1999) and

activating cytotoxic T cells. Cytotoxic T cells express TCRs that bind to antigens and have specialised roles in destroying tumour cells (Martin-Orozco *et al..* 2009), virally infected cells and other damaged cells.

1.2.2.2.2: B cells

Lymphoid B cell development is initiated in the bone marrow from hematopoietic stem cells which undergo differentiation into multipotent progenitor cells and then common lymphoid progenitor cells (Lai and Kondo 2008). In the case of B cell development, differentiation of CLP cells eventually leads to the formation of progenitor-B cells (Pro-B cells) which undergo rearrangements of the H, D and J gene segments of the immunoglobulin heavy chain on the cell surface, named the pre-B cell receptor (pBCR) (Tornberg *et al..* 1998), signals the development of pre-B cells (Meffre *et al..* 2001). Once in the pre-B cell stage, the light chain segments undergo rearrangement till the immunoglobulin IgM expression is enabled. Once IgM is expressed on the outer membrane of the now named immature B cell, the B cell leaves the bone marrow and enters circulation to migrate to the spleen (Sims *et al..* 2005). Once in the spleen, the immature B cell matures, leading to the expression of IgM and IgD on the outer cell surface, and migrates from the cortex, where B cells are most abundant, to the paracortex which is abundant with T cells to be activated.

Activation of B cells requires two activation steps. The first of these involves the binding of the B cell receptor (BCR) with an antigen (Ag), where the antigen is engulfed into the cell through receptor mediated

endocytosis. Once engulfed, the Ag is then digested and complexed with major histocompatibility complex class 2 molecules (MHCII). The second step of activation can occur in two mechanisms; thymus dependent or thymus independent. Thymus dependent activation requires T helper cells which bind to the B cell via the antigen-class II MHC complex, signalling the activation of the T cell. Activation of the T cell then induces a secondary signal that activates the B cell. The other mode of activation is through thymus independent activation whereby certain types of Ag, such as lipopolysaccharides or bacterial flagella, are brought directly to the B cell and induce an activation signal. Once activated, B cells will migrate to the germinal centre where it will undergo mutations and selection (Natkunam 2007) to become centroblasts. The intracellular enzyme activation-induced cytidine deaminase induces hypermutation (Park 2012) that results in changes in the affinity and specificity of the antibodies present on B cells, leading to the differentiation of B cells into memory B cells or plasma cells, the latter of which can either be short lived or long-lived plasma cells. Long lived plasma cells release antibodies that can neutralise pathogens, enhance phagocytosis and activate the complement cascade, while short lived plasma cells are involved in the initial immune response.

B cells undergo a series of stages of selection during development to prevent the formation of self-binding antibodies to ensure successful development of B cells, through positive or negative selection, known as central tolerance (Nemazee 2017). Positive selection of B cells is where B cells that are unable to bind to antigens due to too low an affinity are

not further developed and undergo apoptosis. Negative selection of B cells occurs when self-antigens bind to the BCR on B cells, leading to apoptosis (Eeva and Pelkonen 2004), receptor editing, whereby rearrangements of genes leads to the development of new BCR with altered specificity (Prak *et al..* 2011), and anergy, where B cells are made to be non-reactive (Yarkoni *et al..* 2010). In some cases, the B cell will ignore the signals and continue to develop, known as clonal ignorance.

Though B cells are thought to be antigen presenting cells, it has been proposed they may be able to phagocytose specific pathogens (Zhu *et al..* 2016). Investigations into antimicrobial peptides in B cells have uncovered evidence for alpha defensin expression in B cells (Agerberth *et al..* 2000) . Research into the potential associations between B cells and diseases have found that CD19+ CD5+ B cells, which produce IgA, have an association with primary IgA nephropathy (Yuling *et al..* 2008) though the role of these cells in IgAN is not fully understood. Nevertheless, evidence has been found of hyperreactive B cells in patients with IgA nephropathy (Hale *et al..* 1986).

1.2.2.2.3: NK cells

The remaining lymphocyte, the natural killer (NK) cell, is formed from common lymphoid progenitor cells. Unlike B and T cells, NK cells are part of the innate immune response and are rapid response cells, responding 3 days after initial infection. NK cells are specialised in their ability to respond to virally infected cells. They are unique in their ability to detect self-cells in stress even in the absence of antibodies of MHC

molecules (Ljunggren and Karre 1990) and are therefore crucial in their role of removal of tumours.

NK cells have several functions, the first being like that of the granulocytic neutrophil. They have small granules that contain various antimicrobial peptides such as proteases and perforin (Krzewski and Coligan 2012) and lytic enzymes such as protease. Release of these granule enzymes by the NK cell destroys the pathogen via lytic activity while antimicrobial peptides such as perforin create pores in the cell membrane of the target cell. This allows the granzymes to enter the cell and induce apoptosis or cell lysis. Much like neutrophils, there are reports that NK cells also express alpha defensins in their granules (Obata-Onai *et al.*. 2002), along with antimicrobial peptides that cause lysis of the pathogenic cell.

NK cells also have functions dependent on antibody presentation, known as antibody-dependent-cell-mediated-cytotoxicity. CD16 receptors on the NK cell surface (Romee *et al.*. 2013) bind to antibodies presenting antigens, inducing the release of cytolytic granules, and the eventual induction of apoptosis. Cytokines, such as IL-12, IL-15 and IL-18, are essential in NK function, signalling to the NK cell in the presence of viral pathogens. This interaction induces NK cells to secrete IFN gamma, which in turn activates macrophages, and TNF alpha, which promotes direct NK tumour killing. NK cells' main function is the specialised role to detect cells in a stressed state in the absence of pathogen molecules and destroy them.

1.2.3: Granulocytes

1.2.3.1: Eosinophils

Eosinophils are one of the least common white blood cell in humans, making up 2% of WBC count (Uhm *et al..* 2012). Eosinophil count fluctuates throughout the day, reaching its lowest levels in the morning and progressively increasing throughout the day. Eosinophils are considered as end stage leukocytes, responding directly and most efficiently to allergic and parasitic infections.

Eosinophils are synthesised in the bone marrow from hematopoietic CD34+ stem cells (Uhm *et al..* 2012), a process which is regulated by IL-5. IL-5 aids in the terminal differentiation and proliferation of eosinophils, along with IL-3 and GM-CSF (Sanderson 1992). Once mature eosinophils are produced, they are released in the blood where they persist in circulation for 8-12 hours, with the majority of the cells residing in tissue, particularly in the mucus membranes (Lozewicz *et al..* 1990) such as the ovaries (Brown and Nellor, 1968), lower gastrointestinal tract (Chernetsova *et al..* 2016) and the spleen, where they reside for 8-12 days before apoptosis.

Eosinophils are characterised as granulocytes due to the presence of crystalloid granules in the cytoplasm that store most of the antimicrobial proteins needed for phagocytosis. The granules are made up of a crystalloid core, consisting of major basic proteins 1 and 2, and a matrix that contains eosinophil peroxidases, ribonucleases, eosinophil-

derived neurotoxins and eosinophil cationic proteins (Johansson *et al.*. 2000), all of which are toxic to a range of tissues such as the heart. Eosinophils have further functions upon activation such as degranulation, the release of reactive oxygen species (ROS) and the induction of the release of lipid mediators, cytokines such as IL-2, and TGF-α (Kita 1996), and chemokines (e.g. RANTES). The ribonucleases in the granules that are released during degranulation aid in the action against viruses, while the other granule peptides can create pores to induce cell permeability. Overactivity of eosinophils contributes to the pathophysiology of various disease such as asthma, inducing epithelial damage (Acharya and Ackerman 2014). Eosinophils also have non-cytotoxic functions, in that they can suppress the action of other white blood cells, such as the proliferation of T cells and immunoglobulin synthesis by B cells. Eosinophils are however highly active against parasites such as helminth worms (Gleich and Loegering 1984) and viruses, making them essential in the immune response despite their low cell count.

1.2.3.2: Basophils

Full understanding into the mechanisms by which basophils function has not yet been achieved, mostly due to the very low proportion of basophils in total white blood cell count (<1%) (Karasuyama *et al.*. 2009). They are understood to play an important role in hypersensitivity and anaphylactic reactions (Schroeder 2009). Basophils are known to develop from CD34+ stem cells in the bone marrow, where cell lineage

is determined by the production of GATA-2 and C/EBP alpha (Arinobu *et al..* 2009). Due to their low yield in experimental investigations in the past, basophils were often described as minor cells and were considered a subtype of mast cells due to their similar structure.

In an immune response, basophils release cytokines such as IL-4 (Van Panhuys *et al..* 2011) and IL-13 (Ochensberger *et al..* 1996) that induce the recruitment of phagocytic cells such as eosinophils and neutrophils to the site of infection. Basophils are antigen presenting cells and have IgE receptors present on the outer membrane of the cells (Stone *et al..* 2011) which bind to IgE bound antigens (Malveuex *et al..* 1978). Basophils, once activated, release heparin, an anticoagulant, and histamine, an inflammatory molecule, before performing phagocytosis (Borriello *et al..* 2017). The release of histamine widens blood vessels, increases blood flow and makes blood vessels more permeable, aiding in migration of neutrophils.

1.2.3.3: Neutrophils

The most abundant white blood cell of the immune system is the neutrophil, making up 50-60% of circulating leukocytes (Peake 2002). Being one of the first cells to be recruited to the site of injury (Smith 1994), their highly efficient phagocytic properties can kill potentially life-threatening pathogens in minutes.

Development of neutrophils occurs in the bone marrow from haematopoietic stem cells in a process called granulopoiesis. The process begins with unipotent stem cells known as myelocytes undergoing a series of development stages including the formation of granules, changes in nuclear shape and chromatin condensation to eventually form mature neutrophils which are then released into the blood. In a healthy individual, roughly 10^{11} neutrophils are produced daily (Leiding 2017), with this rate increasing following stress and infection. Once released into the blood, neutrophils circulate in the blood with a life span of 4-10 hours (Summers *et al..* 2010) and, if signalled, enter tissue where they survive for 1-2 days. If they are not signalled they are cleared in order to prevent inflammation. Unusual levels of neutrophils, whether elevated or decreased, has the potential to cause a great impact to human health ranging from patients suffering with repeat infections to some types of cancer (Uribe-Querol and Rosales, 2015). Therefore, granulopoiesis is a tightly controlled process that involves several specific mechanisms (Von Vietinghoff and Ley 2008), the first being the production of granulocyte colony stimulating factor (G-CSF) (Basu *et al..* 2002). G-CSF enhances neutrophil synthesis, stimulating the formation of mature neutrophils from stem cells in the bone marrow. People deficient in G-CSF and/or its receptor suffer with excessively low levels of neutrophils, known as neutropenia, (Dong *et al..* 1994), and a synthetic form of the protein is often administered to cancer patients after chemotherapy to restore the immune system. The counteracting mechanism to this to prevent excessive levels of neutrophils, is through

the release of chemokines (Eash *et al..* 2010) that increase neutrophil retention, and efferocytosis, the process of phagocytosis of neutrophils by macrophages (Martin *et al..* 2014).

Under normal conditions, neutrophils roll along the microvascular walls through low affinity interactions of selectins with endothelial carbohydrate ligands (Sundd *et al..* 2011). During the initial stages of infection, macrophages in the infected tissue release cytokines that signal the endothelial cells to express cellular adhesion molecules (CAMs) such as P and E selectins. Carbohydrate ligands on neutrophils bind to these selectins, which slows the neutrophils down. This slowing process enables chemokines on the surface of the neutrophil to bind to chemokine receptors on the endothelium, leading to stable adhesion, a process added to through integrins (Powner *et al..* 2007). Through a process of integrin binding, neutrophils 'crawl' down the endothelium wall to the site of infection. Neutrophil migration occurs in two potential mechanisms (Kvietys and Sandig 2001). The first mechanism occurs between endothelial cells through the influence of a chemotactic gradient that signals the neutrophil to penetrate the endothelial layer and migrate through connective tissue, a process known as diapedesis (Muller 2013). The second, less understood mechanism is through the travelling of neutrophils through individual cells (Kolaczkowska and Kubes 2013).

Neutrophil function is closely related to their structure. They are characterised by lobular nuclei and the presence of three types of granules; primary azurophilic, secondary granules and tertiary granules.

The most abundant of the granules are the azurophilic granules formed in the promyelocytic stage of development which have a regulatory role in the phagocytosis of neutrophils to terminate inflammation. They are key granules also found in monocytes (Nichols *et al..* 1971) and aid in the phagocytic property of the cell, enabling them to regulate neutrophil count; they are known to degrade inflammatory products (Falloon and Gallin 1986). Neutrophils destroy microbes in several functions including, phagocytosis, ROS and the formation of NETs. Neutrophils are abundant and highly active phagocytes, the process occurring in minutes. The process begins on the activation of opsonic receptors such as complement receptors (Sengelov 1995) which signals the neutrophil to engulf the microbe via the membrane to form a phagosome. This engulfing of the microbe is mediated by intracellular signalling, cytoskeleton rearrangements (May and Machesky 2001) and molecular determinants such as membrane lipids (Kaufmann 2016). Primary azurophilic and secondary granules fuse with the phagosome to form a phagolysosome. The low pH of the phagolysosome aids in the defence against microbes but it is the combined effort of oxidative and non-oxidative defences that destroys pathogens. These components include lytic peptides such as lysozymes (Cieutat *et al..* 1998) and defensins which interact with the pathogen cell wall to induce cell lysis. The rate at which phagocytosis occurs is enhanced through complement and binding to IgG (Hed and Stendahl 1982). In some cases, the targets are too large to engulf or are not fully phagocytosed. This results in frustrated

phagocytosis leading to the release of granule contents outside of the cell leading to tissue damage (Mayadas *et al..* 2014).

Activation of neutrophils also signals the formation of NADPH oxidase from membrane bound flavocytochrome and cytoplasmic components such as Rac (Sheppard *et al..* 2005). The formation of NADPH oxidase causes potassium ion fluxes to occur in response (Nunes *et al..* 2013) and leads to the formation of superoxide from oxygen (Winterbourn *et al..* 2016). Superoxide acts as a precursor for hydrogen peroxide formation, formed by spontaneous dismutation of superoxide (Weiss *et al..* 1981), which is used to generate other oxidants such as HOCl generated by H_2O_2 with Cl^- when neutrophils are stimulated by IgG (Blackburn 1994). Reactive oxygen species damage enzymes leading to metabolic defects in bacterial cells. The final response to microbes is through the formation of NETS, extracellular fibres that enable the neutrophil to kill extracellular pathogens using antimicrobial proteins (Delgado-Rizo *et al..* 2017).

Neutrophils express alpha defensins in abundance. However, though associations between the *DEFA1A3* locus and diseases such as IgAN and periodontitis have been found, the connection by which neutrophils are involved are not fully understood. Investigations into potential changes and differences in neutrophils are essential to understand potential disease mechanisms. However, most investigative work on alpha defensins has been focused on neutrophils, but evidence is not conclusive as to which other white blood cells also express alpha

defensin. The potential association between the locus and disease may only be fully understood if alpha defensin expression in both neutrophils and other white blood cells is fully understood.

1.3: Alpha Defensins

1.3.1: Defensins

Defensins are small cysteine rich antimicrobial peptides roughly 2-6 kDa in size, found in both vertebrates and invertebrates, that are effective against bacteria, fungi and some viruses (Hancock *et al..* 1995). Defensins have similar ancestral history and are characterised into three subsets; alpha, beta and theta, based on differences in structure and function. Theta defensins are rare, circular proteins that are expressed solely in primates (Nguyen *et al..* 2003). Beta and Alpha defensins are common in mammals but are expressed in different cells due to their altered function and structural differences, mainly in the different cysteine residues that are involved in the formation of their cysteine bridges. Beta defensins are secreted by leukocytes and epithelial cells in humans and are widely distributed around the body. Beta defensins are active in interacting with the membrane of pathogens and are involved in the innate and adaptive immune responses. Copy number variation is common in the beta defensin genes and is known to be associated with disease such as psoriasis (Hollox *et al..* 2008).

Human alpha defensins are split into six subsets based on their location in the body and function in the immune response; the peptides are numbered 1 through to 6 and are encoded by five *DEFA* genes (*DEFA1* and *DEFA3-6*). *DEFA5* and 6 are expressed in the Paneth cells of the intestines and are involved in maintaining a balance of microbes in the gut (Ouellette 2006). *DEFA4* encodes human neutrophil peptide 4 (HNP4) found as a minor alpha-defensin in neutrophils (Wu *et al..* 2004) and has roles in anti-adrenocorticotropic hormone activity (Singh *et al..* 1988). *DEFA1* and 3 encode human neutrophil peptides (HNP) 1-3 and have been found to exhibit copy number variation in the locus *DEFA1A3*.

1.3.2: Structure

The structures of HNP1, 2 and 3 are nearly identical (X-$C_1YC_2RIPAC_3IAGERRYGTC_4IYQGRLWAFC_5C_6$) (Lehrer *et al..* 2012) where X is either Alanine in HNP1, Aspartate in HNP3 or absent in HNP2, the latter of which is thought to be a proteolytic product of HNP1 and 3 (Valore *et al..* 1992). Alpha defensins 1-3 (HNP1-3) are cysteine rich 29/30 amino acid long peptides with three disulphide bridges between cysteine resides 1:6, 2:4 and 3:5. The disulphide bridges are crucial in the role of alpha defensins, particularly in dimerization to form pores (Zhang *et al..* 2010) and to neutralise bacterial toxins (Kim *et al..* 2005).

1.3.3: Synthesis

Synthesis of alpha defensins occurs in neutrophil precursors (promyelocytes) during haematopoiesis in the bone marrow. The

prepropeptide, roughly 94 amino acids long, is cleaved into a 19 amino acid signal peptide and a 75 amino acid proHNP. The proHNP is then cleaved by protease to produce HNP (Wu *et al..* 2003). Synthesis of alpha defensins is rapid, matching the rapid production of neutrophils. Neutrophils are produced at a rate of $8x10^8$ PMNs per kg body weight per day (Dale *et al..* 1998). As 4-5ug of alpha defensins are present in $1x10^6$ neutrophils (Ganz 1987), a rough estimate of 3-4 mg of alpha defensins per kg of body weight are produced daily in humans, or 200mg per day for a 70kg adult. Production of alpha defensins halts once the mature neutrophil is formed and leaves the bone marrow (Yount *et al..* 1995) and alpha defensin mRNA can be found to be actively expressed in bone marrow, particularly highest in promyelocytes (Ganz 2007). However, lower levels of mRNA are found to be present in circulating neutrophils, alongside precursors of alpha defensins (Harwig *et al..* 1992) though the reason behind this is not yet understood and assumed to be left over from synthesis in the bone marrow promyelocytes.

1.3.4: Function

Mature HNP1-3 are found in the azurophilic granules of neutrophils (Faurschou and Borregaard 2003), alongside myeloperoxidase, elastase and other antimicrobial peptides. HNP1-3 are in abundance in these primary granules, making up 30-50% of the granule protein (Lundy *et al..* 2005). During phagocytosis, neutrophils undergo degranulation, stimulating the release of peptides present in the azurophilic granules, including alpha defensins. Though they have many

roles and functions in the immune response, the general principle of alpha defensin antimicrobial action is through binding to pathogen cell membranes to induce cell permeability. Defensins have positively charged regions (Ganz 1999) that bind to the anionic membrane of the pathogen, enabling them to insert themselves into the phospholipid membrane. Once there, the peptide dimerises to form pores in the cell membrane, leading to increased cell permeability and eventual lysis of the cell. HNP1 interacts with the bacterial cell wall precursor Lipid II involved in the synthesis of cell walls (Breukink and Kruijff 2006). This interaction inhibits cell wall synthesis, leading to an increased susceptibility to damage of the pathogen. This process is aided by the presence of glycolipids, glycoproteins and glycans on the cell surface of pathogens for which alpha defensins have a lectin-like affinity (Wang *et al..* 2004). Alpha defensins show a level of non-specific binding, whereby they will, if given the opportunity, bind to themselves (Lehrer and Lu 2012). However, alpha defensins remain in the azurophil granules until they reach the site of infection, and therefore their non-specificity is not detrimental to the host.

Alpha defensins can also interact with other cells of the immune system. They interact directly with monocytes by inducing the expression of TNF-α and IL-1β CD64 (Chaly *et al..* 2000) which in turn enhances expression of IgG Fc receptors CD32 and CD64 that are involved in the activation of phagocytosis (Soehnlein *et al..* 2008). They also have an opposing effect on the immune response. Alpha defensins interact directly with macrophages to inhibit protein translation, essentially acting

as a “molecular brake” (Brook *et al.*. 2016) on inflammation to prevent tissue damage to the host.

Alpha defensins are expressed in abundance in neutrophils and can suppress neutrophil apoptosis through the down regulation of the pro-apoptotic protein Bid and the up regulation of the anti-apoptotic protein Bcl-xL to prolong neutrophil lifespan and therefore the immune response (Nagaoka *et al.*. 2010). . However, it is still not fully understood how the gene, protein and diseases, such as IgA nephropathy and periodontitis, are linked in a potential disease mechanism.

1.4: The Human Genome

1.4.1: Variation in the genome

Genomic variation is a generalised term that defines differences in the DNA between individuals. Variation determines differences in physical appearance between populations and individuals, such as hair and eye colour, but can cause functional defects and disease. Structural variation ranges in size and scale, from large scale variation (defined as changes of DNA larger than 3Mb) that can be seen microscopically, to small scale variation (<3Mb) often encompassing single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) (Feuk *et al.*. 2006). Due to availability of technology, initial investigations of variation were limited to changes detectable under a light microscope, through chromosome banding or via fluorescence *in situ* hybridisation (FISH)

(Price 1993). The visible physical changes in the chromosome included abnormalities in the number of chromosomes, i.e. the presence of an extra chromosome or set of chromosomes, or chromosomal misalignment, leading to the loss of sections of DNA or the extension of arms of the chromosome.

In recent years, further advances in technology have enabled the sequencing of whole genomes, leading to the increased accessibility in the detection of small scale variation (or ‘sub microscopic structural variation’). The overall phenotypic effect of variation on an individual is not directly correlated to the size of the genome that is involved. Whilst most variation in humans is neutral or determines commonly variable traits like hair colour and height, even very small-scale variation including SNPs (single nucleotide polymorphisms) can lead to debilitating disease. Variation in the human genome has been of interest in medical research due to the strong association it has with disease and the far-reaching effect variation can potentially have on human health (Reich *et al..* 2002). Advances in experimental approaches have highlighted the proportion of variation naturally occurring in the genome. In the early 21st century, it was assumed that less than 1% of the genome showed variation among individuals (Jorde and Woodling, 2004) but in 2015 the 1000 genomes project found up to 5 million sites in individuals that varied compared to the reference genome (Siva 2008).

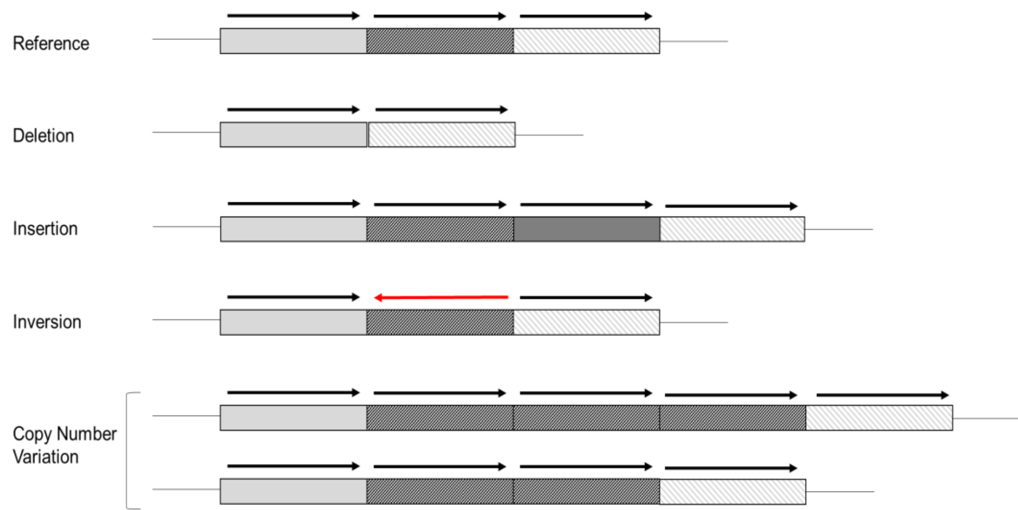


Figure 1.1. Categories of structural variation including deletions, insertions, inversions and two possible outcomes of higher-level copy number variation.

1.4.2: Single nucleotide polymorphisms

The smallest scale variants within the genome are single nucleotide variants which involve the mutation of a single nucleotide base. Single nucleotide polymorphisms (SNPs) are described as single nucleotide variants that have a frequency of >1% (Brookes 1999). The potential effects of a SNP on the genome are dependent on several contributing factors. The first is location, whereby a SNP can be either located within non-coding regions, leading to no loss or change of function or located within coding or gene control regions (i.e. promoter or enhancer regions) that can lead to changes in protein synthesis and function, therefore contributing to disease.

The second factor that affects SNP contribution to phenotype is the effect the SNP has on the coding sequence. Due to the redundancy of the genetic code, the change of one nucleotide in the genome can be synonymous, leading to no change in the amino acid code. However, non-synonymous SNPs, which alter the amino acid code, can lead to dramatic changes in the genome, such as introducing a premature stop codon, known as a nonsense mutation, resulting in a truncated protein. Over the years, SNPs have been well characterised, underpinning large scale genome wide association studies (GWAS) that genotype large sections of the genome to determine differences between individuals and populations. Variation in the genome comes in several forms, and until recently it was believed that SNPs had the largest scale effect on the genome. However, in more recent years, the phenomenon known as copy number variation (CNVs) has been identified and characterised on a larger scale. This has revealed a larger proportion and frequency of CNVs in the genome than was originally understood.

1.4.3: Copy number variants

In recent years the phenomenon of copy number variation (CNV) has been characterised more frequently and in greater depth. CNVs are where a series of duplications, insertions and deletions of more than 1Kb of DNA occur, leading to variable numbers of copies of a gene compared to a reference sequence (Stranger *et al..* 2007). It was originally assumed that CNVs were infrequent in the genome (Redon *et al..* 2006). However, advances in experimental procedures, such as the development of

comparative genomic hybridisation (CGH) enabled further understanding of this mode of variation and how it accounts for a large amount of genetic variation in humans (Freeman *et al..* 2006). CNVs potentially make up more of the genome than previously thought (Stankiewicz *et al..* 2010) both in intronic and intergenic regions (Sebat *et al..* 2004) with 11,000 CNVs overlapping more than 1000 genes (Hurles *et al..* 2008) and accounting for roughly 10% of the genome (Redon *et al..* 2006) (Zarrei *et al..* 2015). Though CNVs span a smaller proportion of the genome than SNPs, due to the potential large-scale of variability caused by CNVs, they are thought to be more likely to underpin evolution and phenotypic diversity (Stankiewicz and Lupski, 2010).

CNVs are characterised into two classes; biallelic and multiallelic, the first being more common and latter less characterised, based on their general structures and mode of variation. Biallelic CNVs are the result of a single duplication or deletion event, resulting in either the repetition or absence of a genomic region. Multiallelic CNVs occur when multiple deletion and duplication events occur at the same locus, resulting in a range of possible copy number outcomes. Studies into CNV consequences have found various loci with associations with disease (Conrad *et al..*, 2010). It is not fully understood if CNVs are causative of disease or how they affect gene expression, though suggested consequences include a negative correlation with gene expression, alteration of gene dosage (Stranger *et al..* 2007) and disruption of coding sequences. Due to the potential pathogenic nature of CNVs, they are being investigated on

larger scales to find associations with disease and potential disease mechanisms (Itsara *et al..* 2009).

1.5: *DEFA1A3*

1.5.1 *DEFA1A3* locus

Located on 8p23.1 is a cluster of genes, *DEFA1* and *DEFA3*, encoding human neutrophil peptides 1 to 3 (HNP1-3 or alpha defensins) (Aldred *et al..* 2005). Gene arrangement at this locus consists of units of both the *DEFA1* and *DEFA3* genes. Prior to the 21st century, it was not fully understood if the genes encoding alpha defensins were in fact separate or were encompassed into one gene (Mars *et al..* 1995). Further investigative research into the locus showed that it exhibited extensive copy number variation and, in 2005, the locus was renamed *DEFA1A3* (Aldred *et al..* 2005) to account for the interchangeable pattern of copies of the structurally similar *DEFA1* and *DEFA3* genes. This variation has strong associations with immune diseases IgA nephropathy (IgAN) (Ai *et al..* 2016) and periodontitis (Munz *et al..* 2017).

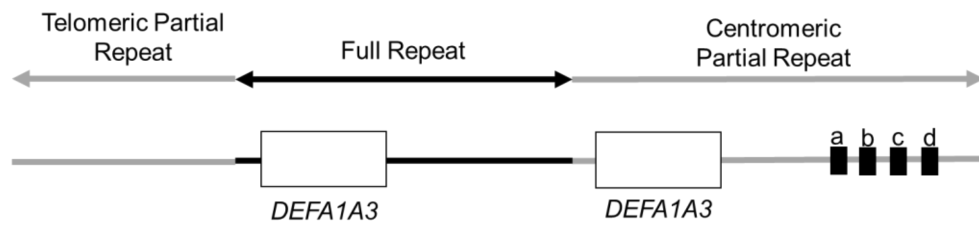


Figure 1.2. Structure of *DEFA1A3* locus on 8p23.1 adapted from Black *et al*.. 2014. The locus consists of two partial repeats surrounding a variable number of full repeats; each "*DEFA1A3*" site can be occupied by the *DEFA1* or *DEFA3* variant of the gene. The schematic shows the four SNPs in the flanking region that tag the five haplotype classes*; a. rs7825750, b. rs6248514, c. rs4300027 and d. rs7826487.*

1.5.2: Variation in populations

DEFA1A3 copy number is variable among individuals in a population. For example, Europeans exhibit variable copy number, commonly ranging from 4 copies to 10 (Khan *et al..* 2013). Whilst copy number is variable within a population, there are differences in copy number between populations. Within the Asian population, China displays with a range between 3 and 11 copies compared to Japan with copies ranging from 4 to 16 (Cheng *et al..* 2013). Repeats within this locus follow a common structure of two partial repeats surrounding a variable number of full repeats of either *DEFA1* or *DEFA3* genes (Black *et al..* 2014). In some cases, however, a small proportion of each population (e.g. 10% in Europeans and 37% in Africans) lack the *DEFA3*

gene completely (Ballana *et al..* 2007). Research into the possible effects this has on individuals revealed that against gram positive and gram-negative bacteria, HNP3 (encoded by *DEFA3*) is less active (Ericksen *et al..* 2005) and expressed twice fold compared to HNP1, encoded by *DEFA1*, suggesting that the single amino acid difference between the genes has some phenotypic consequences. Generally, however, it is not yet clear how the immune response of those lacking the *DEFA3* gene is affected.

1.5.3: Haplotype classes

European variation in the *DEFA1A3* locus has been characterised into 5 main haplotype classes based on modes of inheritance (Black *et al.*., 2014), each of which carries a single copy of a centromeric partial repeat and a variable number of full repeats. The first of these classes is named the 'Reference Haplotype' which is found in the GRCh37/hg19 human reference assembly. It is usually associated with high copy number (4 to 5 per haplotype) one of which is a copy of *DEFA3* in the centromeric partial repeat. The second set of classes are 'Class 1' and 'Class 2' which are similar in structure to one another and tend to have low copy number (2 or 3 repeats per haplotype). The final set are known as 'Exchange 1' and 'Exchange 2' whereby regions of the centromeric partial repeats are replaced with the equivalent region of the full repeat with sequence lengths of 140bp and 1990bp in Exchange 1 and Exchange 2, respectively (Black *et al..* 2014). Frequencies of each

haplotype is variable between populations, to some extent explaining the observed differences in copy number. Despite SNPs being generally poor tags of CNVs, variation in the number of copies of *DEFA1A3* is known to be correlated with several specific SNPs such as rs4300027 (Khan *et al.*. 2013) and rs2738048 (Black *et al.*. 2014). Identification of the *DEFA1A3* haplotypes have given insight into the possible inheritance mechanisms, further developing an understanding of the locus and how variability in copy number can lead to disease.

1.5.4: Association with disease

Studies have identified associations between copy number in the *DEFA1A3* locus and immune diseases such as Periodontitis (Munz *et al.*. 2017) and IgA nephropathy (IgAN) (Ai *et al.*., 2016).

Periodontitis is caused by a dysfunctional inflammatory reaction to the presence of microorganisms in the mouth. Overactive immune responses lead to prolonged inflammation and eventually, tissue damage and the destruction of the periodontium (Darveau, 2010). Association studies have found an association between the *DEFA1A3* locus, in particular the flanking SNPs associated with the locus, and periodontitis including aggressive periodontitis, the most severe and rare form of the disease (Munz *et al.*., 2017). It is not fully understood how variability in the locus effects phenotype, with changes in *DEFA1A3* copy number showing no correlation with alpha defensin expression (Aldred *et al.*.

2005). However, investigations into the disease found that those with chronic periodontitis displayed with elevated levels of antimicrobial peptides including alpha defensins in the saliva (Gorr, 2011), with levels being two-fold and four-fold higher in aggressive and chronic periodontitis patients respectively (Puklo *et al..* 2008); patients also had neutrophils present in the gingival crevicular fluid and junctional epithelium (Dale *et al.*., 2001). However, full understanding of the mechanism underlying the association is yet to be achieved and experimental work looking at different aspects of the gene, protein and disease is ongoing.

IgA nephropathy (IgAN), also known as Berger's disease (Berger 1968) is associated with low copy number of the *DEFA1A3* locus. IgAN is characterised by IgA deposits in the glomerular mesangium of the kidneys and is the most common glomerular disease worldwide (Ai *et al..* 2016) Due to variability of the disease, both clinically and pathologically, the mechanisms of IgAN are not fully understood. Patients with the disease display elevated levels of galactose deficient IgA1 (Gd-IgA1), leading to the formation of circulating immune complexes that deposit into the mesangium. This leads to cytokine release, chemokine secretion and the migration of phagocytic cells to the kidney, causing the destruction of the mesangium. Although elevated levels of serum Gd-IgA1 is associated with the disease, it is not necessarily all that induces IgAN (Reily *et al..* 2014). Investigations into disease associated loci showed that, particularly in the Chinese population, the *DEFA1A3* locus and other loci were associated with susceptibility to the disease (Li *et al..* 2015) (Qi *et al..* in 2015). Strong associations with low *DEFA1A3* copy

number and IgAN have been found (Ai *et al..* 2016). Much like with periodontitis, investigations into potential disease mechanisms of alpha defensins are ongoing.

1.6: Project aims

Investigations into the *DEFA1A3* locus have found associations with autoimmune diseases, revealing it as a risk locus for IgAN and periodontitis. Understanding into the disease mechanisms by which alpha defensins, encoded by *DEFA1A3* genes, are associated with these diseases is incomplete, and investigations into the expression of alpha defensin in cells other than neutrophils have remained inconclusive. Expression databases have been primarily focused on whole blood RNA (Carithers *et al..* 2015) and conclusions from investigations of alpha-defensin expression in separated white blood cell types have been variable, with some literature showing good evidence of expression in mononuclear cells (Villani *et al.*., 2017), particularly in monocytes (Mackewicz *et al..* 2003), while others show expression in T cells and NK cells (Obata-Onai *et al..* 2002). Investigations into variation in the *DEFA1A3* locus have identified associated variability in the properties of blood cells other than neutrophils (Astle *et al..* 2016), setting the foundation of investigations in this project

The aims of this project are to determine which white blood cells are expressing alpha defensins and if this expression is variable between

individuals depending on genotype. We will specifically examine expression levels of alpha defensin in cells other than granulocytes, such as in the mononuclear cell subtype, to determine if they are potential contributing factors that may aid in the mechanism which causes the associations with IgAN and periodontitis.

CHAPTER 2: MATERIALS AND METHODS

2.1: MATERIALS

2.1.1: SAMPLES

2.1.1.1: Blood samples

Blood samples were taken from 25 volunteers of European descent. Copy numbers of the individuals were provided by Paula Necsoiu from her MRes project. Genomic DNA for each individual was provided to be used for genotyping assays.

2.1.1.2: Macrophages

Macrophage samples were taken from 2 individuals and matured from isolated monocytes for 7 days by Sirina Muntaka from the Martinez-Pomares research group, before RNA isolation.

2.1.1.3: Monocytes

Monocyte cDNA samples were provided from previous work on isolated monocytes stimulated with LPS.

2.1.2: GENERAL REAGENTS

2.1.2.1: ACK lysis buffer

1X ACK (ammonium chloride and potassium) lysis buffer was prepared with final concentrations of 155mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM EDTA.

2.1.2.2: 1X PBS

1X PBS (phosphate buffered saline) was prepared using 1X PBS tablet (Oxoid) per 100ml of distilled water.

2.1.2.3: 10X LD PCR Mix

10X LD (low dNTP) PCR mix contained 50mM Tris-HCl, 12.5mM ammonium sulphate, 125ug/mL BSA, 1.4mM Magnesium chloride, 7.5mM 2-mercaptoethanol and 200ul of each dNTP (Khan *et al.* 2013).

2.1.2.4: 10X TBE

10X TBE (Tris-borate-EDTA) buffer contained final concentrations of 900mM Tris base, 890mM boric acid and 27.7mM EDTA.

2.1.2.5: Agarose gel

Agarose gel contained final concentrations of 0.5X TBE (using 10X TBE as above) and 0.5ug/ml ethidium bromide. Agarose was added depending on concentrations required in % (g per 100ml).

2.2: METHODS

2.2.1: Separation of blood cells

2.2.1.1: Histopaque separation

To separate blood samples into cell fractions, 12ml of Histopaque-1119 (density: 1.119g/mL) (Sigma) was gently underlaid under 12ml Histopaque-1077 (density: 1.077g/mL) (Sigma) to create a sharp density gradient. 20 ml of diluted blood (1:1 in 1X PBS) was overlaid onto the gradient and centrifuged at 700 x g for 30 minutes at 23°C resulting in two distinct opaque white blood cell layers separated based on cell density, the upper layer consisting of mononuclear cells and the lower of granulocytes, with erythrocytes in the lowest layer. The separated mononuclear (MN) and granulocyte (GRN) cell fractions were extracted using a widened tip then washed twice in 10mL 1X PBS and centrifuged at 500 x g for 5 minutes to remove excess Histopaque. Blood smears were prepared from separated cells (See Materials and Methods section 2.2.1.2) and RNA was isolated from cell fractions immediately after separation (See Materials and Methods section 2.2.2.1).

2.2.1.2: Staining of blood smears

Thin blood smears were prepared using 10µl of each cell suspension and left to air dry on glass microscope slides. After drying of blood smears, samples were stained by incubating samples in 0.5mL Leishman's staining solution (A4277 AppliChem) for 2 minutes and then an additional 10 minutes after the addition of 1mL distilled water.

Samples were washed in distilled water, air dried and visualised under light microscopy at X100 magnification after mounting with DPX mounting medium (Fisher Scientific).

2.2.1.3: Blood counts

Cell blood counts were done using a Marienfeld Neubauer counting chamber with a depth of 0.100mm. Samples were diluted (1/200) in 1X PBS and cell counts per µl were calculated using the equation:

$$cells\ per\ \mu l = \frac{Total\ cells\ counted^{*}}{Counted\ area^{**}\ x\ Chamber\ depth^{***} x\ Dilution\ factor}$$

* = Total of cells counted in all squares. ** - Area of total squares. Each smaller square has a volume of $0.04mm^2$ and larger squares volume of $1mm^2$.

2.2.1.4: Buffy Coat preparation

Buffy coat samples were prepared by centrifuging 10mL undiluted blood samples at 2000 x g for 10 minutes, resulting in layering of blood cell types by density, the upper layer consisting of human plasma, the centre layer containing all white blood cells (WBC), known as the buffy coat (BC), and the lowest layer erythrocytes. The buffy coat layer was removed and incubated in 1mL of ACK lysis buffer to lyse any remaining erythrocytes. Samples were then spun at 300 x g for 5 minutes and washed in 1X PBS. Thin smears were prepared, and slides left to air dry.

Smears were then used for immunocytochemistry staining (See Materials and Methods section 2.2.6)

2.2.2: Detection of *DEFA1A3* expression

2.2.2.1: RNA isolation

250µL of each cell fraction were incubated with 0.75mL of TRIzol (Invitrogen) and 0.15mL chloroform for 5 minutes at 23°C after emulsification. Samples were then centrifuged at 12,000 x g for 15 minutes, forming three distinct layers. 250µL of the upper clear aqueous layer containing RNA was extracted and mixed with 0.375mL of isopropanol. The remaining two layers were discarded.

The isopropanol suspension was then incubated at 23°C for 10 minutes and then spun at 12,000 x g for 10 minutes, resulting in the formation of a small white pellet containing the RNA. The supernatant was discarded and 0.75ml of 75% ethanol was added, the samples vortexed to resuspend the pellet and spun at 7500 x g for 5 minutes. The supernatant was discarded, and the pellet was left to air dry for 10 minutes. 50µL of distilled water was added to the pellet and RNA samples were heated at 60°C for 10 minutes, put on ice immediately and then stored at -80°C. RNA samples were then used for cDNA synthesis (See Materials and Methods section 2.2.2.2).

2.2.2.2: cDNA synthesis

cDNA synthesis was done via the Tetro cDNA synthesis kit (Bioline) using RNA samples from fractionated blood cells. A master mix was prepared using 1μL Oligo (dT) (used for general PCR) or Random hexamer primers (in the case of qPCR analysis), 4μl 5xRT buffer, 1μL 10mM dNTP mix, 1μL Ribosafe RNAse inhibitor and 1μL of Reverse transcriptase (200u/μl). 10μL total RNA was added and 2μL DEPC-treated water was added to bring the volume to 20 μL. The samples were then heated for 30 minutes at 45°C. Due to the distance of the qPCR probes relative to the poly-A tail, cDNA samples used for qPCR were prepared using random hexamers (1μL) and samples were preheated for 10 minutes at 25°C before being incubated at 45°C for 30 minutes. The reaction was terminated by incubating at 85°C for 5 minutes. The samples were either stored at -20°C or used immediately for PCR (See Materials and Methods section 2.2.2.3) or qPCR (See Materials and Methods section 2.2.5).

2.2.2.3: *DEFA1A3* PCR

For detection of expression of *DEFA1A3* in cellular fractions, a master mix of PCR was made containing 1x LD, 0.5μM each of primers DefRes4F (ATCCTTGCTGCCATTCTCC) and DefRes4R (TCCCTGTAGCTCTCAAAGCA), 0.5U Taq DNA polymerase and made up to 19μL for each sample. 1μL of cDNA was then added to make a

volume of 20µL and run under PCR conditions of 35 cycles of 30s at 95°C, 30s at 60°C and 2 minutes at 70°C. Samples were then run alongside a 100bp DNA ladder on a 1.5% agarose gel (w/v) for 1.5 hours at 110V and visualised under UV. Genomic DNA was used as control, giving a fragment of 898/902bp. cDNA PCR gives a predicted product of 322bp. To determine viability and condition of cDNA, primers DefRes4F and 4R were replaced with primers coding for housekeeping gene *SDHA*, *SDHA*F (GCAACAGAAGAAGCCCTTTG) and *SDHA*R (CAGCCACTAGGTGCCAATCT) and run under the same conditions. *SDHA* cDNA PCR products gave a fragment of 306bp.

2.2.3: Expression of *DEFA3*

5µl of *DEFA1A3* PCR product was digested in 10µl containing 1U *Hae*III at 37°C overnight. Digest products were then run on a 1.5% gel (w/v) for 1.5 hours at 110V and visualised under UV. Undigested product gave a band at 322bp with a constant *Hae*III site producing fragments of 288bp and 34bp. The *DEFA1* gene has a site for *Hae*III that cleaved the 288bp product into two lots of 144bp, a site that is not present in the *DEFA3* gene.

2.2.4: Haplotype identification

Haplotype identification was done on 25 individuals of known copy number using the SNP data generated as described in sections 2.2.4.1-2.2.4.3. Copy numbers were determined by Paula Necsoiu during her MRes project and used in this project for genotypic comparisons.

2.2.4.1: Reference proxy SNP rs4300027 [ref Khan *et al.* 2013] and Class 1 proxy SNP rs7826487 [ref Black *et al.* 2014] assays

To make an alternative, extended PCR product that included the flanking region, a master mix was made using 1μM of primers TagSNPsF (AAGCTGTCTGTGTGTCTGGT) and RepSpec2RB (GAACCCCCGTCACCCATT), 1x LD mix, 1U of Taq polymerase, made up to 19μl with H_2O. 1μL of input DNA was added and cycled with conditions of 37 cycles of 95°C 30 sec/ 57°C 30 sec/ 70°C 3 minutes. To confirm a successful PCR, 3μl of the PCR product was run on a 3% agarose gel alongside a 100bp DNA marker for 2 hours at 110V and visualised using a UV illuminator to reveal a band at 1409bp (hg19 chr:6879639-6881047, figures 2.1 and 2.2).

For the assays used to detect SNPs rs4300027 and rs7825750, 5μl of the TagSNP-RepSpec PCR products were digested with 2.5U *Hin*fI and *Ssp*I respectively. Digestions were run overnight at 37°C and digestion products run on 2% agarose gels alongside 100bp DNA marker for 2 hours at 110v and visualised using a UV illuminator.

AAGCTGTCTGTGTGTCTGGTgttgaggattgcagcaggtttat
tttacaataaaccataaggaaatagcttccttgagctgtttta
aaatatgttccctaaattcttttattatttttatagaaagaa
aggtttctgttctctcctgttaggtctaggatgggtaacattt
tgaccaacagactacatggaactagatgccatgctgttttctg
ggatcaggcgctagtgaactggtgtcttttctatctctcttt
ggatggtgctgtggtctgagacattttcctctgaaattcata
tgttgaagtcttagaccctaggatgatagcattaaaagaaagg
gtctttttggaaatgaggaggtcagcatggtggagtcctcatg
aatggaatgagtgcccctgtagaagaggtcccagagagctcct
tcattccttccaccagatgaagacacaataagaagatgttgta
tatgaaccaaaaagctggttctcaccagacactgattctgctg
ccaccttgatttggacttcttagcctccacagggattccgaa
tggtggcagaagttagggtgtggccatgcggtggaggttgga
acaaagaagatgaagggtcactgttgtcccggaggacagagcg
ctgagaggtagtgagggtgaccttccaggcaatattagagtaa
cttagttcacgacattttgaatttttttagcagtccacatgt
gcttagagggctggtgcctttggagcccagataccatgcttgg
aggaagactaagcatcccacagggagaggaactgagcccacct
gcaaggatgggctagagaacactgagcaaccagctttctagga
aaaaagaaaactctgatttgcaatgtttgtaaatttctgtggt
taaaatgctcccagctatagacagtttaagaattatcacacaa
aaactcctccctcatgagctggcctgatctgaccccagcacat
cacagggtctcatccttcagctttctcagagtttccagctgag
ccaacaccacctgccacctgtgcacgagtgtcctggccctgaa
attttcagatctcagcagaacctctcctcttatgcccgtggaa
ggatccaaaccccaattgcaaatgtgtgagtgaagacgtgatc
atgctgtttcaatccactactttctgtggtgtcttttcgcaca
gtcctagatgaacagaaggcacggtcttggtgagaagttgaat
gtgtgcatttttttgtgtgtgtaaatctcagcctctctataata
ttgttgaagtaggacagaaccctctcaccttatttccaaagtg
tcacaaagagcccatcttaatggcagcgtggaattgtggactc
tttggagtgactgaaGAACCCCCGTCACCCATT

Figure 2.1. Sequence of 1409bp product from rs4300027 PCR with *Hinf*I restriction sites (GANTC) underlined. rs4300027 is highlighted in yellow, showing the variable SNP site (c/t).

AAGCTGTCTGTGTGTCTGGTgttgaggattgcagcaggtttat
tttacaataaaccataaggaaatagcttccttgagctgtttta
aaatatgttccctaaattcttttattatttttttatagaaagaa
aggtttctgttctctcctgttaggtctaggatgggtaacattt
tgaccaacagactacatggaactagatgccatgctgttttctg
ggatcaggcgctagtgaactggtgtctttttctatctctcttt
ggatggtgctgtggtctgagacattttcctctgaaattcata
tgttgaagtcttagaccctaggatgatagcattaaaagaaagg
gtctttttggaaatgaggaggtcagcatggtggagtcctcatg
aatggaatgagtgcccctgtagaagaggtcccagagagctcct
tcattccttccaccagatgaagacacaataagaagatgttgta
tatgaaccaaaaagctggttctcaccagacactgattctgctg
ccaccttgatttggacttcttagcctccacaggggattccgaa
tggtggcagaagttagggtgtggccatgcggtggagggttgga
acaaagaagatgaagggtcactgttgtcccggaggacagagcg
ctgagaggtagtgagggtgaccttccaggcaatattagagtaa
cttagttcacgacattttgaatttttttagcagtccacatgt
gcttagagggctggtgcctttggagcccagataccatgcttgg
aggaagactaagcatcccacagggagaggaactgagcccacct
gcaaggatgggctagagaacactgagcaaccagctttctagga
aaaaagaaaactctgatttgcaatgtttgtaaatttctgtggt
taaaatgctcccagctatagacagtttaagaattatcacacaa
aaactcctccctcatgagctggcctgatctgaccccagcacat
cacagggtctcatccttcagctttctcagagtttccagctgag
ccaacaccacctgccacctgtgcacgagtgtcctggccctgaa
attttcagatctcagcagaacctctcctcttatgcccgtggaa
ggatccaaaccccaattgcaaatgtgtgagtgaagacgtgatc
atgctgtttcaatccactactttctgtggtgtcttttcgcaca
gtcctagatgaacagaaggcacggtcttggtgagaagttgaat
gtgtgcatttttttgtgtgtgtaaatctcagcctctctataata
ttgttgaagtaggacagaaccctctcaccttatttccaaagtg
tcacaaagagcccatcttaatggcagcgtggaattgtggactc
tttggagtgactgaaGAACCCCCGTCACCCATT

Figure 2.2. Sequence of 1409bp product from rs7826487 (chr8: 6880925) PCR with *Ssp*I restriction sites (AATATT) underlined. rs7826487 is highlighted in yellow, showing the variable SNP site (a/g).

2.2.4.2: Class 2 proxy SNP rs7825750 assay

Using 0.5µl of the PCR products from the TagSNP-RepSpec PCR, a secondary PCR was run using 1 µM of primers TagSNPs-F (AAGCTGTCTGTGTGTCTGGT) and C2FnuR (CATGTAGTCTGTTGGTCAAAATGCT) , 1U of Taq polymerase and 1x LD. A PCR with conditions 12 cycles of 95°C 30sec/55°C 30sec/ 70°C 30 seconds were run. 5µl of the products were then digested in 1U *Fnu*4HI and digested at 37°C overnight. Digested products were then run on 2% agarose gel (w/v) for 2 hours at 100V.

```
AAGCTGTCTGTGTGTCTGGTgttgaggattgcagcaggtttat
tttacaataaaccataaggaaatagcttccttgagctgtttta
aaatatgttccctaaattcttttattatttttatagaaagaa
aggtttctgttctctcctgttaggtctaggatgggtAGCATTT
TGACCAACAGACTACATG
```

Figure 2.3. (hg19 chr8:6879639-6879828) Sequence of 190bp product from rs7825750 (Chr8: 6879803) PCR with *Fnu*4HI restriction site (GCAGC) underlined. rs7825750 is highlighted in yellow, showing the variable SNP site (c/t) while the pink-highlighted nucleotide is the nucleotide introduced to engineer a *Fnu*4HI site in the presence of the c allele. Primer binding sites are indicated in uppercase

2.2.4.3: Secondary PCR for Exchange 1 proxy SNP rs62487514 assay [ref Khan *et al.* 2013]

A 10µl secondary PCR was done using 0.5 µl of the TagSNP-RepSpec PCR products, 1 µM of primers Exch1F (ATCTCTCTTTGGATGGTG) and Exch1R (GGTTCATATACAACATCTTCTTAAT) 1U of Taq polymerase and 1x LD. A PCR was run for 12 cycles of 95°C 30s/50°C 30s/70°C 30s. Digestions of 5µl of the PCR products with 1U of *Tsp*509I were incubated overnight at 65°C and run on a 3% agarose gel for 2 hours at 110V and illuminated using UV.

```
ATCTCTCTTTGGATGGTGctgtggtctgagacattttcctct
gaaattcatatgttgaagtcttagaccctaggatgatagcatt
aaaagaaagggtctttttggaaatgaggaggtcagcatggtgg
agtcctcatgaatggaatgagtgcccctgtagaagaggtccca
gagagctccttcattccttccaccagatgaagacacATTAAGA
AGATGTTGTATATGAACC
```

Figure 2.4. (hg19 chr8:6879887-6880119) Sequence of 233bp product from rs62487514 (chr8:6880094) PCR with *Tsp*509I restriction site (AATT) underlined. rs62487514 (C/A) is highlighted in yellow, showing the variable SNP site while the nucleotide highlighted in pink is an introduced nucleotide to create a *Tsp*509I site if the variable site is A. Primer sites are indicated in uppercase.

2.2.5: Real time PCR

Using the random primed cDNA, multiplexed qPCR master mixes were made containing 1µL of the TaqMan expression assay probes for *DEFA3* (detecting both *DEFA1* and *DEFA3*) and *SDHA* (Applied Biosystem 7300 TaqMan gene expression assays, ID numbers; *DEFA*: Hs0041408_m1, *SDHA*: Hs00188166_m1) with 10ul of 2X TaqMan gene expression assay master mix, 1ul of cDNA and 7ul of H_2O. Reactions were prepared in triplicate for each sample of cDNA from granulocytes, mononuclear cells and whole blood. Standards were made using an initial concentration of 20ng/ul and a series of serial dilutions of 1:2 to produce standards of concentrations 20ng, 10ng, 5ng, 2.5ng and 1.25 ng per µl. Samples were run on an Applied Biosystems 7500 Fast thermal cycler under standard conditions: 50°C 2mins/95°C 10min then 40 cycles of 95°C 15s/60°C 1min. Fluorescence was detected using the standard *7500 Fast* software to determine Ct. To determine efficiency of PCR, Ct values for standards were put on a log graph and %Eff was calculated. Statistical analysis of qPCR data was done using Pearson's correlation coefficient to determine correlation between expression and copy number. Significance level was set at <0.05.

2.2.6: Immunocytochemistry

Blood smears prepared using buffy coat were fixed in 4% paraformaldehyde in PBS. To reduce background staining, slides were then incubated for 10 minutes in 3% H_2O_2 in 70% methanol to remove any endogenous peroxidase activity. Cells were then permeabilised using 0.5% saponin in 1XPBS for 20 minutes. To maintain pores in cell membranes created by saponin, subsequent reagents contained 0.1% saponin. To block any non-specific Fc receptor binding of primary or secondary antibodies, cells were blocked in 10% human plasma in 1X PBS for 20 minutes. Cells were incubated for an hour with 1:100 mouse primary mAb anti-human human neutrophil peptide 1-3 (Clone D21, Hycult Biosciences) and were washed three times in 0.1% saponin in 1X PBS, before being incubated for an hour with 1:200 peroxidase conjugated secondary anti-mouse Ab (A4416 Sigma). Once washed three times, cells were stained using DAB substrate, leaving a dark brown stain where HRP is active, and therefore presumably alpha defensin is present. To identify cell types based in morphology, slides were counter stained using haematoxylin counter stain at a dilution of 1 in 10 to stain nuclei blue.

CHAPTER 3: RESULTS

3.1: Genotyping

To relate the expression of alpha defensins in white blood cells to copy number and flanking SNP variants, flanking SNP genotypes were determined in 25 individuals of known copy number (See Materials and Methods section 2.2.4). Though copy number reveals a certain level of variability between individuals, flanking SNP haplotype classes reveal more about underlying similarities in ancestry and inheritance. Haplotypes with similar copy number show less similarity regarding ancestry, than those with similarities in haplotype structure and other features of the locus, such as the presence or absence of *DEFA3*. The assignment of genotypes was done using the experimental approaches developed in Black *et al.*. 2014, to type four SNPs present in the centromeric flanking region of the locus (hg chr:6879639-6881047) that tag the five main haplotype classes.

Haplotype class	SNP			
	rs4300027	rs7826487	rs7825750	rs62487514
Reference	T	A	T	C
Class 1	C	G	T	C
Class 2	C	A	C	C
Exchange 1	T	A	T	A
Exchange 2	C	A	T	C

Table 3.1. SNP haplotype classes. Alleles of SNPs tagging each of the five haplotype classes determined in Black *et al.*. 2014.

To assign the genotypes to the individuals, the alleles of the four flanking SNPs of the *DEFA1A3* locus were determined (Table 3.1), enabling the assignment of haplotype classes to the individuals (See Materials and Methods section 2.2.4). The four flanking SNPs rs4300027, rs7826487, rs7825750 and rs62487514 were genotyped using restriction enzymes *Hin*fI, *Ssp*I, *Fnu*4HI and *Tsp*509I, respectively (See Materials and Methods section 2.2.4). To do this, the centromeric flanking region of the locus was initially amplified using primers TagSNPsF and RepSpec2RB, producing a fragment of 1409bp at the centromeric end of the locus, and the resulting product was then digested directly using *Hin*fI and *Ssp*I (See Material and Methods section 2.2.4.1). A secondary PCR was digested with *Fnu*4HI and *Tsp*509I (See Materials and Methods, sections 2.2.4.2 and 2.2.4.3). The resulting banding patterns demonstrated the genotype of the individual (Table 3.1).

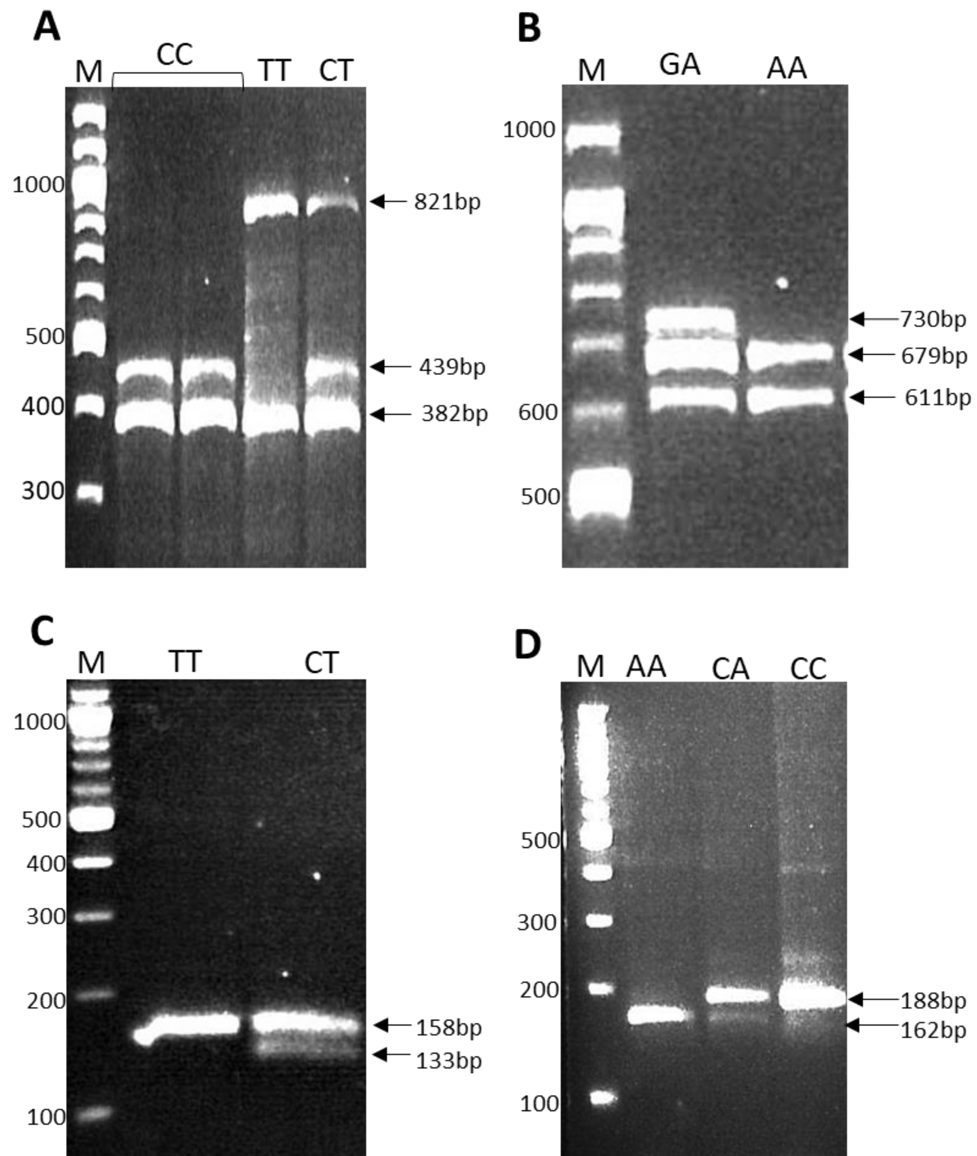


Figure 3.1. Restriction enzyme assay digest products for genotyping SNPs rs4300027 (A), rs7826487 (B), rs7825750 (C) and rs62487514 (D). All digestion products were run with a 100bp ladder DNA marker (Indicated as lane labelled M) at 110V for 2 hours on variable percentage agarose and illuminated using UV; A. Samples from TagSNP-RepSpec PCR were digested with *Hin*fI overnight at 37°C and then run on a 2% agarose gel (w/v) resulting in CC (Lanes

1 and 2) and TT homozygotes (Lane 3), and CT heterozygotes (Lane 4). B. Samples were digested overnight at 37°C with *Ssp*I and run on a 2% agarose gel (w/v) resulting in GA heterozygotes (Lane 1) or AA homozygotes (Lane 2). No individuals sampled displayed as GG homozygotes. C. Samples from TagSNPsF-C2FnuR PCR were incubated with restriction enzyme *Fnu*4HI overnight at 37°C. Digest products were then run on a 2% agarose gel (w/v) resulting in either TT homozygotes (Lane 1) or CT heterozygotes (lane 2) with no CC homozygotes. D. Samples from Exch1F-Exch1R PCR were incubated with enzyme Tsp509I overnight at 65°C and digested products run on a 3% agarose (w/v), resulting in AA homozygotes (Lane 1), CA heterozygotes (lane 2) and CC homozygotes (lane 3).

The 1409bp TagSNPsF/RepSpec2RB fragment produced in the initial PCR was digested using *Hin*fI which had five restriction sites (5'-GANTC-3'), one of which contained the variable SNP rs4300027 (Figure 2.1 in Methods section 2.2.4.1). When rs4300027 is a T allele, however, this recognition site is lost, therefore turning 5'-GAATC-3' into 5'-GAATT-3', creating an uncut product with a length of 821bp (Figure 3.1A) (See Methods and Materials section 2.2.4.1).

To determine the individuals with a Class 1 (C1) haplotype, the same 1409bp fragment from the initial PCR was digested with restriction enzyme *Ssp*I. In the 1409bp length fragment, there was a constant restriction site present in all alleles, creating fragments of 730bp and

679bp; A alleles have an additional site for this enzyme 5'-AATAAT-3' which cuts the 730bp fragment into 630bp and 119bp (Figure 2.1 Methods Section 2.2.4), but which is lost in the presence of the G allele (GATAAT) that tags the C1 haplotype. In the presence of a G allele, therefore, the resulting restriction products are 730bp + 679bp, but in the presence of the A allele the lengths are 679bp, 611bp and 119bp (Figure 3.1B). From the 25 individuals sampled, only two outcomes were apparent, GA heterozygotes or AA homozygotes (Figure 3.1B), with no C1 homozygotes (GG).

To determine the SNP alleles of Class 2 (C2) and Exchange 1 (E1) classes, two secondary PCR products were produced using mismatched primers. The first of these, used to determine the allele of SNP rs7825750 that tags C2 haplotypes, produced a product of 190bp. The mismatched primers replaced the A nucleotide at the 3' end of the primer binding region with a G nucleotide, therefore producing the *Fnu*4HI site 5'-GCNGC-3' when in the presence of the C allele at this SNP, which is lost in the presence of the T allele (See Materials and Methods section 2.2.4.2.). The separation of the products from this digestion produced fragments of uncut 158bp or cut 133bp (Figure 3.1C), of which the 25 samples presented as either TT homozygotes or CT heterozygotes (Figure 3.1C). The same approach was used to determine the final SNP rs62487514 using primers Exch1F and Exch2R. These primers replaced a nucleotide with an A at the 3' end of the PCR primer, creating the restriction site 5'-AATT-3' for *Tsp*509I in the presence of the A allele (See Materials and Methods Section 2.2.4.3). The resulting fragments were

either uncut 188bp or a cut product of 162bp (Figure 3.1D), indicating either AA homozygotes, CA heterozygotes or CC homozygotes.

A similar approach was applied to determine *DEFA3* expression of the 25 samples using PCR of cDNA and restriction enzyme *Hae*III which produced restriction fragments depending on the presence or absence of a specific recognition site (See Materials and Methods Section 2.2.3).

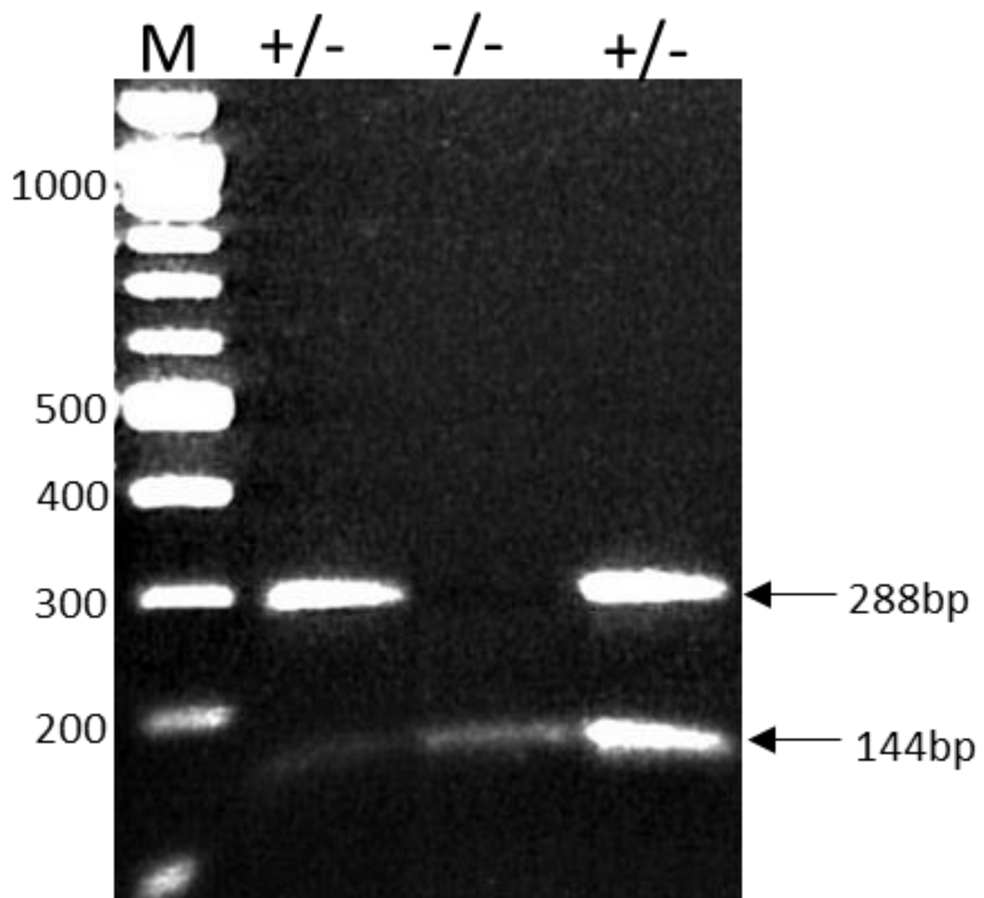


Figure 3.2. Restriction enzyme assay distinguishing *DEFA1* from *DEFA3* transcripts in cDNA. Agarose gel showing outcomes of *Hae*III restriction enzyme digest for the presence or absence of *DEFA3* expression. Samples from DEFRes4F-DEFRes4R cDNA PCR

were digested overnight at 37°C with *Hae*III and run on a 1.5% agarose gel (w/v) for 1.5 hours at 110V and visualised in UV. Shown are the two outcomes from the 25 samples. Individuals displaying a positive result for *DEFA3* and *DEFA1* (+/-) presented with 2 bands at 288bp and 144bp (Lanes 1 and 3). Individuals that did not express *DEFA3* displayed one band at 144bp (Lane 2).

Using the PCR products from the DEFRes4F/R primer pair, the 322bp length product was cut at the constant *Hae*III site to produce fragments of 288bp and 34bp. The larger fragment was cut in the presence of the variable *DEFA1*/*DEFA3 Hae*III site, producing a fragment length of 144bp in the presence of *DEFA1* and an uncut fragment in the presence of *DEFA3* (Figure 3.2). To assign the haplotypes of the 25 individuals, the resulting SNP allele results were collated, from which each individual's haplotype composition could be determined; previous experience in European samples shows that nearly all observed combinations of SNP genotypes can be simply explained as some combination of the five haplotypes shown in Table 3.1. Table 3.2 shows the deduced haplotypes along with the results of the *DEFA3* assay and known copy numbers.

Sample	SNP				Haplotye	Haplotype	Genotype	DEFA1A3	Copy number*
	rs4300027	rs7826487	rs7825750	rs62487514					
AN18001	CC	GA	TT	CC	CGTC	CATC	C1/E2	DEFA1	5
AN18002	CC	AA	CT	CC	CACC	CATC	C2/E2	DEFA1A3	8
AN18003	TT	AA	TT	CC	TATC	TATC	REF	DEFA1A3	10
AN18004	CT	GA	TT	CC	CGTC	TATC	C1/REF	DEFA1A3	7
AN18005	CC	GA	CT	CC	CGTC	CACC	C1/C2	DEFA1A3	6
AN18006	TT	AA	TT	AA	TATA	TATA	E1	DEFA1	6
AN18007	CT	GA	TT	CC	TATC	CGTC	REF/C1	DEFA1	8
AN18008	CT	AA	TT	CC	CATC	TATC	E2/REF	DEFA1A3	6
AN18009	CT	GA	TT	CC	CGTC	TATC	C1/REF	DEFA1	7
AN18010	CC	GA	CT	CC	CGTC	CACC	C1/C2	DEFA1A3	5
AN18011	CT	GA	TT	CC	CGTC	TATC	C1/REF	DEFA1	6
AN18012	TT	AA	TT	CC	TATC	TATC	REF	DEFA1A3	9
AN18013	CC	AA	CT	CC	CATC	CACC	E2/C2	DEFA1A3	4
AN18014	CT	AA	CT	CC	TATC	CACC	REF/C2	DEFA1	7
AN18015	TT	AA	TT	CC	TATC	TATC	REF	DEFA1A3	8
AN18016	CC	GA	CT	CC	CGTC	CACC	C1/C2	DEFA1	6
AN18017	CT	AA	CT	CC	TATC	CACC	REF/C2	DEFA1A3	11
AN18018	CT	AA	CT	CC	TATC	CACC	REF/C2	DEFA1	7
AN18019	CT	GA	TT	CC	CGTC	TATC	C1/REF	DEFA1A3	7
AN18020	CT	AA	CT	CC	TATC	CACC	REF/C2	DEFA1A3	5
AN18021	CC	GA	TT	CC	CGTC	CATC	C1/E2	DEFA1	**
AN18022	CT	AA	TT	CC	TATC	CATC	REF/E2	DEFA1A3	6
AN18023	CC	AA	TT	CC	CATC	CATC	E2	DEFA1A3	6
AN18024	CT	AA	TT	CC	TATC	CATC	REF/E2	DEFA1A3	7
AN18025	TT	AA	TT	CC	TATC	TATC	REF	DEFA1A3	9

Table 3.2. Table displaying the outcomes of the four combined SNP assays and the assigned haplotypes for the 25 individuals. Also displayed are the *DEFA3* presence/absence outcomes ('*DEFA1*' indicating *DEFA1* only and '*DEFA1A3*' indicating both *DEFA1* and *DEFA3*) and the known copy number of the individuals provided by Paula Necsoiu. Highlighted in grey are the samples not chosen for further analysis. **Copy number for sample not available.

The resulting haplotype classes show that the most common of classes in the 25 individuals was the reference haplotype with a frequency of 0.4 (Table 3.3). Copy numbers for the 25 individuals investigated ranged from 4 copies to 11 copies, with one individual in

either extreme (Table 3.3.). This agrees with the observed frequencies of the haplotypes in the European population with the reference haplotype being the most common (Black *et al..* 2014). However, the frequencies of the remaining classes are less obviously in keeping with the observed population frequencies in Black *et al.*., but some deviation is to be expected due to the sample size.

Haplotype	Number of haplotypes in 25 individuals	Frequency	Frequency of haplotypes taken from *Black et al 2014*
REF	20	0.4	0.36
C1	10	0.2	0.12
C2	9	0.18	0.32
E1	2	0.04	0.11
E2	9	0.18	0.08

Table 3.3. Table showing the relative frequencies of the haplotype classes in the 25 volunteer samples. Homozygotes were counted as two separate occurrences so that total number of haplotypes =2n (n being the number of individuals).

The opposite is true for the frequency of those not expressing *DEFA3* with a frequency of 36% (9 samples out of 25. Table 3.3) that, when compared to the previously observed 10% in the European population (Aldred *et al.*. 2005), shows a considerable difference in frequency. Again, this difference is not unexpected due to the small sample size.

3.2: Qualitative expression of alpha defensin genes in white blood cells

Expression of alpha defensins in neutrophils is known and well characterised (Faurschou and Borregaard *et al..* 2003). Alpha defensins are expressed in neutrophils in abundance, making up to 50% of the azurophil granules. However, investigations into alpha defensin expression in other WBCs have had variable results with some papers finding expression in monocytes but not T cells (Mackewicz *et al..* 2003) and NK cells (Obata-Onai *et al..* 2002). Understanding of the mechanism by which the *DEFA1A3* locus, and therefore alpha defensins, are associated with diseases IgAN and periodontitis has not been fully established. This be may related to the fact that the majority of investigations have been focused on neutrophil action, a cell that is important in the origin of periodontitis but not thought to be associated with aetiology of IgA nephropathy. Should expression of alpha defensin be found to be abundant or variable in cells other than neutrophils, alternative disease mechanisms could be indicated. To do this, experimental investigation in this project was originally focused on looking at the expression of alpha defensins in WBC types, initially separating granulocytes from mononuclear cells. Once the genotypes for the selected 25 individuals had been determined, expression of alpha defensin in white blood cells of a selected subset of 10 samples was examined through reverse-transcriptase PCR.

3.2.1: Separation into cell fractions

To compare expression of alpha defensin of the cell types, whole blood was separated using a density gradient into two fractions of granulocytes (GRN) and mononuclear cells (MN), the first of which contains neutrophils, eosinophils and basophils, with some contaminating red blood cells (See Materials and Methods section 2.2.1.). The mononuclear cell fraction contained monocytes and lymphocytes, the latter being in abundance due to naturally occurring cell counts in human blood, as well as platelets.

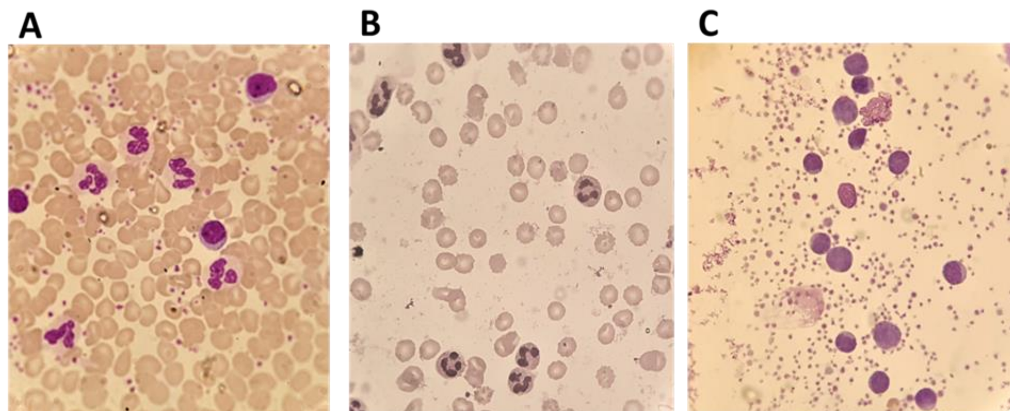


Figure 3.3. Sections of microscope slides of cell preparations; Whole blood smear (A), Granulocyte cell fraction (with red cells) (B) and Mononuclear cell fraction (with platelets) (C). Slides were stained using Leishman's stain and visualised at x1000 magnification. Microscope slide pictures presented are representative of the 25 samples investigated.

Blood smears were prepared for whole blood (WB, figure 3.3A), GRN (Figure 3.3B) and MN (Figure 3.3C) and stained using Leishman's stain (See Materials and Methods Section 2.2.1.2.) and visualised under light microscopy. Generally, the morphology of cells was unchanged in the whole blood compared to the separated cells of all samples investigated (3.3B and 3.3C compared to 3.3A). The granulocyte fraction maintained cell morphology and had an abundance of erythrocytes in the cell fraction (Figure 3.3B) though this was not of any concern due to the absence of RNA in erythrocytes. Similarly, the MN cell fraction also contained large amounts of platelets, but morphology was intact (Figure 3.3.C). To determine if cell fractions were contaminated, differential cell counts were done on separated fractions.

Sample	GRN count in MN cell fraction (%)			
	C1	C2	C3	Avg
AN18001	1	2	0	1.00
AN18002	1	1	1	1.00
AN18003	7	0	3	3.33
AN18005	1	2	1	1.33
AN18006	0	0	0	0.00
AN18007	0	0	1	0.33
AN18008	0	1	5	2.00
AN18010	2	1	1	1.33
AN18012	0	0	0	0.00
AN18013	1	2	2	1.67
AN18014	4	1	2	2.33
AN18015	0	0	5	1.67
AN18016	0	1	2	1.00
AN18018	1	3	3	2.33
AN18019	1	1	0	0.67
AN18020	0	1	1	0.67
AN18021	0	1	0	0.33
AN18022	3	1	3	2.33
AN18024	1	1	1	1.00
AN18025	1	3	1	1.67

Table 3.4 Table displaying the contamination counts of granulocytes in mononuclear cell fraction of the 20 samples investigated. Counts were done in triplicate and averages taken, displaying contaminations of <4% in all samples.

Any contaminating cells were counted per 100 cells on the slide in triplicates and a percentage was calculated, showing an average contamination levels of 1.3% (95% CI 0.94 to 1.66).

3.2.2: Expression of alpha defensin using *DEFA* PCR

To examine the expression of alpha defensins in the WBC fractions qualitatively, synthesised cDNA was used as input to a PCR with *DEFA* primers spanning exons 2 and 3 of the locus, producing a product of 322bp and was run on an agarose gel (See Materials and Methods section 2.2.2.). To confirm results of *DEFA* PCR, a pair of primers was also used for the reference gene *SDHA*, which is known to be expressed in abundance in several cell types, including white blood cells (Ledderose *et al.*. 2011).

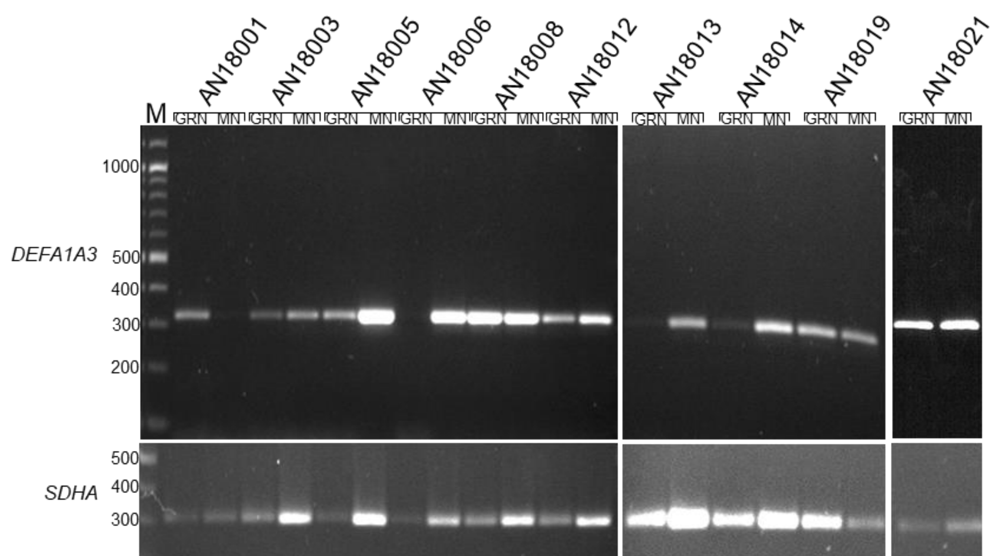


Figure 3.4. Agarose gels showing PCR products from *DEFA1A3* PCR (predicted product size 322bp) and housekeeping gene *SDHA* (predicted product 306bp) PCR of 10 samples using separated granulocytes (GRN) and mononuclear cells (MN). Expression level of *DEFA* PCR is of the same order as *SDHA* expression, showing

expression in all samples, although faint in some samples (AN18001 Mononuclear and AN18006 Granulocytes).

Alpha defensins appeared to show expression in both cell fractions (Figure 3.4). At first glance, there appears to be variability of expression among different cell types between individuals. However, the intensity of the bands, once evaluated relative to the intensity of bands in the *SDHA* PCR, shows approximately proportionate expression, likely pertaining to variability in RNA concentrations and quality (Figure 3.4). All individuals appear to show at least some expression of alpha defensin mRNA in both cell type fractions, however faint. This unexpected expression of alpha defensin in mononuclear cells formed the basis of further investigation.

3.2.3: Expression in macrophages and monocytes

Once expression was found in both the mononuclear cells and granulocytes of the 10 individuals, the expression of alpha defensin in cell subtypes from the mononuclear cell fraction was investigated to determine which cells are expressing alpha defensin. To do this, qualitative PCR was undertaken using the *DEFA* primers in exons 2 and 3. This was done on 4 macrophage samples and 30 monocyte samples independent of the 25 samples investigated. This was run alongside a *SDHA* PCR as a control and reference PCR (See Materials and Methods section 2.2.).

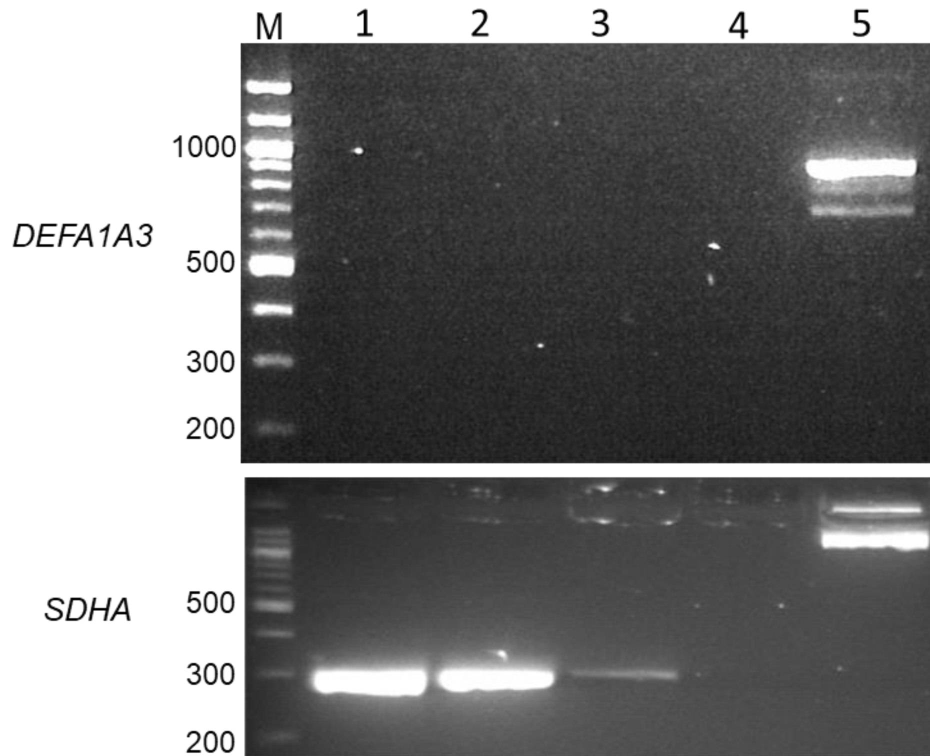


Figure 3.5. Qualitative analysis of *DEFA1A3* expression in cDNA from purified macrophages derived from peripheral blood monocytes. Agarose gel of PCR product from *DEFA1A3* PCR and reference gene *SDHA* PCR of 4 macrophage samples (Lanes 1- 4). Lane 5 is positive PCR control using human genomic DNA, from which an 898/902bp product is predicted, contrasting with the 322bp product expected from cDNA. PCR products were run on a 1.5% gel (w/v) for 1.5 hours at 110V and show positive results for reference gene *SDHA* for samples 1-3 with no *DEFA1A3* expression in all cells.

Of the four macrophage samples, 3 showed successful RT-PCR, indicated by the presence of a band on the *SDHA* gel (Figure 3.5). The negative result in lane four can be assumed to be due to unsuccessful

PCR from the absence of enough or reasonable quality RNA. However, for all the remaining samples in lanes one to three (Fig 3.5) there appeared to be no *DEFA* expression in the macrophage samples despite clear expression of *SDHA*.

To look at other cell types within the mononuclear cell fraction, 30 archived cDNA samples from a previous project on isolated and LPS-stimulated peripheral blood monocytes were analysed with the same *DEFA* primers in exons 2 and 3.

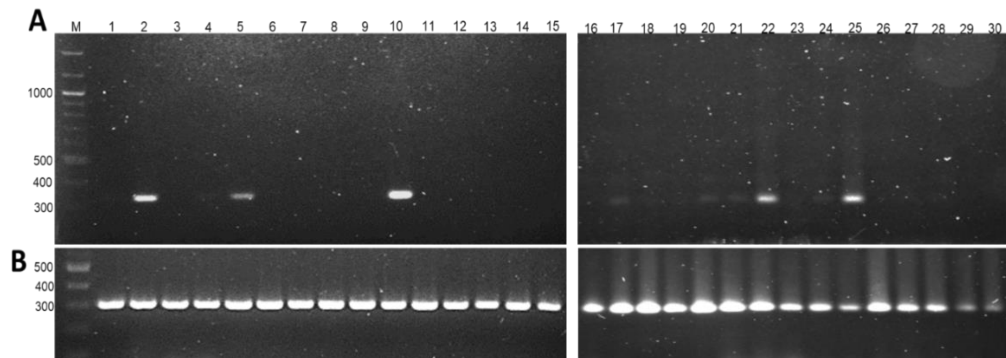


Figure 3.6. PCR of cDNA from stimulated monocytes. Agarose gel of PCR products from *DEFA1A3* PCR (A) and *SDHA* PCR (B) of 30 cDNA samples from monocytes. PCR products were run on 1.5% agarose gel (w/v) for 1.5 hours at 110V. All samples show successful PCR with *SDHA* expression. 5 out of 30 samples (Lanes 2, 5, 10, 22 and 25) show clearly detectable levels of expression of *DEFA1A3*. Some samples (Lanes 16, 20, 21 and 24) show very faint expression of *DEFA1A3*.

Of the 30 samples, all proved to have relatively good quality cDNA with successful PCR outcomes from the *SDHA* products (Fig 3.6B). Of the 30 samples, 5 samples (Lanes 2, 5, 10, 22 and 25 in Fig 12A) showed clearly detectable *DEFA* expression. Some samples showed very faint bands (Lanes 17, 20, 21 and 24 Fig 3.6A). However, this variability in expression could not be related to genotypes as this was unknown for the 30 individuals investigated but we can confirm that appears to be variability in the expression of alpha defensin in stimulated monocytes.

3.3: Quantitative expression of alpha defensin

Detection of alpha defensin expression using conventional PCR methods was able to reveal if RNA was present or absent in the cell fractions. However, this did not reveal the relative expression of alpha defensin between the two cell types or the quantitative expression. To determine the relative expression of alpha defensin in granulocytes and mononuclear cells, real time PCR (or qPCR) was done using a TaqMan-based real-time PCR assay for *DEFA* and *SDHA* in 20 of the 25 volunteer samples. qPCR enabled the determining number of cycles required for fluorescence (inversely correlated with the number of copies of that gene in the PCR) to cross the detection threshold (known as Ct values). Once given Ct values, comparisons were made relative to *SDHA* values of the same sample. Delta Ct was calculated for mononuclear cells and granulocytes to determine the number of cycles required for *DEFA1A3* to cross the threshold compared to *SDHA*.

3.3.1: Expression of alpha defensin in granulocytes compared to mononuclear cells

The initial use of the quantitative PCR (delta Ct) data was to determine the difference between the relative expression of alpha defensin mRNA in mononuclear cells and granulocytes. The general PCR data showed that expression was present in both cell types, but the amount of expression was variable between the cell types in each individual (See Materials and Methods section 2.2.5). However, the relative amounts of expression between the individuals were not comparable. Therefore, using ΔCt values, whereby the Ct values of the *DEFA* expression in each sample was compared to the *SDHA* expression in the same cell type, we compared the relative expression of *DEFA* in GRN compared to MN cells in 19 individuals. To determine if there was a batch effect, an independent samples t-test was conducted to compare the means of $2^{\Delta ct}$ of GRN and MN in the two batches. There was not a significant difference in the GRN means for batch 1 and batch 2 (student t-test, $P>0.05$). The opposite was true for the MN means for the two batches, which were significantly different and showed distinct batch effect ($P<0.05$). Therefore, for qPCR analysis, GRN data could be collated and compared, while MN data was analysed in two separate batches.

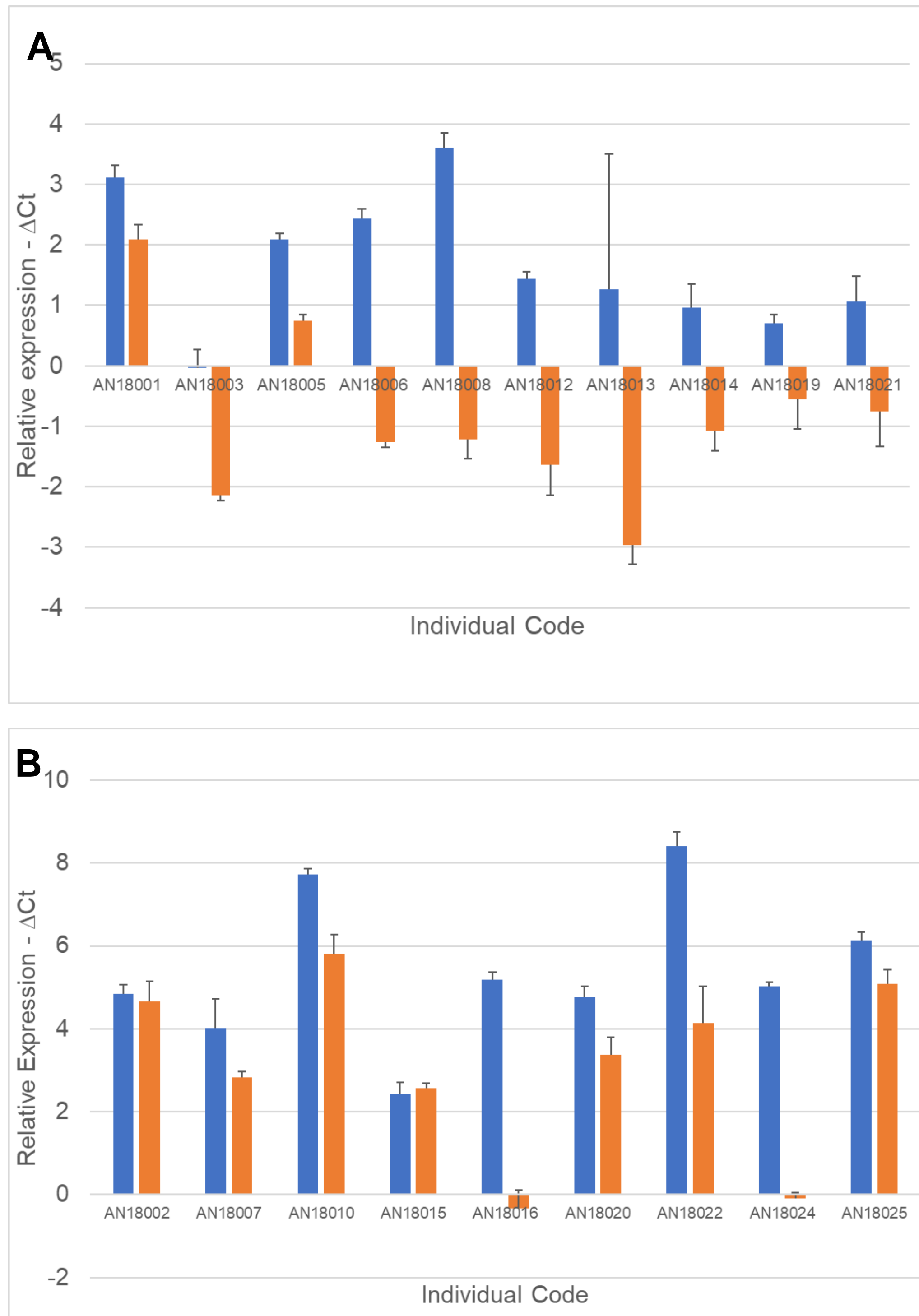


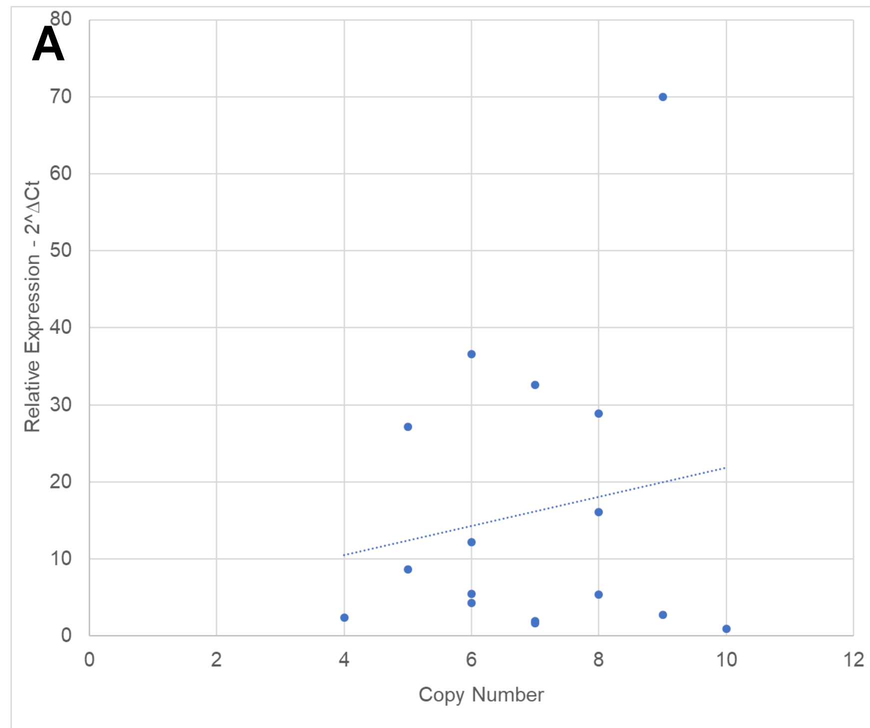
Figure 3.7 Bar charts showing qPCR data of ΔCt values for batch 1 (A) and batch 2 (B). Delta Ct values were calculated (*SDHA* Ct-*DEFA* ct) to determine the relative expression of alpha defensin in GRN

(Blue) and MN (Orange) cell types. SEM values are standard error bars.

The initial comparison looked at the relative expression of *DEFA* expression against SDHA expression in the GRN and MN fractions in each individual in two batches, batch 1 being the initial 10 samples investigated using qualitative PCR (Figure 3.7A) and batch 2 with 9 samples investigated using quantitative PCR only (3.7B). Across both batches, all individuals except for AN18003 showed relatively higher levels of alpha defensin expression of *DEFA* compared to *SDHA* in the GRN fraction (Figure 3.7A and B). Due to the distinct batch effect evident in the MN cell fraction, the two batches could not be compared to one another. In the first batch, only two samples (AN18001 and AN18005 Figure 3.7A) displayed elevated levels of expression of *DEFA* compared to *SDHA* in the MN cell fraction, with the remaining samples showing lower *DEFA* expression than SDHA (Figure 3.7A Orange). This pattern of expression is not present in the second batch of samples (Figure 3.7B) which shows expression of alpha defensin in MN cells in all samples to be more than that of *SDHA*, other than in the MN cell fraction of samples AN180016 and AN18024. However, the resulting data did show expression to be present in all of the mononuclear cells, though in some cases to a lesser extent.

3.3.2: Expression of *DEFA1A3* against copy number

While expression of alpha defensin mRNA was, in the GRN cell fraction, greater than that of the MN cell fraction, there was a level of variability between the individuals investigated. Therefore, the association of this variability in expression and the genotype of the individuals was investigated. Using the $2^{\Delta ct}$ values obtained from the qPCR, comparisons could be made to determine if there was any variability in expression of alpha defensins in WBCs. More specifically, comparison of expression and copy number was done to find any correlation. This was done using $2^{\Delta Ct}$ against copy number to show relative expression compared to genotypic variation.



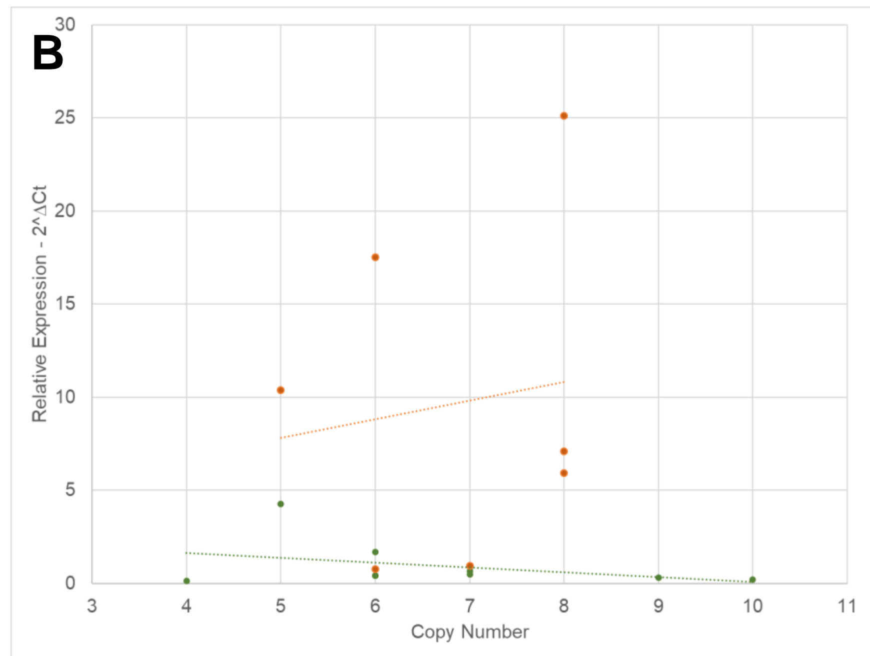


Figure 3.8. Scatter graph of $2^{\Delta Ct}$ against copy number for GRN cells using collated data from both batches (A) and MN cells (B) with samples in two batches; batch 1 (Green) and batch 2 (Orange). Pearson's correlation coefficient was calculated to determine correlation between copy number and relative expression of GRN

(Correlation coefficient = 0.16) and MN (Correlation coefficient: batch 1 = -0.37 batch 2 = 0.14).

Due to the lack of batch effect evident in the GRN cell fraction, the data across both batches were collated and compared to CN. When comparing expression of *DEFA* to copy number, the GRN cells show a positive correlation (Figure 3.8A). In order to determine significance of correlation between alpha defensin expression and CN, a regression test was conducted and found no significant effect of CN on *DEFA* expression in granulocytes ($F_{1,14} = 0.39$, $p > 0.05$). When comparing the MN fraction, the samples were investigated in two batches that shows two distinct patterns of association with copy number. Batch 1 (Figure 3.8B Green) shows negative correlation with copy number while batch 2 shows a positive correlation (Figure 3.8B Orange). When determining significance of this association with copy number, there was no significant effect of copy number on *DEFA* expression for batch one ($F_{1,7} = 1.08$, $p > 0.05$) and batch two ($F_{1,5} = 0.09$, $p > 0.05$) of mononuclear cells.

3.4: Detection of alpha defensin protein in white blood cells

Using PCR and qPCR enabled the identification of expression of alpha defensin RNA in the cells. However, this did not give any indication of expression of alpha defensin protein in the cell fractions. To determine if the cells were expressing alpha defensin protein, peroxidase-coupled immunocytochemistry (ICC) staining was undertaken. This used a mouse monoclonal antibody specific to alpha defensin proteins in humans, and secondary staining with a peroxidase conjugated anti-mouse IgG secondary antibody that resulted in brown staining in the presence of the protein (See Materials and Methods section 2.2.6). It was expected that the resulting stain would be strong in neutrophils, due to the presence of alpha defensin protein in abundance in the azurophil granules. However, the approach was focused on identifying if there would be any staining in the mononuclear cells, to determine if they also presented with alpha defensin protein.

On the other hand, however, the antibody stain might also have provided us with an insight into the type of cell harbouring alpha defensin protein. Counterstaining of the cells using haematoxylin aided in the identification of the specific cells showing positive alpha defensin protein expression. This was of particular interest in the mononuclear cells as it had the potential to provide insight into whether lymphocytes or monocytes were expressing alpha defensin protein in the mononuclear cell fraction.

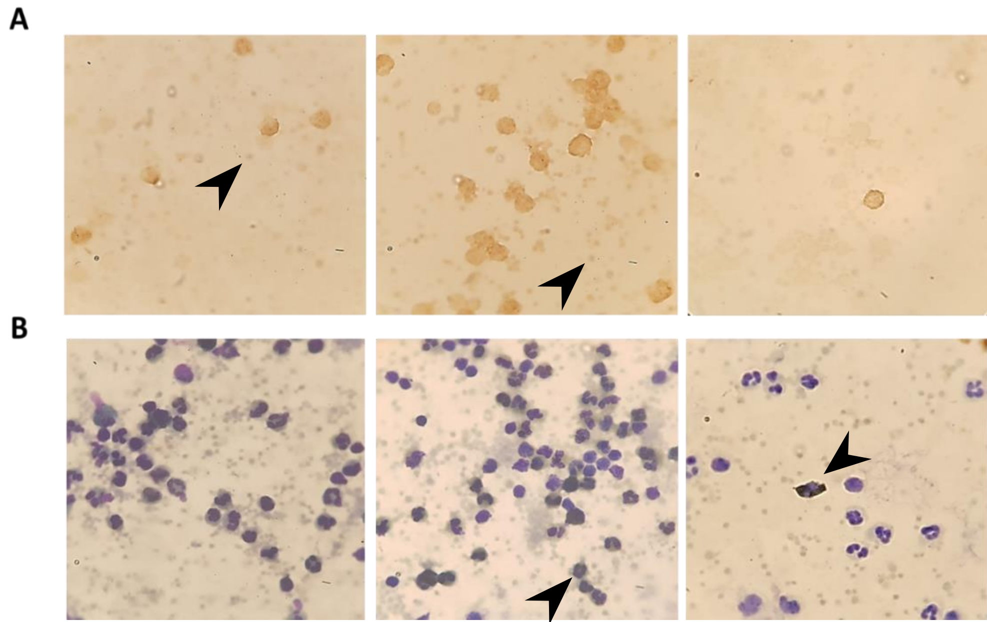


Figure 3.9. Immunocytochemistry staining of buffy coat. Buffy coat samples were incubated with 1:100 primary antibody (Hycult) and 1:200 HRP conjugated anti-mouse secondary antibody. A. Samples were stained using DAB substrate. Black arrows indicated platelets that appear to have brown DAB staining. B. Samples were then counter stained using blue counter stain from haemacolor dye kit, turning brown DAB stain dark brown-black colour. Black arrows indicate cells that appear to show positive DAB staining and lobular nuclei.

Stained buffy coat samples exhibited inconsistent staining across all neutrophils on the slide, even those in close proximity. Morphological detail was lost in the staining process, particularly in nuclear staining, making identification of the cells that were positive difficult to identify. Counter staining with haematoxylin was found to obscure DAB staining.

Though nuclear staining was difficult, some of the cells showing positive expression displayed characteristics of granulocytes, presumably neutrophils, due to the presence of a lobular nucleus (Figure 3.9B). Therefore, clear expression of alpha defensin protein was found in only neutrophils in all samples investigated (brown stained cells in 3.9A and indicated by arrows in Fig 3.9B). However, when comparing the staining present in other cells in the buffy coat, there is some staining present in the platelets in the majority of cell fractions (Indicated by arrows in Fig 3.9A). While platelets are not known to synthesise alpha defensin protein, this indicates the presence of either background staining, or alpha defensin protein. No staining was found to be present in any white blood cells other than neutrophils, though this may have been due to the difficulties in the staining process, indicating no alpha defensin protein was clearly detected in cells other than neutrophils.

CHAPTER 4: DISCUSSION

The aim of this project was to investigate the effect of variability in the *DEFA1A3* locus on alpha defensin expression in white blood cells. The initial approach to tackling this question was to examine the expression of alpha defensin RNA in separated white blood cell fractions to determine if expression was variable depending on genotype and copy number. The data consistently showed evidence of alpha defensin RNA present in both granulocyte and mononuclear cell fractions (See Results sections 3.2 and 3.3). When comparing expression of *DEFA1A3* mRNA relative to *SDHA*, the granulocyte fraction appeared to express proportionately higher levels than mononuclear cells (See Results section 3.3). However, the level of RNA present in the granulocyte fraction was likely to be left over from alpha defensin synthesis in promyelocytes during haematopoiesis (Faurschou and Borregaard 2003). When investigating quantitative data, the expression of alpha defensin in mononuclear cells appeared to be at a lower level than the reference gene *SHDA* (See Results section 3.3). The cDNA used in the qPCR analysis was synthesised using random hexamer primers (See Methods section 2.2.2.2). Though this might be advantageous in the case of low quality RNA, the resulting data from the qPCR analysis might not have come from translation-competent mRNA transcripts and the fluorescence signal may be produced from incompletely processed or non-standard transcripts incapable of directing synthesis of alpha-defensin peptides. On the other hand, however, when taking into consideration the qualitative data from the agarose gel analysis of RT-

PCR products, the cDNA was synthesised using oligo(dT) primers (See Methods section 2.2.2.2.), demonstrating that the signal derives from polyadenylated RNA. Though there is a disadvantage of potentially incomplete reverse transcription using oligo(dT) primers in samples with reduced RNA quality, the main advantage of using these primers was that they prime from the Poly(A) tail of mRNA. Therefore, the cDNA products used in the general PCR would only contain DNA molecules that had been synthesised using polyadenylated mRNA. Also, the primers used in both qualitative and quantitative PCR analysis of cDNA crossed a splice site. Therefore, it can be confirmed that the samples investigated, including the mononuclear cell fraction, did in fact have spliced and polyadenylated alpha defensin mRNA present

Previous reports of the expression of alpha defensin in mononuclear cells have been inconclusive and contradictory. mRNA expression has been found in isolated monocytes but was specifically excluded in T cells (Mackewicz *et al..* 2003). By contrast, other studies have demonstrated *DEFA1A3* expression in NK cells (Obata-Onai *et al..* 2002) or in T cells (Chalifour *et al..* 2004). On the other hand, extensive research has found little to no expression of alpha defensins in monocytes and dendritic cells (Villani *et al..* 2017). Though alpha defensin RNA was found in the mononuclear cell fraction, one possible explanation is that the RNA comes from granulocytes contaminating the mononuclear fraction. Histopaque separation, though effective, can incur some cross-contamination between the separated cell fractions, mainly in the mononuclear cell fraction being contaminated with granulocytes

such as basophils (Feldman and Mofelesky 1987). This was of particular concern due to the relatively high mRNA level apparent in the granulocyte fraction. Had the mononuclear cell fraction been contaminated, the resulting RNA levels in the mononuclear fractions could have been attributable to granulocyte contamination and not be representative of RNA levels present in the mononuclear cells. To ensure the mRNA expression was not due to possible contamination, cell counts revealed relatively low proportions of granulocyte contamination from the separation process (<3%), insufficient to explain the level of RNA observed (See Results section 3.2.1).

However, the data from the qPCR showed a distinct batch effect in the mononuclear cell fraction, with two separate patterns of variability corresponding to the two groups making up the 19 samples analysed by qPCR (batch one of 10 samples and batch two of 9 samples) (Section 3.3). This may have been due to several factors, the most likely being the condition of mRNA, and therefore cDNA, of the samples. This is evident in the fact that the granulocyte fraction appeared to show no evidence of batch effect. With the expectedly high levels of alpha defensin RNA in the granulocyte fraction, a loss of RNA quality would be overcome by the presence of large concentrations of *DEFA* mRNA compared to the mononuclear cell fraction. RNA quality has a known impact on the measurable gene expression in qPCR (Vermeulen *et al..* 2011). Confirmation of reduced RNA quality using comparable data such as RIN (RNA integrity number) values (Schroeder *et al* 2006) was not achieved in this project due to time constraints. Had the RNA quality been

compromised, this may have had an overall effect on the quality and concentration of cDNA used as the template for qPCR probes. Had this affected particular genes such as *DEFA3*, this would have had given falsely low values of *DEFA3* compared to *SDHA* (as is the case in mononuclear cells of batch one) or in the opposite case, whereby levels of *DEFA3* would seem comparably higher than *SDHA* (batch two). A way in which this could be investigated is using another reference gene and comparing the relative expression of alpha defensins to that. Other than *SDHA*, a common reference gene used in WBC investigations is *TBP* (Zhang *et al..* 2005).

A number of contributing factors can lead to batch effects, from environmental conditions, such as room temperature, to experimental differences, such as incubation times or the integrity of RNA purification reagents. Variation in cell processing conditions could lead to the change in cell morphology, particularly in blood cells such as neutrophils which have a short lifespan. A way to combat this is to ensure individuals are sampled and investigated together, if possible. Should this not be possible, however, measures should be taken to avoid variation in factors that lead to batch effects, such as maintenance of temperatures and using exact incubation times.

When comparing expression of alpha defensin mRNA in white blood cells against genotypic variation, there appeared to be no evidence of an association. Variability of RNA expression was found in isolated and stimulated monocytes from a previous study, a possible output to

investigate in future research. However, due to the lack of information on the *DEFA1A3* genotypes of the monocyte samples, associations with copy number or haplotype class could not be analysed. In the case of the variability found in the qPCR, when analysed against copy number, expression in granulocytes and mononuclear cells showed no significant correlation with copy number (P>0.05) when normalised against *SDHA*. The lack of significance in which the associations show indicates that variability in the expression of alpha defensin is independent of copy number. When investigating the expression of alpha defensin mRNA compared to haplotype classes, we found that the frequencies of heterozygotes and homozygotes was too small to do a statistically powerful comparison.

Though a 25-sample cohort gave rise to a substantial body of practical work, a larger cohort would have been more powerful in analysis of variability in expression. A larger sample size would have had the potential to highlight any lower frequency variation that was not identified in these investigations. Though the genotypes determined in the 25 individuals were generally representative of the population, the potential to make comparisons between haplotype classes was limited. It would have been beneficial to have some similarities in genotypes of different individuals in the cohort to identify any similarities present between the haplotype classes. The genotypes available in the 25 individuals showed very few homozygotes for flanking haplotypes. Similarly, in the case of copy number, comparisons would have been more effective had there been more individuals with the extremes of copy number. The range of

copy number in the individuals investigated in the qPCR was from 4 copies to 11 copies, with only one individual for either end of the range.

The resulting data from this project showed both the granulocyte and mononuclear cell fractions express alpha defensin mRNA but only granulocytes, specifically neutrophils, express alpha defensin protein at levels detectable by the immunocytochemical method used in this project. However, there was some evidence of positive DAB staining in platelets. The separation process resulted in contamination of erythrocytes in the granulocyte fraction and thrombocytes in the mononuclear fraction (See Results Section 3.2 Fig 3.3). The focus of this project was in white blood cell expression, and contamination via other cells was not of urgent concern. Although it is not likely, it is possible that the platelets present in the mononuclear cell fraction accounted for some, if not all, the expression of alpha defensin. Therefore, while a focus on expression in monocytes vs lymphocytes is evidently the clear avenue to pursue, expression of alpha defensins in platelets also should be considered. The mononuclear cell fraction had consistent evidence of mRNA but appeared to show no detectable alpha defensin protein expression, suggesting mononuclear cells are not synthesising alpha defensin protein in the cell. This may be due to the absence of the necessary components required to induce alpha defensin synthesis in NK cells, such as engagement with bacterial PAMP (Chalifour *et al.*. 2016). This suggests that the mRNA present in the mononuclear cells is not utilised in protein synthesis unless activated. However, the experimental approach used in this project was detection of a

horseradish peroxidase-coupled secondary antibody with DAB substrate staining, for which sensitivity is a limiting factor. This may have accounted for the lack of detectable staining. This could be overcome using alternative more sensitive staining processes such as fluorescence. It was evident that not all neutrophils appeared to show staining, most likely related to the gentle nature of saponin permeabilization, a factor that may have limited the internalisation of the antibodies required for staining in both mononuclear and granulocytic cells. The samples investigated in the antibody staining were buffy coat samples. This was adopted because of the effect that more prolonged separation methods had on the morphology of the cells. Staining separated cells (i.e. granulocytes and mononuclear cells) appeared to lead to loss of morphology when ICC staining. Alternative methods in which cells could be separated should be considered, as this may aid in the identification of cells expressing alpha defensin protein.

The way in which previous studies in the literature looking at alpha defensin expression sampled and processed the blood was variable depending on the study, and those factors may contribute to the differences between the outcomes of those studies and this work. This variability in sampling included the use of heparinised collection tubes (Mackewicz *et al.*. 2003) compared to EDTA tubes (Obata-Onai 2002); EDTA was the anticoagulant used in this project. This may have had an effect on the expression of alpha defensin mRNA in cells, due to the potential stimulatory effects from the components in the collection tubes. There is the possibility that the cells investigated in this project were

unintentionally stimulated through the collection and separation process, stimulating the production of alpha defensin mRNA immediately after collection, but without time to generate alpha defensin protein. The sample processing could have had an effect on the expression in the WBCs based on separation procedures such as using density gradients like the ones used in this experimental work (Chalifour *et al.*. 2004) compared to separation of individual cell types by FACS (Villani *et al.*. 2017). Considering the length of time of sampling and processing prior to RNA isolation, this could have had a detrimental effect on the level of expression of mRNA or alpha defensin protein. Neutrophils are most effectively analysed quickly due to the short lifespan of the cells that leads to loss of morphology, a factor that could have affected the staining process in ICC staining and in the isolation of RNA from dead or dying cells. Because time frames are not included in the corresponding literature, comparison of time courses between this experimental investigation and published literature is not easily made.

The outcomes from this investigation have provided a number of avenues on which future investigations can focus. The first is to continue similar investigative approaches as used in this project (such as expression analysis techniques) but to look at cells after they are separated further. The separation of mononuclear cells into lymphocytes and monocytes can be done through negative selection using antibody-coated magnetic beads (Miltenyi *et al.*. 1990) or through FACS to separate cells into subtype (Villani *et al.*. 2017). In contrast to neutrophils, working with mononuclear cells in future studies would be advantageous

due to the ability to culture them *in vitro*. This would enable the identification of the specific cell types expressing alpha defensin mRNA and may give insight into the possible mechanisms by which *DEFA1A3* is associated with IgAN and periodontitis. Secondly, there may be a number of environmental factors that could induce or prevent the expression of alpha defensins that could be relevant to the association with disease. In investigations where expression was identified in mononuclear cells, NK cells were in fact stimulated using IL-2 and IL-15 (Obata-Onai *et al.*. 2002) which increased alpha defensin expression, even if only slightly in the case of IL-2. The cells investigated in this project were not purposely stimulated by any factors to induce mRNA production. However, due to the variability in sampling methods of this project compared to those used in the literature, cells may have been stimulated unintentionally. Therefore, in future work separated cells could be deliberately and specifically stimulated using factors such as LPS and PHA and then compared to unstimulated cells.

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