CHARACTERISING THE PEPITEM PATHWAY IN PATIENTS WITH ATHEROSCLEROSIS

By

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Abstract:

Atherosclerosis is an asymptomatic disease which is regarded as one of the most fatal diseases. However, the mechanism of the immune response is not well understood. There is accumulated evidence supporting the idea that inflammatory response initiates the disease. A new novel peptide has been discovered in our lab which down-regulates T cell recruitment during inflammation called PEPITEM (Peptide Inhibitor of Trans Endothelial Migration). We are interested in testing the action of PEPITEM on PBL isolated from atherosclerosis patients. We first demonstrated that PEPITEM did not affect the levels of adhesion of PBL from either diseased or healthy donors. Interestingly however, we did observe that PBL isolated from atherosclerosis patients adhere more readily than those isolated from healthy control subjects. Therefore, we studied the surface expression of certain adhesion molecules and chemokine receptors on the PBL of atherosclerosis patients. We found significantly higher surface expression of Beta-receptor family (Beta-1 and Beta-2) and PSGL-1 receptors in some PBL subsets in atherosclerosis patients. In addition, we looked at the effect of PEPITEM and adiponectin (AQ) treatment on the migration of PBL and we revealed for the first time based on our knowledge that there was no effect of treatment on PBL isolated from atherosclerosis patients.

These observations will contribute to understanding the potential therapeutic applications of PEPITEM on atherosclerosis.

Dedicated to my previous Supervisor who told me 4 years ago "YOU ARE NOT READY FOR A PhD".

Acknowledgement:

I want to begin by expressing my sincere acknowledgement to my advisor, Professor Ed Rainger who gave me the opportunity to venture on this research project under his guidance and supervision. He was the source of my motivation, encouragement and support throughout the period of my graduate studies. From him, amongst other things, I have learned to write and present my research data for publications and presentations in scientific journals and conferences.

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Last, but certainly not the least, my admiration and heart-felt gratitude to my wife Asma for her unconditional love, support, and care and to my daughter Lana and son Maalik and Dana.

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List of Abbreviations:

ApoE: Apolipoprotein E

AQ: Adiponectin

AR1: Adiponectin receptors 1

AR2: Adiponectin receptors 2

BMI: Body mass index

T1D: Type 1 diabetes

T2D: Type 2 diabetes

CD: Cluster of differentiation

CVD: cardiovascular disease

DP-1: Prostaglandin receptor 1

DP-2: Prostaglandin receptor 2

FTY720: Fingolimod

GPCR: G-protein coupled receptor

HDL: High density lipoprotein

HMW: High molecular weight

BSA: Bovine serum albumin

EAE: Experimental autoimmune encephalomyelitis

EC: Endothelial cells

FoxP3: Forkhead box P3

HEV: High endothelial

KO: knock-out

HUVEC: Human umbilical vein endothelial cells

PBS: Phosphate bufferedsaline

PBSA: Phosphate bufferedsaline plus albumin

APCs: Antigen presenting cells

DCs: Dendritic cells

NK: Natural killer

PBL: Peripheral blood lymphocytes

PBMC: peripheral blood mononuclear cells

TCR: T cell receptor

Th1: T helper 1

Th2: T helper 2

Treg: regulatory T cells

RBC: red blood cells

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

eNOS: Nitric oxide synthase

ICAM-1: Intercellular Adhesion Molecule 1

IFN-γ: Interferon-gamma

Ig: Immunoglobulin

IL: Interleukin

JAM-A, B, C: Junctional adhesion molecule A, B, C

LFA-1: Lymphocyte function-associated antigen 1

MAPK: Mitogen-activated protein kinases

MCP-1: Monocyte chemotactic protein

NF- κB: nuclear factor kappa-light-chain-enhancer of activated B cells

PGD2: Prostaglandin D2

PSGL-1: P-selectin glycoprotein ligand-1

RANTES (CCL5): Regulated upon Activation, Normal T-cell Expressed, and Secreted

S1P: Sphingosine-1-phosphate

S1PR: Sphingosine-1-phosphate receptor

SLOs: Secondary lymphoid organs

SPHKs: Sphingosine-1-phosphate kinases

TNF-α: Tumour necrosis factor alpha

TLR: Toll-like receptor

VCAM-1: Vascular cell adhesion protein 1

VE-cadherin: Vascular endothelial cadherin

VLA-4: Very Late Antigen-4

PECAM1: Platelet endothelial cell adhesion molecule 1

α-1: alpha-1

α-4: alpha-4

β-1: Beta-1

β-4: Beta-4

CCR5: C-C chemokine receptor type 5

CXCR3: Chemokine receptor CXCR3

CX3CR1: CX3C chemokine receptor 1

ATP: Adenosine triphosphate

Bp: base pair

cDNA: Complementary deoxyribonucleic acid

dNTPs: Deoxyribonucleotide triphosphate

Da: Dalton

PCR: Polymerase chain reaction

gDNA: genomic deoxyribonucleic acid

Ct: Threshold cycle

PB: Pacific blue

MFI: Mean fluorescence intensity

FITC: Fluorescein isothiocyanate

APC: Allophycocyanin

APC-Cy7: Allophycocyanin Cyanine 7

PE: Phycoerythrin

PECy7: Phycoerythrin Cyanine 7

PerCpCy5.5: Peridinin-chlorophyll-protein complex tandem with Cyanine 5.5

CHAPTER1: GENERAL INTRODUCTIO

1. The cellular constituents of blood and the process of their derivation

Blood is composed of a liquid component called plasma, which contains many solutes, and a solid component, composed of blood cells. These cells can be broadly sub-classified as red blood cells (RBCs), white blood cells (WBCs), and platelets. From the embryological point of view, the production of blood cells (haemopoiesis) starts in the yolk sac at day 14 post-fertilization, whereas in the second and third trimester, the liver and then the bone marrow become the major sites of haemopoiesis. After birth until ≈4 years of age, the marrow in all bones is called red bone marrow, because haemopoiesis is taking place; however, by age 25 haemopoiesis is limited to the ribs, sternum, vertebrae and pelvis (Rodak et al., 2002).

1.1. Platelets

Platelets are not considered true cells because they lack a nucleus and therefore DNA. They are derived from bone-marrow resident megakaryocytes and their life span in the circulation is approximately 10 days (Harrison, 2005). Quiescent platelets circulate within a physiological range of 150 to 400 x 109 /L (Rodak et al., 2002). Platelets play an important role in many physiological functions in the body such as haemostasis, wound healing and inflammation (Rodak et al., 2002) however, activation is essential for their efficient function, for example during haemostasis in vascular injury. In this context activation promotes responses such as adhesion, aggregation and secondary messenger production which work together to form a thrombus (or clot) to prevent blood loss (Rodgers, 1999). In the context of pathology, platelets have been shown to interact directly with intact monolayers of endothelium, for example in models of atherosclerosis, where they may promote the

recruitment of inflammatory leukocytes to the diseased vessel wall thereby propagating the disease process.

1.1. Red blood cells (RBC)

RBCs are non-nucleated discoid cells about 2-2.5 μm thick and 6-8 μm in diameter. Their main function is oxygen transport between the lungs and the body's tissues, which is facilitated by haemoglobin. The life span of RBCs in the circulation is ≈120 days, and as they age they are removed in the spleen for destruction by the reticulo-endothelial cell system. The normal range of RBCs counts in males is 4.6 to 6.2 x106/μl , whereas in females it is 4.3 to 5.6 x 106/μL (Tefferi, 2003). These numbers account for between 35-45% of blood volume, the haematocrit. As well as gas transport, RBCs play an important role in inflammation being responsible for the margination of WBCs to the vessel wall during the process of recruitment of these cells to the inflamed tissue (see below for details). RBCs also possess the Duffy antigen receptor for chemokines (DARC), which promiscuously binds a number of inflammatory chemokines (Hadley and Peiper, 1997). The binding of chemokines to DARC does not activate signalling pathways in RBCs, as it is not a G protein coupled receptor in these cells (Comerford et al., 2007). Rather, it is thought that DARC regulates the plasma concentration of biologically active chemokines by acting as a sink for their removal from the circulation and by neutralisation of their function. (Fukuma et al., 2003) (Lee et al., 2006).

1.2. White blood cells

The production of white blood cells is called leucopoiesis and it occurs in different locations depending on age; for example, it takes place in the yolk sac during early embryogenesis, then in the spleen later in ontogeny. In adult life lymphoid tissues (i.e. thymus, spleen and lymph nodes) and the bone marrow are the major sites for leucopoiesis

(Dalton et al., 1993). White blood cells (WBCs) form a heterogeneous population, with sub-populations being derived from distinct progenitor pathways (Rodak et al., 2002). These are generally classified into two major families: 1) polymorphonuclear leukocytes (PMN); 2) mononuclear cells.

1.2.1. Polymorphonuclear leukocytes (PMN)

In adults PMNs are formed exclusively in the bone marrow where they are stored and from which they are continuously released. They make up to 50%-70% of the total circulating WBCs count, and the production and their release into the blood can be increased in response to leukopoietic signals generated during infection and/or inflammation (Bagby, 2007). In general, these cells are highly granular in appearance due to the numerous vesicular bodies within their cytoplasm which has given rise to their alternative name of granulocytes. Based on their staining characteristics, granulocytes are subdivided into 3 groups: neutrophils, eosinophils and basophils (Figure 1-1). Neutrophils are the predominant subset of circulating granulocytes composing 40-65% of the total WBC count. Eosinophils and basophils are relatively rare in the circulation; eosinophils account for 1% - 4% of total blood count and basophils for 0.5% -1.0% (Bagby, 2007). These cells are intrinsically linked to the innate immune response. Indeed, neutrophils are generally the first leukocytes recruited during inflammation and/or infection, where their phagocytic capacity and powerful array of cytotoxins form the first line of defence against invading micro-organisms. Eosinophils are also stored in the bone marrow and can be mobilised rapidly from this depot. They are also recruited to tissues, although they are most prevalent during infection with multi-cellular parasites, e.g helminths and roundworms (Dalton et al., 1993). They also play important roles in the exaggerated responses of the immune system in allergic conditions. Basophils express

receptors have a high affinity for the Fc regions of IgE and hence have important roles in protection from multi-cellular parasites. They are important mediators of allergic responses including anaphylaxis (Rodak et al., 2002).

1.2.2. Mononuclear cells

Mononuclear cells can broadly be divided into two major classes; monocytes and lymphocytes and both of these classes are also heterogeneous in nature (Figure 1-2). Thus, monocytes, which constitute 2% to 11% of the total blood count (Bagby, 2007), are again divided into at least two further sub-populations depending upon the expression of surface markers. Therefore, all monocytes are positive for CD14, however, ≈10% of these cells are also marked by positive expression of CD16. The CD16+ cells may again be divided into 2 populations which are marked by the presence or absence of the chemokine receptor CCR2 (Geissmann et al., 2003). Upon recruitment to tissue, monocytes differentiate into macrophages or dendritic cells which play important immune regulatory roles. Whether subsets show a preference in their differentiation pathways is unclear and there is limited data on the functional distinctions between the subpopulations of monocytes (Imhof and Aurrand-Lions, 2004). The literature in this area is complicated by the preponderance of studies conducted on mice, which show a different profile of monocyte subset distribution. Moreover, in mice, sub-sets are identified by surface markers distinct from those in humans. It is probable that there is limited concordance between the functions of monocyte in these two species. Indeed, a recent comparison of genes expressed by monocyte subsets in humans and in mice demonstrated that far more genes were differentially expressed than were expressed in common (Ingersoll et al., 2010)

Lymphocytes represent the most complex of the leukocyte families. They constitute ≈30% of the total leukocytic blood count, but their numbers are expanded during immune responses (Bagby, 2007). The lymphocytic system evolved in the vertebrates to support the adaptive immune response and through time has developed a cell based system with the plasticity to respond to millions of distinct molecular variations (antigens), the facility to discriminate these from 'self', and the ability to generate a cellular memory of this process (see below for details). Lymphocytes can be generically sub-divided based on their origin of maturation; cells derived from bone marrow are called B-cells, whereas those derived from thymus, after seeding of this organ with progenitor cells from the bone marrow, are called T-cells (Rodak et al., 2002). However, within this classification it should be appreciated that there are many sub-divisions of B- and T-cells, which have distinct regulatory or effector functions, some of which are discussed in greater detail below.

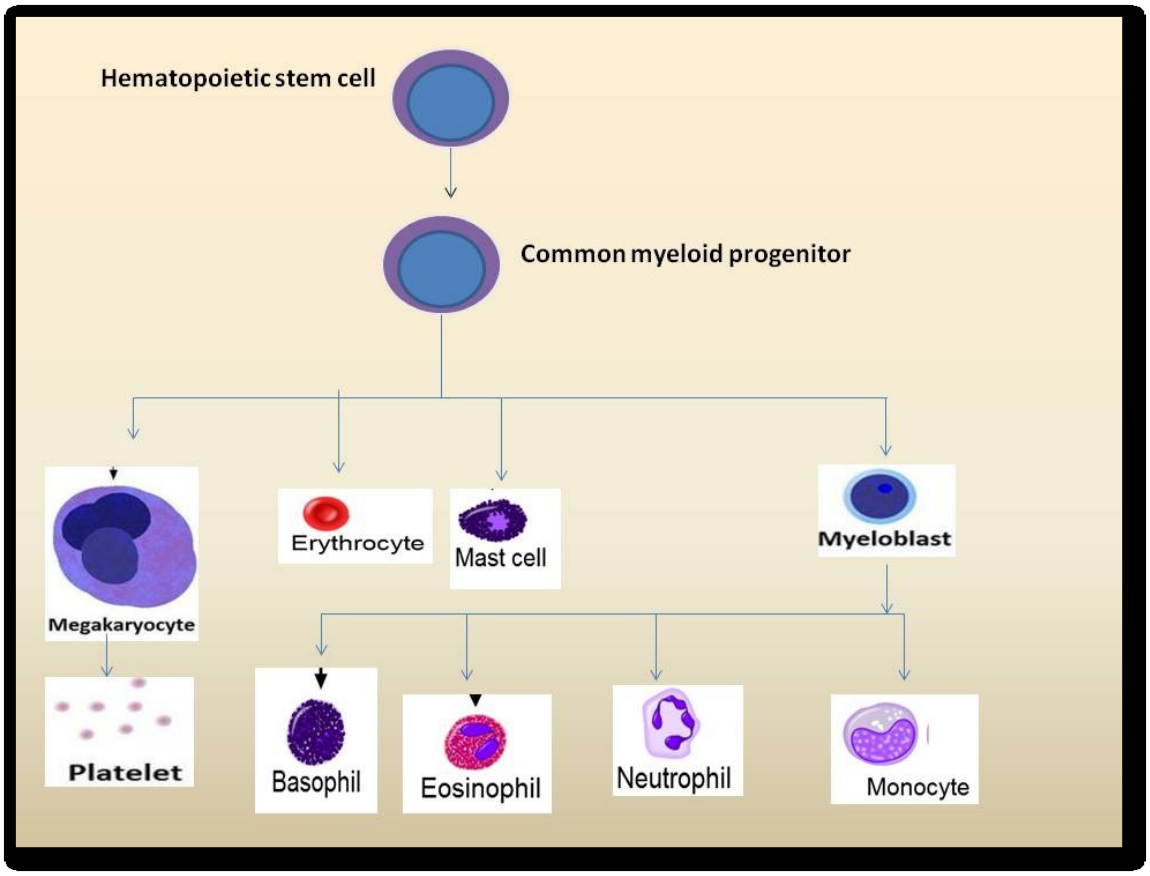


Figure 1-1: Haematopoiesis (The development of myeloid progenitor cells).

Hematopoietic stem cell is the earliest leucopoiesis precursor; it matures in bone marrow to common myeloid progenitor cells when it undergoes stimulation and mitosis. Because of the complexity of humoral factors such as interleukins, cytokines, and receptors expression of precursors surface, myeloid progenitor gives arise to different types of granulocytes (Rodak et al., 2002).

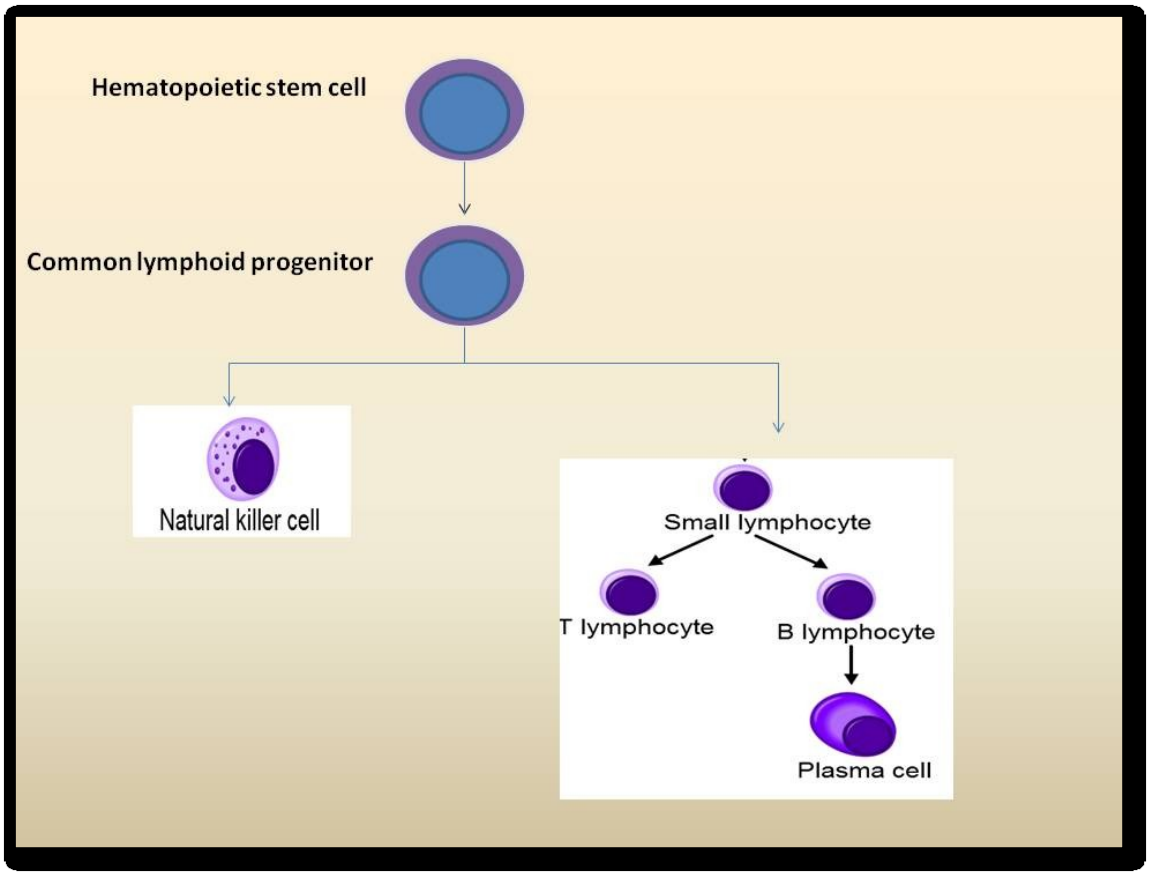


Figure 1-2 Haematopoiesis (The development of lymphoid progenitor cells).

Stem cell is the earliest leucopoiesis precursor; it matures in bone marrow to common lymphoid progenitor cells when it undergoes stimulation and mitosis. Common lymphoid progenitor cell matures to several cell types. The thymus and bone marrow participate in lymphocytes maturation and differentiation (Rodak et al., 2002).

2. The blood vascular system:

The blood vessels transport blood all over the body. Vessels carrying oxygenated blood from the heart are called arteries, while vessels carrying deoxygenated blood back to the heart are called veins. Microvascular capillary beds bridge the two and are the sites of gas and metabolite exchange from blood to tissues and vice versa.

2.1. The arterial circulation

The arterial circulation is a high pressure system, and arteries are thick walled with significant muscular and elastic structures conferring strength and maintaining vessel integrity (Rodak et al., 2002). There are three layers in the artery wall from the inner layer to the outer: the tunica intima, the tunica media and the tunica adventitia. The adventitia is composed of irregular fibres of connective tissues, fibroblasts and adipose deposits. In large arteries an independent circulation, the vaso vassorum, enters the vessel wall from the adventitia and provides vascular support for cells far removed from the blood circulating within the lumen of the artery. The tunica media is bounded by the internal and external elastic lamina and is composed of concentric layers of smooth muscle cells which are interspersed between lamina of elastic tissue. In muscular arteries, the former dominate the structure of the medial layer, while in elastic arteries the later are the major structural components. The intimal layer lies between the endothelial cells lining the lumen of the vessel and the internal elastic lamina (Clark, 2005). The importance of these layers to the pathogenesis of atherosclerosis will be discussed in greater detail below.

2.2. Arterioles

Arterioles are the smallest vessels on the arterial side of the circulation. They are well endowed with SMC and are reactive to many vasoactive molecules which regulate their diameter. As such, blood flow in capillary beds can be tightly regulated. Indeed, arterioles are otherwise known as the resistance vessels of the vasculature, as changes in resistance (due either to diltion or constriction) regulate the rate of blood flow to the 4th power of the radius of the vessel (i.e. there are large changes in resistance and blood flow for relatively modest changes in vessel radius) (Carola et al., 1990). Indeed, dilatation and constriction of the arterioles determine the level of peripheral resistance in the vasculature. As a result, arterioles regulate blood pressure as SMC responds to vasoactive substances such as angiotensin II (Ang II) and nitric oxide (NO) (Clark, 2005). The arteries and arterioles, besides providing a conduit for dissemination of leukocytes, do not ordinarily play a role in the trafficking of leukocytes to the tissues.

2.3. Capillaries

Capillaries are relatively simple vessels comprising of an EC layer on a basement membrane. In unspecialised vascular beds the EC form a continuous monolayer, however in organs with specialised function (e.g. endocrine tissues or the liver) the EC monolayer may be discontinuous or the EC may possess fenestrated sieve plates, which maximise contact between blood-borne solutes and the tissue parenchyma (Clark, 2005). As stated above, capillaries are the sites of metabolite and gas exchange between tissues and the blood. However, in some organs such as the lung and the liver, capillaries may be major sites of extravasation for leukocytes during immune surveillance and/or inflammation (Vonandrian et al., 1991).

2.4. The venous circulation

Venules collect blood from the capillaries for delivery to veins and thus to the heart, thereby completing the vascular circuit. Being a low pressure system, the venous vessels lack a robust medial layer. However, as they carry blood to the heart, often against gravity, many have none-return valves along their length to avoid movement of blood in a retrograde direction. Veins generally rely on the contractions of adjacent skeletal muscles (peristalsis) as well as the opening and closing of the intra-luminal valves, to facilitate blood flow back to the heart. Although large veins do not play a role in leukocyte trafficking to the tissues, post-capillary venules play a very important role in this process. In most tissues these vessels are the sites at which leukocytes traffic from the blood to the tissues during inflammation. This process is aided by the structural and functional characteristics of these vessels. The luminal endothelial cells are highly responsive to inflammatory stimulation and in response express molecules, which support leukocyte trafficking (see below for details). Being part of the low pressure venous circulation, blood flows relatively slowly and these vessels are marked by low wall shear stresses, a necessity for efficient interaction of circulating leukocytes with the endothelium. The rheological properties of blood flow in these vessels allow red cells to aggregate and form rouleaux, a process which facilitates margination of the leukocytes to the vessel wall. Lastly, migrating leukocytes encounter relatively few obstacles during transit to the tissue, having to cross the EC monolayer, the EC basement membrane and the single layer of SMC–like pericytes.

3. The lymphatic vasculature

The lymphatic system is separate from the blood vasculature, but its vessels are found in all body organs, except the central nervous system and non-vascular tissue such as the Cartridge (Schulte-Merker et al., 2011). The primary function of the lymphatic system is to reabsorb the extravasated tissue fluid and return it into the blood in order to maintain the interstitial fluid balance (Figure 1-3). It also plays an essential role in the packaging of dietary lipids in to chylomicrons (lipoprotein associated lipid particles) and then transporting these from the small intestine to the blood (Oliver and Srinivasan, 2008). Animal studies also suggest that lymphatic vessels help in cholesterol clearance by participating in Reverse Cholesterol Transport (RCT) (Martel et al., 2013). Importantly however, the lymphatic system also plays a crucial role in the adaptive immune response. Lymph nodes are the sites at which foreign antigen is delivered and presented to B and T lymphocytes which initiates the adaptive immune response. This process is achieved by supporting the migration and transport of leukocytes from the tissues to the lymph nodes (see below for details). Although the lymphatic system is discrete from the blood vasculature, it separates from a common vascular structure during early ontogeny (Wilting et al., 2004).

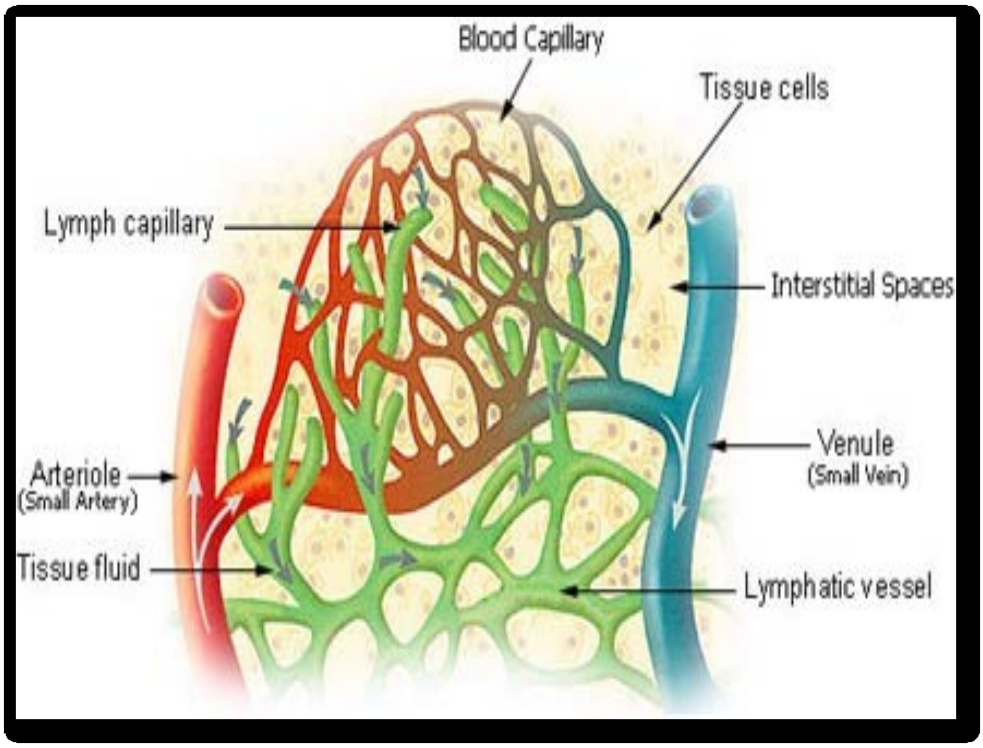


Figure 1-3 Lymphatic vasculature.

The lymphatic system is separate from the blood vasculature, but its vessels are found in all body organs, except the central nervous system and non-vascular tissue such as the Cartridge. The primary function of the lymphatic system is to reabsorb the extravasated tissue fluid and return it into the blood in order to maintain the interstitial fluid balance (Smith, 1997-2015).

4. Innate immunity

Even though the innate and adaptive immune systems work together, it is important to identify the specific characteristics of each system because innate immune responses are non-specific and are not intensified by previous exposure to infectious organisms, while the adaptive immune response is exquisitely targeted against specific antigens and is intensified upon secondary exposure by establishing populations of 'memory' cells (Abbas et al., 2012).

Innate immunity is a non-specific response to infection or injury which acts quickly against invading pathogens. It includes physical and chemical barriers and utilises the inflammatory response to deliver effector cells from the blood to the tissue within a time frame ranging from minutes to hours. The major cellular constituents of the innate system are neutrophils and monocytes which are phagocytic cells capable of recognising and rapidly eliminating foreign agents (Iwasaki and Medzhitov, 2004). The immune system recognizes the foreign invaders via signals which are released either from the invaders or in the case of viral infection from the damaged tissue. Signals that are released from foreign invaders are called pathogen-associated molecular patterns (PAMPs) whereas the ones from damaged tissue are called damage-associated molecular pattern molecules (DAMPs) (Tang et al., 2012). When the immune system recognises PAMPs, it initiates immune response immediately in order to eliminate the pathogen and this is why in some articles PAMPs are called signal 0 (Akira and Hemmi, 2003). After pathogen recognition via PAMPs, Toll-like receptors (TLRs) become activated in combination with pattern recognition receptors (PRRs) in order to alert the immune system of to the existence of a pathogen (Tang et al., 2012).

5. The adaptive immune response

There are three basic strategies by which adaptive immune responses fight foreign pathogens (Abbas et al., 2012). Antibodies which are generated after interactions with B-cells, T-cells and antigen presenting cells bind to a pathogen and then disable its normal function and prevent infection of other cells. Antibody binding also activates the complementary system which leads to the destruction of pathogens by lysis. Antibody binding also 'opsonises' cells, marking them for efficient subsequent clearance and destruction by phagocytes. Phagocytes engulf and ingest pathogens with help from Th (T helper cells) which also reinforces the microbicidal effect of phagocytes. Cytotoxic T Lymphocytes (CTLs) destroy infected host cells through recognition of foreign antigen associated with MHC class 1 by the T-cell receptor.

There are three levels of the adaptive immune system; the afferent arm (Antigen recognition), the efferent arm (lymphocyte activation), and immunological memory. The afferent arm enables the system to distinguish between what is self and what is foreign antigen. Importantly this system is able to identify and respond to millions of distinct antigens, thereby conferring specificity to each response. The efferent arm delivers the molecular and cellular response against the antigen whereas the effector cells of the adaptive response are composed of antigen presenting cells and lymphocytes (O'Gorman and Donnenberg, 2008). Importantly, although single B-cell and T-cell clones only recognise a single antigen, collectively they are able to recognize a wide variety of antigens due to the hyper variability of their antigen recognition receptors, i.e. antibody on the B-cell membrane and the T-cell receptors (TCR) on the surface of T lymphocytes (Alberts et al., 2002).

The immunological memory amplifies and accelerates the immune response against secondary exposure to the same antigen (Vesely et al., 2011).

5.1. Antigen recognition

APCs degrade the antigen proteins into peptides and display them on the cell surface bound to major histocompatibility complex molecules (MHC). MHC molecules are display molecules specific to the adaptive immune system. Once activated by the inflammatory response and having acquired foreign antigen, DCs migrate from tissues to the lymph nodes via the afferent lymphatics. Here, they reside in the node where they can present MHC-antigen complexes in association with co-stimulatory molecules to naïve T and B lymphocytes which traffic through these organs (Paul, 2012).

5.2. Lymphocyte activation

Naïve T lymphocyte activation takes place after peptide–MHC molecule recognition by the T-cell receptor. This recognition is crucial to the efficiency of immune response. Accessory signals such as the B7-1 and B7-2, which are delivered from the APC to the T-cell upon ligation of MHC, ensure a robust and specific activation of specific T-cell clones without significant involvement of T-cells with antigen recognition profiles inappropriate for responding to the specific invading pathogen (Akira and Hemmi, 2003). Although some of these T-cells will become antigen specific cytoxic T-cells, others facilitate in the activation of B-cells and production of the humoral response, i.e. synthesis of antigen specific antibodies. These helper T- cells are essential to B-cell responses. The displaying of antigen peptide on class II MHC molecules by APCs activates helper T-cells and induces the expression of costimulatory ligands such as CD40L and B7 molecules. Then, due to a chemokine gradient, the activated helper T-cells migrate toward the lymphoid follicle where they interact with B-

cells which leads to their activation because of the presence of CD40L and some cytokines secreted from helper T-cells. Finally, a foci of extra-follicular B-cells is formed in the lymph node medulla in which B-cell derived plasma cells differentiate and begin to secrete antibodies (Gray and Skarvall, 1988).

The activation of B lymphocytes results in antibody mediated humoral immunity as these cells differentiate into antibody producing plasma cells. CD4+ helper T-cells can be important for the activation of B-cells, although there are mechanisms that allow B-cell activation in the absence of T-cells (T-cell independent activation). Due to the B-cells' activation, B-cell clones expand and some of their progeny differentiate into cells called Plasma cells. Plasma cells secrete antibodies which have the same antigen binding site as the B-cell receptors (cell surface antibodies) which originally recognized the antigen during the first encounter (van Essen et al., 2000). Pathogen proteins stimulate the production of different classes of antibodies (IgG, IgA, IgE) from a single B-cell clone.

Antibodies classes have specific roles in different tissues. For example, IgA is important in mucosal protection as it is removed from the blood and secreted into the mucosa by epithelial cells. IgG plays an important role in protecting the new-born. It can cross the placenta allowing transmission of immunity from the maternal circulation, and after birth it provides immunity to the baby because it is present in the colostrum. The half- life of most of these antibody classes is a few days, however, IgG has a half-life measured in weeks and can therefore deliver longer lived protection (Figure 1-4). Importantly, plasma cells can remain in the bone marrow for years releasing antibodies and conferring extremely long-lived and specific protection against pathogens (Alberts et al., 2002).

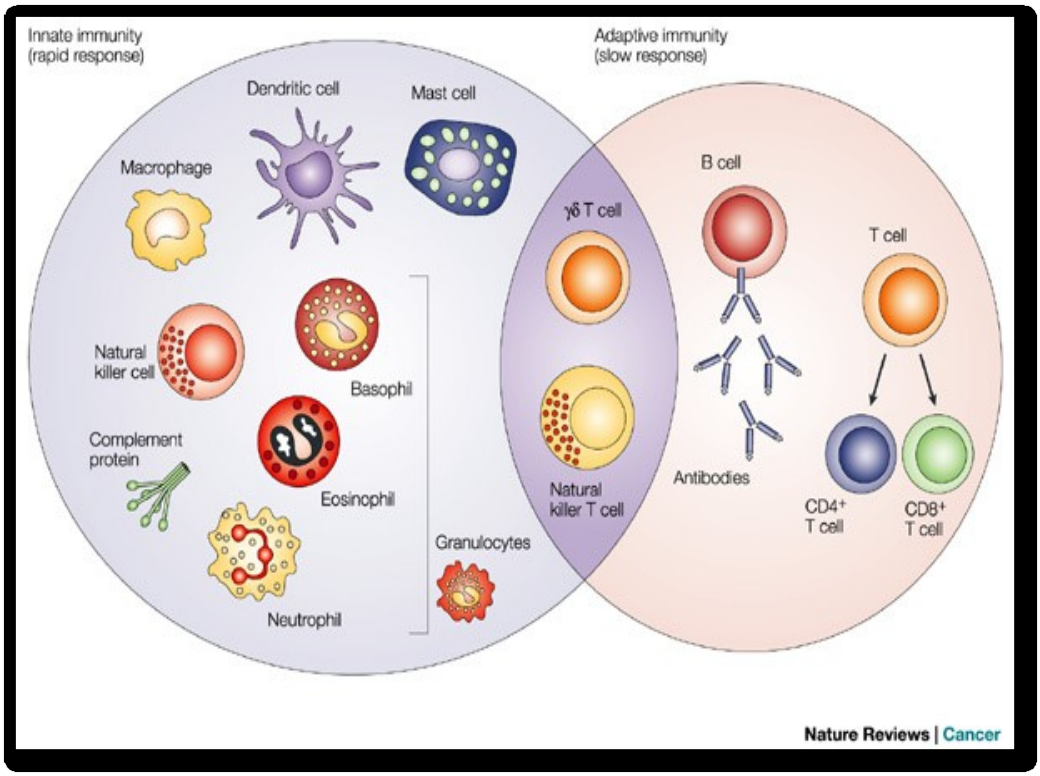


Figure 1-4: Innate immunity vs Adaptive immunity.

Even though the innate and adaptive immune systems work together, it is important to identify the specific characteristics of each system. Innate immunity is a non-specific response to infection or injury which acts quickly against invading pathogens. The major cellular constituents of the innate system are neutrophils and monocytes which are phagocytic cells capable of recognising and rapidly eliminating foreign agents. Adaptive immune response is exquisitely targeted against specific antigens and is intensified upon secondary exposure by establishing populations of 'memory' cells (Dranoff, 2004).

6. Immunological memory

After the elimination of a pathogen, a contraction of the specific immune response is required. Thus, clonal expansion of lymphocyte effectors stops and established colonies are depleted in order to re-establish homeostasis. Some plasma cells migrate to the bone marrow where they may reside for years producing specific antibodies which are released into the circulation, thereby ensuring long lived immunity (Alberts et al., 2002). Moreover, a small number of memory B-cells differentiate which can recirculate and provide a long-lived memory population that is available for rapid expansion should reinfection occur (Paul, 2012). The immune response mediated by T-cells against antigen, generates specific memory T- cells which could persist for life. The long survival of memory T-cells and their swift activation is attributed to many unique characteristics; the number of memory T-cells for a certain antigen is greater than that of naïve cells for the same antigen. In addition, memory T-cells express a group of proteins called anti-apoptotic proteins such as Bcl-2 and Bcl-xL. These molecules enable memory T-cells to survive after the elimination of an antigen and in the absence of stimulatory signal of T-cell proliferation (Figure 1-5).

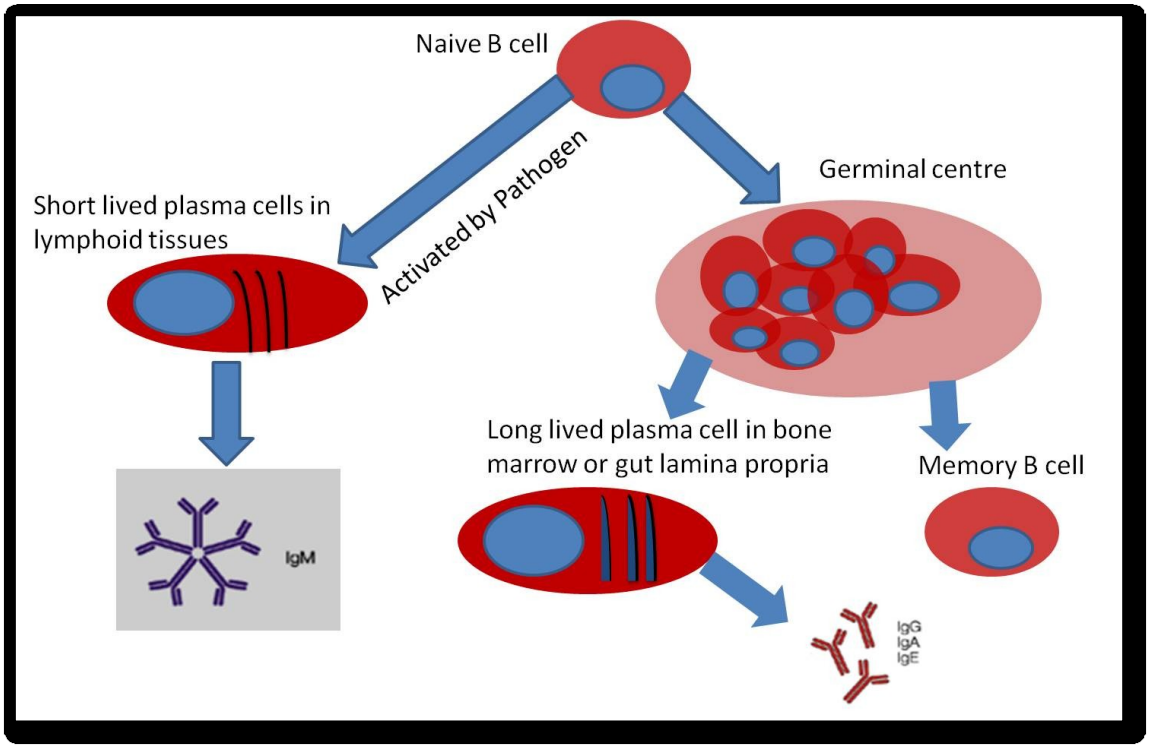


Figure 1-5: Immunological memory.

Memory B-cell and effector B-cell development is a two stages process. The first stage is the production of plasma cells which predominantly generate IgM; they are also called short-lived plasma cells. They are generated after the primary response and are located mainly in the lymph node medulla. The second stage is the production of memory B-cells and the seeding of the other type of plasma cells which are called long-lived plasma cells which are located in the bone marrow (Gray, 2002).

7. Inflammation and leukocyte recruitment

Inflammation is an integral component of the immune response. It has been characterised since antiquity by four cardinal signs; Dolor (Pain), Rubor (redness), Tumor (swelling), and Calor (heat) (Carola et al., 1990). More recently loss of tissue function has been added as a fifth cardinal sign of the inflammatory response (Lawrence et al., 2002). The main goal of inflammation is to expedite repair of damaged tissue, either by aiding in sterilisation of infected tissue and/or by promoting the repair of infected or damaged tissue. This complex process requires the integration of many cellular and molecular mechanisms, but the principal aspect of inflammation that is of interest in the context of this thesis is the trafficking of immune cells from the blood, across the endothelial cells and other structural components of the blood vessel wall and then into inflamed tissue (Luu et al., 2013).

During inflammation, cytokines such as tumour necrosis factor-alpha (TNF-α) Interleukin-1β (IL-1β) and interferon (IFN-γ) are released from stromal cells at the site of injury/infection where they act locally on endothelial cells to induce gene expression programmes that support leukocyte trafficking. These agents are thought to derive primarily from tissue macrophages, although other cells such as fibroblasts are quite capable of synthesising inflammatory cytokines (Pober and Cotran, 1990b). Other vaso-active substances such as thrombin and histamine can also induce adhesion receptor expression more quickly (in minutes) than cytokines (in hours). Some bacterial products, e.g. lipopolysaccharide (LPS or endotoxin) can also directly activate endothelial cells with the same consequences for leukocyte recruitment.

As a result of endothelial cell activation, leukocytes are recruited to the vessel wall and activated locally so that they can penetrate the tissue in a precisely targeted fashion (Ley

et al., 2007). During inflammation, leukocytes transmigrate through postcapillary venules into inflamed tissue. These vessels are marked as sites of low shear stress which facilitates leukocytes attachment to the vessel (Springer, 1995). Specialised receptors of the selectin and immunoglobulin super family (IgSF) families are unregulated on the activated endothelial cells of these venules and mediate the first contact between leukocyte and the vessel wall. (McEver et al., 1989).

During inflammation, vessels dilate due to the release of Prostacyclin (PGI) and nitric oxide (NO) which relax vascular smooth muscle. As a result, blood flow and the delivery of leukocytes to the site of injury increases. Experimental studies have shown that vasodilatation is very important to inflammation (Pober and Cotran, 1990a). As a consequence of vasodilatation, plasma leakage increases, so elevating the haematocrit inside the micro-vessels which leads to change in blood rheology. The low shear stresses and increased haematocrit allow aggregation of red blood cells in rouleaux which force white blood cells to the vessel wall in a process termed margination, thereby increasing the rate of leukocytes-vessel wall collisions and maximising the localised recruitment of leukocytes (Schmid-Schönbein et al., 1980).

Inflammatory cytokines such as TNF-α and IL-1β modulate the expression of endothelial adhesion molecules which are essential to initiate the leukocyte adhesion cascade. This cascade is a multi-step process by which leukocytes enter the inflamed tissue to carry out the immune response. The cascade consists of four main steps as seen in the figure 1-6 below: step 1- Capture and rolling; step 2 - Activation and firm adhesion; step 3- Endothelial transmigration; step 4 - Basement membrane migration (Ley et al., 2007).

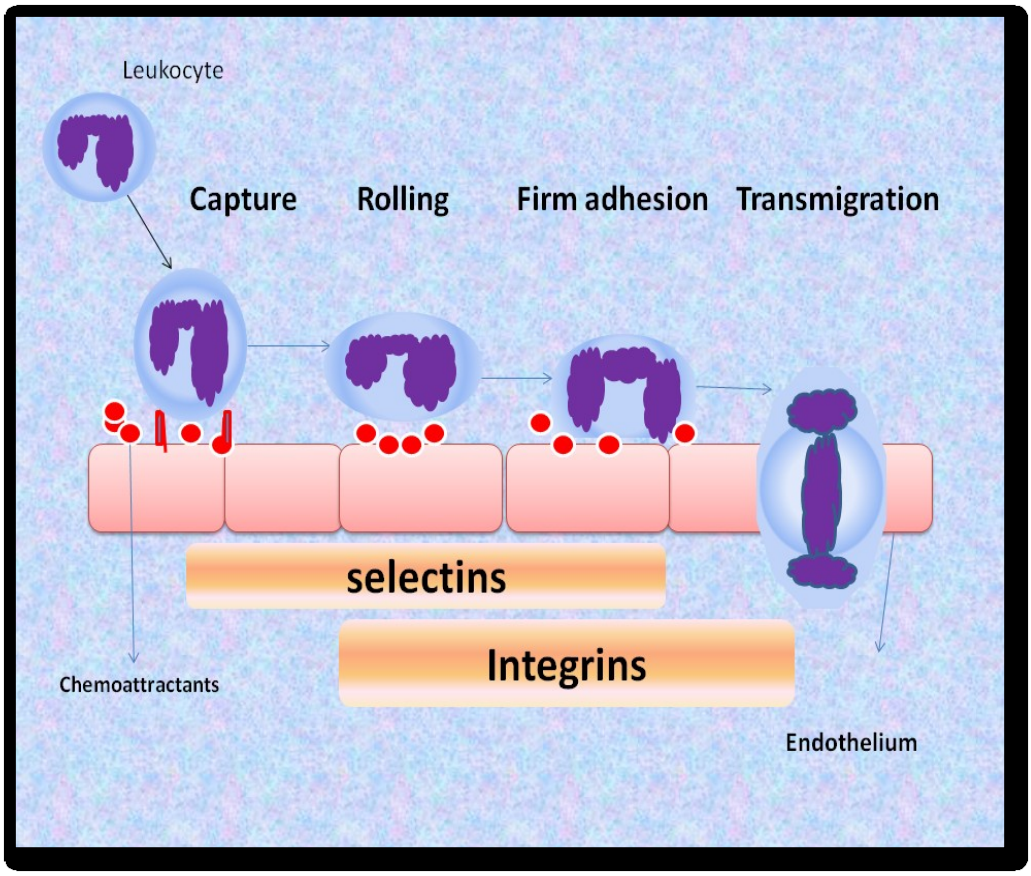


Figure 1-6: Leukocyte Adhesion Cascade modified from (Ley et al., 2007).

This cascade is a multi-step process by which leukocytes enter the inflamed tissue to carry out the immune response. During inflammation, cytokines such as tumour necrosis factor-alpha (TNF-α) Interleukin-1β (IL-1β) and interferon (IFN-γ) are released from stromal cells at the site of injury/infection where they act locally on endothelial cells to induce gene expression programmes that support leukocyte trafficking. As a result of endothelial cell activation, leukocytes are recruited to the vessel wall and activated locally so that they can penetrate the tissue in a precisely targeted fashion. Finally, leukocytes transmigrate through postcapillary venules into inflamed tissue. These vessels are marked as sites of low shear stress which facilitates leukocytes attachment to the vessel.

Step-1: Capture and rolling

The molecular processes that support the initial interactions between blood borne leukocytes and the endothelial cells lining post capillary venules (the site in the blood vasculature where leukocytes are recruited during acute inflammation) are well described (see Figure 1-6 for a schematic representation of the steps in the leukocyte recruitment process). Therefore, in response to the localised production of inflammatory mediators, such as histamine, or the cytokines tumour necrosis factor-α (TNF), Interleukin-1-beta (IL-1β) and interferon-gamma (IFN-γ), activated endothelial cells decorate themselves with specialised adhesion receptors that form strong but short-lived bonds with counter ligands on the leukocyte surface (Huo et al., 2000) Thus, the selectins and the immunoglobulin super-family (IgSF) member, vascular adhesion molecule-1 (VCAM-1), are capable of tethering leukocytes from flowing blood (Barreiro et al., 2002). The sequential formation and dissolution of these bonds also supports a characteristic and dynamic form of adhesion, referred to as rolling (Lawrence et al., 1995). Rolling adhesion does not require leukocyte activation. However, leukocyte migration through the vessel wall and into the inflamed tissue is still dependent upon the receipt of an activating stimulus (Lawrence et al., 1995, Muller, 2009)

P selectin is found in the α granules of platelets and also in Weibel-Palade bodies of endothelium and is released from these granules stores within minutes in response agents such as histamine or thrombin (Butcher, 1991). Endothelial cells also express P selectin over slower time scales in response to cytokines such as TNF. Whether this requires de novo protein synthesis or mobilises granule stores is not clear. E selectin is induced on endothelial cells as a result of stimulation of cytokines IL-1 or TNF or endotoxin (Bevilacqua et al., 1994, Butcher, 1991). It requires de novo synthesis and in vitro expression peaks at around 4-6

hours post-stimulation and wanes by 24h. L selectins are constitutively and highly expressed on most circulating leukocytes (Bevilacqua and Nelson, 1993).

The counter receptors for selectins are sialylated carbohydrates (Bevilacqua and Nelson, 1993), in particular glycoproteins which carry the sialyl Lewis X (sLeX) motif (Smith, 2008). The major ligand for P selectin is P-selectin glycoprotein ligand 1 (PSGL1); it was initially described as a ligand for activated platelets, although E-selectin can also bind this molecule. (Ley et al., 2007). Specific ligands for E-selectin have also been identified, and endothelial cell-borne ligands for L-selectin have also been described, although it is not clear whether these are expressed on human post-capillary venules during inflammation (Hidalgo et al., 2007). They are, however, expressed in other vascular beds such as the HEV of lymph nodes, where L-selectin plays a critical role in trafficking to these organs.

Interestingly, the interaction between selectins and their ligands requires shear stress to sustain adhesion (Ley et al., 2007). Indeed, an important feature of selectin bonds is the catch bond phenomenon, where the strength of the adhesive interaction with the ligand increases with increasing shear stress. During leukocyte adhesion, selectin bonds are strengthened when shear stress is applied and weakened when the shear stress is absent leading to detachment of the rolling cells (Phillipson et al., 2006). During capture and rolling, there is a rapid translation of the adhesive zone between leukocyte and endothelium cell along the vessel wall. Therefore, there is a need for rapid adhesive bond formation and breakage (Chen et al., 1997). The forward motion of leukocyte increases the force applied on bond formation until it balances the hydrodynamic by force which leads to the leukocytes stopping. Finally, the rolling leukocytes undergo a jerky rolling motion because of the failure of the weakened bond in the rear and at the same time a front bond is formed as the leukocytes roll forward (Sundd et al., 2011).

There is some evidence indicating that ligation of selectins can signal into leukocytes and mediate their activation. Therefore, human neutrophils rolling on E selectin becomes a stationary adherent due to integrin activation via p38 MAPK (mitogen-activated protein kinase) pathway (Simon et al., 2000) (Ley et al., 2007).

Furthermore, leukocyte rolling can be mediated by other receptors. The most common one in inflammation is the IgSF molecule VCAM-1 which, like selectins, is expressed by de novo synthesis in EC upon exposure to inflammatory cytokines. The counter ligand(s) for VCAM are integrins. Integrins are heterodimeric molecules consisting of α and β subunits linked together by non covalent bonding. The α and β subunits have N terminal portions which join together to form a ligand binding site (Smith, 2008). The major ligand for VCAM-1 on leukcoytes is the α4β1 integrin, also called the very late antigen-4 (VLA4). This is present on many mononuclear leukocytes, e.g. monocytes and some lymphocyte subsets, however, it is absent on human neutrophils. A fuller description of the integrins utilised for leukocyte recruitment and their function is provided in the section below.

Step-2: Activation and firm adhesion

In response to inflammatory cytokines, endothelial cells synthesise and present activating stimuli on their surface. During rolling, leukocytes are exposed to these stimuli which trigger activation of the β1 and β2 integrins which mediate leukocyte arrest (Campbell et al., 1998). Chemokines (chemotactic cytokines) are the major activating signals synthesized and transported to the luminal surface by the activated endothelium (Ley et al., 2007). In some disease models chemokines from other cells such as platelets can be deposited on the endothelium. CCL5 (CCL chemokine ligand 5) and CXCL4 (CXC-chemokine ligand 4) have both been described to be deposited by platelets on to the endothelium leading to the

arrest of rolling monocytes (von Hundelshausen et al., 2005). Chemokines have high affinity for specific receptors borne by the leukcoytes, and these can oligomerize and make heterophilic interactions that modulate leukocyte responses. Thus, for example, monocytes arrest triggered by CCL5 can be amplified by CXCL4 (von Hundelshausen et al., 2001). Chemokines are bound and presented on the endothelial cells surface polysaccharides units on glycosaminoglycan molecules (GAGs) which may protect the chemokine from cleavage by proteases released at the site of inflammation. (Ley et al., 2007).

The adhesive activity of integrins is controlled by an inside-out signalling process. In response to stimulation by chemokines, the leukocyte alters the activation status of the integrin molecules from a confirmation that does not recognise ligand to one that is competent to bind ligand (Harburger and Calderwood, 2009). Changes in integrin function may also include surface clustering which alter the affinity of receptor-ligand interactions (Smith, 2008). IgSF molecules and matrix proteins are the predominant ligands for integrins. In the context of leukocyte trafficking across EC the former are the important ligands. Intercellular adhesion molecule (ICAM) is a major counter ligand for the β2 integrins (αLβ2 and αMβ2; LFA-1 and MAC-1 respectively), while α4β1 is the major ligand for VACM-1. Both of these IgSF molecules are upregulated on endothelial cells in response to cytokine stimulation (Smith, 2008).

Step-3: Endothelial transmigration

Transendothelial migration into tissue is the last phase in leukocyte emigration. There are two known mechanisms that support this process. Firstly, leukocytes may undergo paracellular migration, i.e. migration between the junctions of adjoining endothelial cells. (Schenkel et al., 2002),(Phillipson et al., 2006). Alternatively migration can occur by the

transcellular route, whereby leukocytes migrate through the body of the endothelial cells. (Phillipson et al., 2006). As discussed above, leukocyte transmigration is triggered by the chemo-attractants which are presented on the luminal surface of activated endothelium, a process dependent upon activation of integrin molecules. When integrins bind their endothelial ligands they stimulate the formation of endothelial projections called transmigratory cups. The surface of these cups are rich in VCAM-1 and ICAM-1 and they are organised by the movement of cytoskeletal proteins (Carman and Springer, 2004); (Barreiro et al., 2002). Indeed, cytoplasmic signalling events leading to cup formation are triggered by the ligation of ICAM-1 by integrins which leads to the translocation of apical ICAM-1 into caveolae and regions rich in F-actin (Millan et al., 2006); (Cinamon et al., 2004) (Figure 1-7).

- Transcellular and paracellular migration

During paracellular migration a number of molecules at the junction of endothelial cells appear to be important regulators of the process. For example ICAM1, ICAM2, members of the junctional adhesion molecule family (JAMs A, B, and C), CD99 and PECAM1 (CD31) have been ascribed regulatory roles. Endothelial junctions also appear to support transendothelial migration through loosening of the intercellular junctions by reorganising local adhesion receptors such as VE-cadherin. As an example, ESAM (endothelial cell-selective adhesion molecule) loosens such junctions by interfering with the homotypic binding of VE-cadherin (Wegmann et al., 2006).

The transcellular pathway appears to occur in restricted circumstances. For example, it appears to be common for leukocyte trafficking into the central nervous system (CNS) through the blood brain barrier and has been observed in some other inflammatory situations (Feng, 1998); (Engelhardt and Wolburg, 2004). Studies in HUVECs showed that only ~5–

20% of migrating cells used this pathway (Carman and Springer, 2004). However, in our own laboratory, and after 20 years of observing of neutrophil, monocyte and lymphocyte migration across EC of varying origin, transcellular migration has not been witnessed (personal communication Prof. Ed Rainger). Since migration proceeds through the cell body, a gateway is required for passage across the cytoplasm, which is an organelle that has been termed as the vesiculo-vacuolar organelle (VVO) (Dvorak and Feng, 2001). Until now, VVOs have not been seen in vitro (Ley et al., 2007).

- Basement membrane migration

In fact there are three barriers encountered by transmigrating leukocytes: the endothelial cells that have just been discussed, the basement membrane of the endothelial cells, and the pericytes (Ley et al., 2007). Passage across endothelial cells occurs rapidly i.e. within 2-5 minutes, however, passage across the basement membrane can take considerably longer i.e. 5-15 minutes. The nature of the basement membrane, which is deposited by both endothelial cells and pericytes, appears to be essential in regulating the process of migration with deposition of structures that can either assist or inhibit transmigration. The endothelial basement membrane consists of a network of protein, predominantly made up of collagen type IV, laminin-8 and laminin-10, linked together by basement membrane proteins such as heparin sulphate and proteoglycans like perlecan-1 and nidogen-2 (Hallmann et al., 2005). Studies on unstimulated mouse cremasteric venules showed areas of low expression of matrix proteins within the basement membrane, where the expression of vascular laminins was low. This suggests that these may be areas that enable the efficient exit of neutrophils (Wang et al., 2006) and T lymphocytes (Sixt et al., 2001). Studies on IL-1β stimulated mouse cremasteric venules revealed that the transmigration of neutrophils temporarily enlarges these areas which co-localise with gaps between pericytes (Wang et al., 2006). (Wang et al., 2006).

These areas of low BM expression are permeable to chemo-attractants released from extravascular tissues and may be important for generating a chemotactic gradient into the tissue. Indeed, heparin sulphate which binds to chemokines may serve as a depot of such chemotactic agents (Miyasaka and Tanaka, 2004).

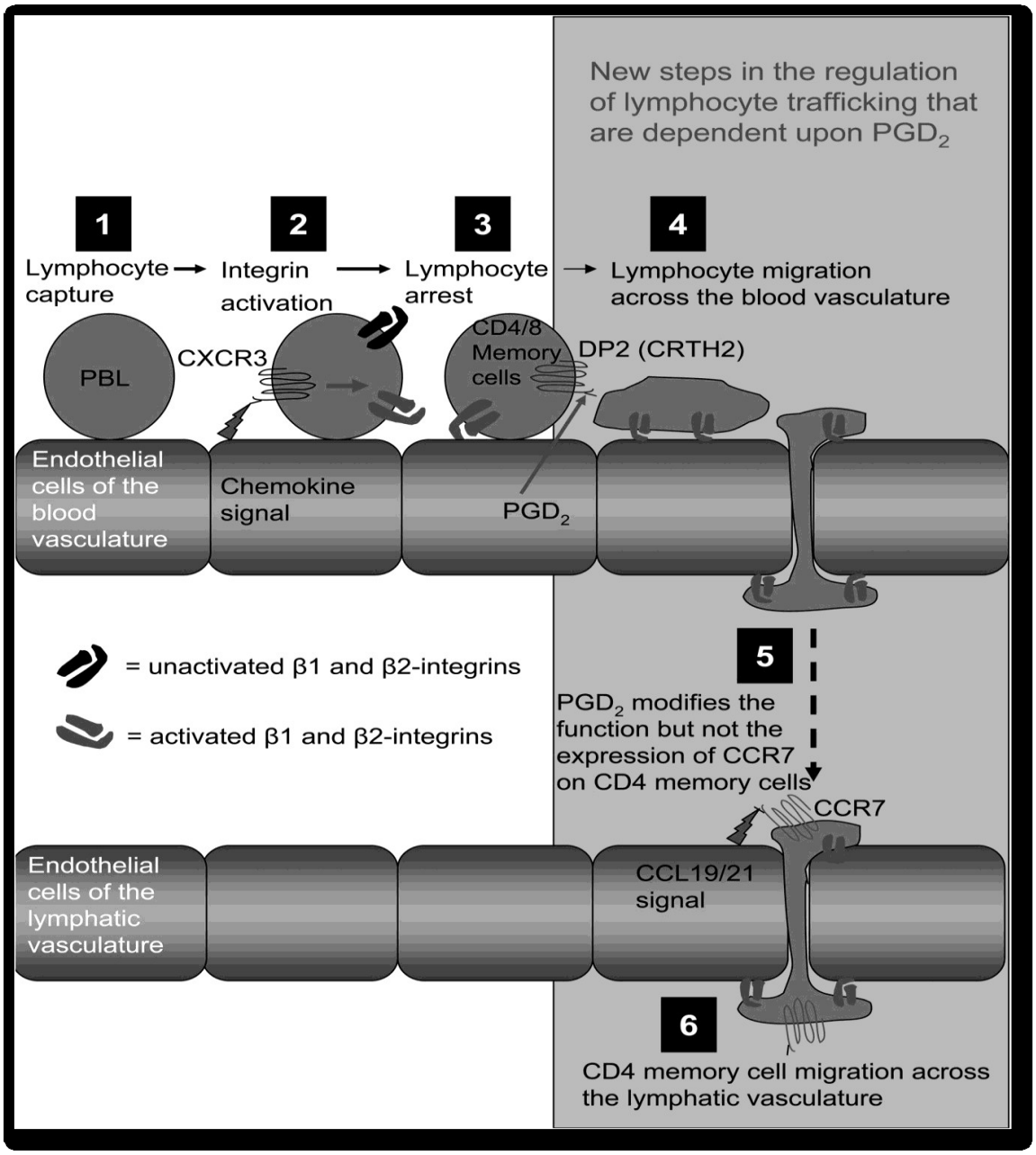


Figure 1-7: the Regulation of CD4+CD45RO+ T-cell trafficking across blood vessels endothelium (BVE).

BVE is stimulated by Cytokine (TNF and IFN) which leads to memory T-cell recruitment. Then T-cells receives stimulating chemokines (CXCL9–11) which is necessary for integrins activation which subsequently causes T-cell activation and immobilization on BVEC surface. After that, the DP-2 receptor is stimulated by PGD2 which is required for T-cell transmigration through the BVEC monolayer. The lymphocyte chemokine receptor CCR7 function is promoted by PGD2 signals which leads to CD4+CD45RO+ T-cells transmigration across the lymphatic vasculature in response to CCL19 and CCL2 (Ahmed et al., 2011).

8. Chronic inflammatory diseases:

In the 1980s researchers from the University of Washington noticed the presence of macrophage, T-cells, and IFN-γ in diseased tissue, and since then it has been widely accepted that inflammation is an important contributor to chronic diseases such as diabetes, cancer, and atherosclerosis (Couzin-Frankel, 2010, Romano et al., 1997). The main distinguishing feature of chronic inflammation is the persistence of the inflammatory response. This may be due to prolonged production of pro-inflammatory mediators such as interferon-gamma (IFN-γ) and tumour necrosis factor α (TNF- α) (Davies et al., 2001). Alternatively, the inflammatory response may escape the resolution programmes that ordinarily play a role in terminating the process. Thus, it could be argued that inflammation starts as an acute response which then transforms into chronic disease with altered patterns of leukocyte recruitment. The mechanism of this transformation is not clearly understood, but there is data suggesting that the IL-6/soluble IL-6 receptor (sIL-6R) complex is a key factor in this process in diseases such as arthritis (Gabay, 2006). In fact, in vivo studies have demonstrated a significant role of IL-6 in leukocytes recruitment by regulating the expression of inflammatory chemokines and adhesion receptors on endothelium (Romano et al., 1997).

8.1. Atherosclerosis and inflammation:

Atherosclerosis is a disease that takes decades to develop, during which period it is asymptomatic. However, upon initiation of athero-thrombotic disease which leads to heart attack and stroke, it manifests into the disease with the most fatalities in the westernised world. Many suggest that inflammation is the driving force for the atherosclerotic plaque progression. The mechanisms of disease development are multifactorial. However, most studies now agree that monocytes recruited to the artery wall are vital in the induction of the atherosclerotic plaque. Monocytes that are recruited to the vessel wall ingest cholesterol from oxidized low density lipoproteins that are deposited in athero-prone areas of the arterial circulation. These subsequently form foam cells which release inflammatory mediators, attracting vascular smooth muscle cells along with other immune cells which contribute to the formation of plaque (Figure 1-8) (Glass and Witztum, 2001). The process of monocyte recruitment is poorly understood, but intervention in this process is considered a good area for developing strategies to treat and prevent cardiovascular disease. The inflammatory nature of atherosclerosis is marked by the mediators that can be found in the circulation of patients with established diseases. These markers are classified into endothelial activation markers (ICAM-1 and CD40L, soluble VCAM-1), non-specific inflammatory factors (TNF-α, interleukin-1, -6 and -18), and thrombotic endothelial dysfunction factors (PAI-1 Plasminogen Activator Inhibitor-1) (Balanescu et al., 2010). In addition the acute phase protein and nonspecific marker of inflammation, C-Reactive Protein (CRP), appears to correlate with the risk of having a heart attack, with progressively greater risk being evident for every milligram increase above 1 mg/l up to 20 mg/l (Kondo et al., 2005). Interestingly, there does not seem to be a direct link between CRP or other inflammatory markers and the burden of plaque (Balanescu et al., 2010). Other studies have demonstrated the relationship between carotid

intimal thickening and the circulating levels of adhesion molecules such as sICAM-1 and sVCAM-1 (Kondo et al., 2005). Nevertheless, there are other trials that reject this association based on a lack of correlation (Luc et al., 2003).

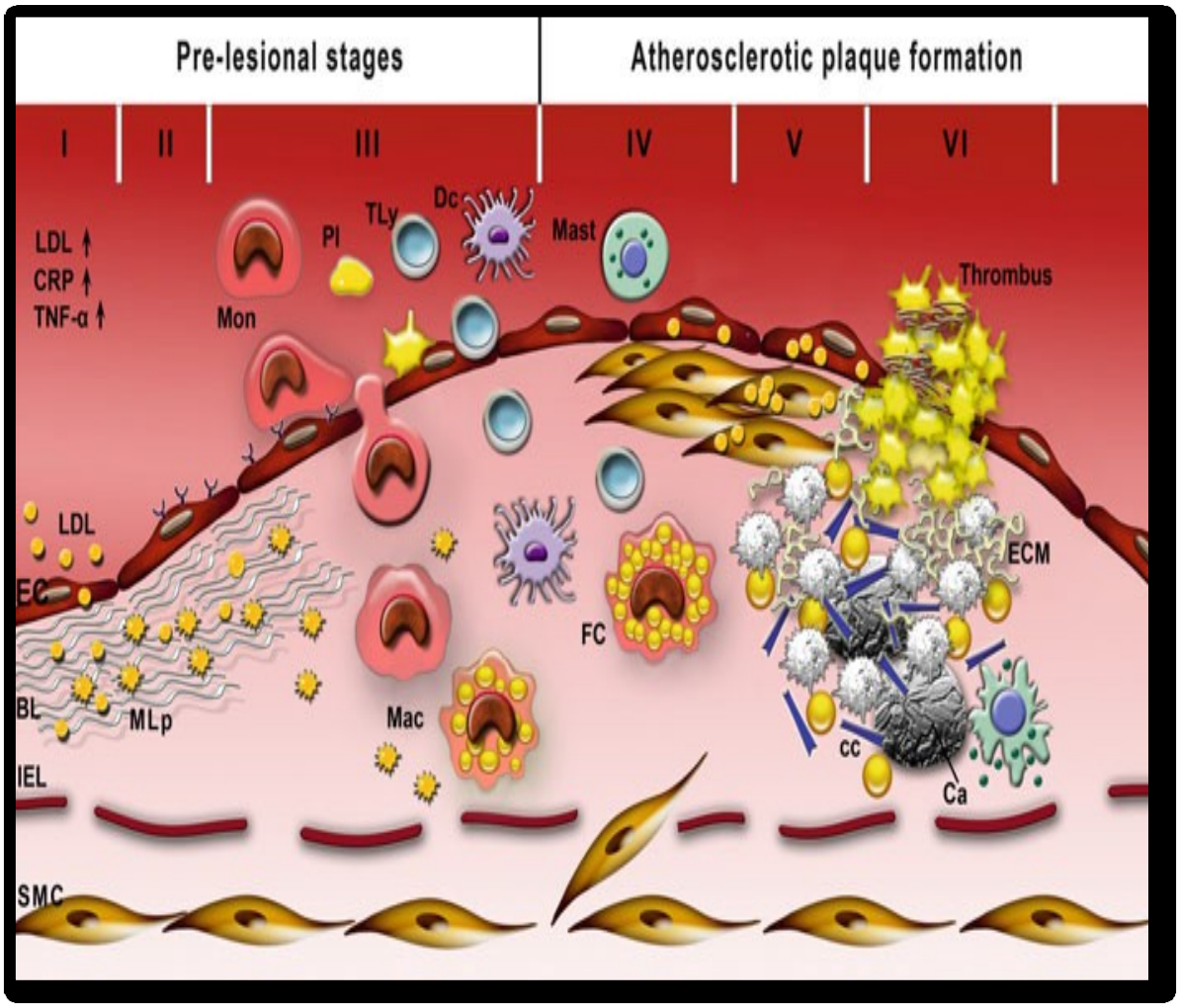


Figure 1-8 Atherosclerosis progression stages (Simionescu and Sima, 2012).

LDL (Low-density lipoprotein), CRP (C - reactive protein), Mon (Monocytes), EC (Endothelial cell), MLp (Lipoproteins), Pl (Platelets), TLy (T lymphocytes), Dc (Dendritic cells, Mac (Macrophages), FC (Foam cells), SMC (Smooth Muscle Cells), ECM (Extracellular matrix), cc (cholesterol crystals), Ca (Calcification cores, BL (Basal lamina).

8.1.1. The stages of plaque development in atherosclerosis:

Atherosclerosis is a progressive disease marked by plaque formation, and is the focal development of lesions within arterial walls. It affects vessels supplying a number of organs, but is always restricted to the arterial circulation. Initiation, progress and eventual fate of atherosclerotic plaque is determined by complex interactions between resident cells of the arterial wall (EC and SMC), blood components (in particular T-lymphocytes, monocytes, dendritic cells, mast cells) and pro-inflammatory mediators secreted by them such as chemokines, cytokines, enzymes (proteases). (Holme et al., 2012). Classical epidemiology suggests that vasoconstrictor hormones implicated in hypertension, products of glycoxidation associated with hyperglycaemia, pro-inflammatory cytokines, dyslipidaemia, and smoking are some of the factors which make a person vulnerable to Atherosclerosis (Holme et al., 2012).

- Stage 1: Modulation of Constitutive Functions of Endothelial cells

Due to their anatomical position, endothelial cells are greatly affected by any changes in blood physiology or interstitial fluid homeostasis. Thus, the constitutive functions of EC are modulated as a result of changes in homeostatic pathways regulating such processes as lipid metabolism (hyperlipidemia), glucose metabolism (hyperglycemia), and inflammation. There can also be secondary effects of these disturbances on the localised environment in the artery wall, for example, in experimental models of atherosclerosis changes in the barrier functions of EC leads to a prominent increase in transcytosis of lipoproteins into the artery wall in experimental models of atherosclerosis (Vasile et al., 1989). In response to the modifications in permeability there is a resulting deposition of plasma LDL within the intima. This explains the close positive correlation, experimentally established, between permeability of aortic LDL and the cholesterol accumulation in a particular segment. Animal (Faggiotto et

al., 1984) (Simionescu et al., 2004) and human models (Kruth, 1984) (Tirziu et al., 1995) of atheroma formation show that the LDL enclosed in Tunica intima becomes chemically modified by oxidation to generate oxidatively modified lipids (MLp). MLp(s) are heterogeneous structures with high levels of unesterified cholesterol and can often be visualised as aggregated or fused lipoprotein particles in experimental models (Tirziu et al., 1995) (Kruth, 1984). Importantly aortic permeability for LDL predicts the localisation of cholesterol-induced atheroma formation in experimental models of disease. (Nielsen et al., 1992).

Studies have long indicated that there is an increase of circulating oxidised LDL level in the plasma of patients with cardiovascular diseases (CVD) (Holvoet et al., 1998). In addition, it has been reported that oxidised LDL is used to distinguish between patients with coronary artery disease (CAD) from healthy subjects (Toshima et al., 2000). In a study by Huang, H et al, it was reported that the plasma level of oxidised LDL is 6 times higher in acute myocardial infarction (AMI) patients than in healthy subjects (Huang et al., 1993). This report suggests that oxidised LDL could be a reliable risk factor for CVD.

A circulating pentraxin significant in the native human immune response, known as C-reactive protein also acts as a stable plasma biomarker for low-grade systemic inflammation. There remains a persistent risk of cardiovascular events in association with plasma levels of the CRP, especially in patients having stable angina and established CVD (Tsimikas et al., 2006). The EC also changes phenotypically as a consequence of this low grade systemic inflammation. The EC adopts a secretory phenotype, having multiple copies of the rough endoplasmic reticulum, Golgi apparatus, centrioles and numerous caveolae. The EC also undergoes functional changes, generating a hyperplasic multi-layered basal lamina which can entrap MLp (Tsimikas et al., 2006) (Simionescu, 2007). An altered response of the vessel wall

to external stimuli is also observed because of the disruption caused in myo-endothelial junctions and gap junctions between neighbouring SMC due to the proliferation of the basal lamina and the ECM.

- Stage 2: Endothelial cell Dysfunction

Endothelial cell dysfunction is demonstrated through the alterations in anti-adhesive and anti-thrombogenic surfaces usually presented by EC to flowing blood. In the early stages of atherosclerosis in experimental models, surface changes are manifested by the expression on the EC membrane of new or additional cell adhesion molecules, such as ICAM-1, E-selectin, and P-selectin, VCAM-1 and chemokines such as CX3CL1 and CCL5, CCL2 (Monocyte chemoattractant protein-1;MCP-1) and CXCL8 (interleukin-8 ;IL-8), all of which can promote the recruitment of inflammatory leukocytes such as monocytes (Simionescu, 2007, Boisvert et al., 1998). Notably, expression of these molecules is also evident on human atheroma (Haley et al., 2000).

- Stage 3: Robust Inflammatory Reaction

This stage is characterized by the adhesion and recruitment of monocytes into the arterial intima using the molecular pathways previously described. Platelets also appear to play a major role in initiating plaque formation by assisting in the recruitment of blood monocytes. Indeed, platelets appear to participate in all stages of development of atherosclerosis, in addition to their role in arterial thrombosis, which is the terminal and symptomatic stage of atherosclerotic disease. In vitro studies have shown that ECs present on their surface von Willebrand factor (vWf) (Ohashi et al., 2010). The recruitment and adherence of platelets to the intact EC surface is supported by platelet glycoprotein Ib (GPIb). It now seems probable that pro-inflammatory chemokines (CXCL4 [platelet factor -4],

RANTES [CCL5] are deposited on the ECs by activated platelets as a result of this adhesion. (Davì and Patrono, 2007). These molecules are potentially responsible for promoting monocyte binding to the vessel wall and supporting their migration into the intima.

- Stage 4: Recruitment of other leukocytes during atherogenesis

In the early stages of atherosclerotic plaque formation CD4+ T-cells along with antigen-presenting dendritic cells and some CD8+ T-cells can be found in the intima (Hansson, 2005). Circulating T-cells are probably recruited by the same pathways as monocytes, although definitive information on this is lacking. Within the intima antigens like MLp, it may be possible to induce T-cell proliferation within the plaque (Packard et al., 2009). In addition, type 1 helper T-cells (Th1) have been reported to become activated and to express and secrete a large array of cytokines (Romagnani, 1991). These agents may contribute to macrophage activation and the potentiation of the inflammatory response (Monney et al., 2002).

B-cells have also been associated with diseased sections of the artery, but perhaps surprisingly were first found within the adventitia (Majesky et al., 2011). Immunoglobulin-positive cells have also been found within atherosclerotic plaques (Majesky et al., 2011). The immune response during the development of the atherosclerotic plaque may be directed by B-cells and their immunoglobulin products may perform protective functions during the plaque progression according to evidence from animal models of disease (Simionescu and Sima, 2012).

The role of polymorphonuclear neutrophils (PMN) was thought to be insignificant in atherosclerosis until recently. However, a relationship between the numbers of circulating ‘activated’ PMN and the extent of coronary artery disease, as well as their presence in lesions,

has been reported in human disease and animal models (Soehnlein et al., 2008). How PMN contribute to the disease process is not clear, but release of oxidants and pro-inflammatory mediators in the vessel wall may promote or amplify the recruitment of other inflammatory cells, or additionally contribute to the oxidation of LDL (Soehnlein et al., 2009). Others have suggested that the activity of PMN in mature plaques may contribute to both destabilization and vulnerability, precursors of rupture and induction of infarction (Drechsler et al., 2010). Interestingly, aneutrophilia induced by hypercholesterolemia in murine models of atherosclerosis has been reported. In this model PMN were being recruited during the early stages of atherosclerosis which indicates a role in the initiation of disease. A significant effect of PMN in atherosclerosis in this murine model was strongly indicated by the blunting of lesion progression in ApoE-/- mice by depletion of circulating PMN (Soehnlein et al., 2009).

Although the ApoE-/- model indicates a possible role for PMN in atherosclerosis, these cells are still exceedingly rare in human plaques. This is surprising as the adhesion molecules and chemokines required for their recruitment are present on the endothelial cells of atherosclerotic lesions. It may be that rapid apoptosis of the PMN within the plaque may be the cause of the lack of PMN found in atherosclerotic lesion (Zernecke et al., 2008). Indeed there is a possibility that apoptosis is induced by the cytotoxic effect of free fatty acids released from modified lipoproteins within the artery wall (Lux et al., 2009).

In monocytes that are recruited to the intima, a program through which scavenger receptors (i.e. SR-B1 and CD-36) are up regulated is thought to be responsible for their differentiation into cholesterol loaded foam cells. In the lesion monocyte/foam cell borne scavenger receptors are required to assimilate MLp, although their physiological roles also include uptake of advanced glycosylation end-products, anionic phospholipids along with apoptotic cells. The switch of differentiated monocytes into cholesterol-loaded foam cells is

facilitated by this non-regulated uptake of the MLp. In the earliest of lesions, the fatty-streak, accumulations of foam cells are the major pathogenic alterations in the artery wall. However, foam cells can be identified at all stages of plaque progression. (Tiwari et al., 2008).

- Stage 5: Fibrous Plaque Formations.

There is now significant evidence that indicates that the intimal thickening termed the fatty streak is the most likely precursor of symptomatic coronary disease. Hence, fatty streak lesions can be found in children in similar locations as to where advanced plaques are found in adults, although interestingly fatty streaks are known to be able to regress (Velican and Velican, 1985). Areas of intimal thickening that develop into more advanced plaques may be either eccentric or diffuse in nature and both of these patterns of disease can occur adjacent to the other. The eccentric type of intimal thickening tends to be distinct and involves up to half of the circumference of the arterial wall. The locations for such thickenings include branch points and areas of disturbed blood flow (Velican and Velican, 1985). Molecular messages are sent by inflammatory cells through the factors they secrete within the plaque which reinforce the inflammatory response. However an important aspect of plaque progression is the recruitment of vascular smooth muscle cells (SMC) into the advancing plaque, with the clonal accumulation of SMC within the intima being reported (Simionescu, 2007).

The migration of the SMC from the media into the intima through the partially degraded internal elastic lamina is a very significant step in the formation of the plaque. It is worth highlighting, however, that sources of intimal SMC other than the media have been postulated. These include circulating bone marrow-derived circulating progenitor cells and vascular progenitor cells present in the adventitia of the artery. An important aspect of the SMC biology, in the context of atheroma formation, is the phenotype of the cells within the

intima. These SMC have a secretory phenotype. Secretory SMC are structurally and functionally different form the contractile SMC of the media. They are hyperplastic and form multi-layered basal lamina and extracellular matrix which is enriched in collagen bundles and fibrils (Louis and Zahradka, 2010). Secretory SMC also releases inflammatory cytokines and chemokines (e.g. CCL2 and CXCL8) which again reinforces the likelihood of inflammatory process (Louis and Zahradka, 2010). These changes are probably an adaption for survival in the alien environment of the arterial intima, however, they then appear to contribute to disease progression (Simionescu et al., 2004). Interestingly, in hyperlipemic hamsters intimal SMC can accumulate lipid droplets and adopt a foam cell-like phenotype (Simionescu, 2007).

Fibrofatty lesions may develop to a size large enough to significantly occlude blood flow in the artery and this can produce symptomatic disease, or angina pectoris, which is a pain across the chest caused by ischemia of the myocardium usually upon increased metabolic demand as a result of mild exercise or even from stressful situations. A similar situation is evident in claudication of the extremities (usually the legs), when disease in the femoral arteries leads to compromised blood flow to skeletal muscle (Condorelli and Brevetti, 2002). In peripheral vascular disease (again prevalent in the legs), loss of perfusion in the extremities leads to localised hypoxia and ulceration of the skin. This can lead to complications such as gangrene as the compromised perfusion of the tissues fails to deliver immune cells and proteins to the affected area (Magnoni et al., 1996, Regensteiner et al., 1993).

- Stage 6: Plaque Rupture and Arterial Thrombosis

Plaque rupture or ulceration is the terminal stage of cardiovascular disease although the precise mechanisms supporting these processes are yet to be described. In ulceration, endothelial cells become fragile and prone to erosion. Loss of EC leads to exposure of the

ECM which has an abundance of pro-coagulant and pro-thrombotic constituents (Simionescu and Sima, 2012). Upon contact between these agents and circulating blood there is rapid thrombus formation. (Simionescu, 2007). Plaque rupture may also be caused due to the mechanical stress generated by arterial blood flow on the cap of vulnerable plaques. Vulnerable plaques are marked by a high content of lipid and foam cells. They also exhibit thinning of the fibrous cap. (Tabas et al., 2010). Cap thinning may occur due to reductions in the synthesis of ECM by cells such as SMC. However proteolytic digestion of the extracellular matrix by matrix metaloproteinases (MMPs) can also contribute to thinning of the fibrous cap. (Berliner et al., 1995) (Simionescu, 2007). Other proteases (tryptase and chymase) are proposed to operate after secretion by mast cells that are located in the rupture-prone shoulder regions of the plaque. The result of such ulceration or rupture is the formation of a platelet-rich mural thrombus and initiation of thromboembolic disease. (Brown and Goldstein, 1990).

The blockage of vessels with thrombus is linked with ischemic episodes e.g. cerebral infarction and acute coronary syndrome. Occlusive thrombi are reported to cause around 80% of sudden coronary death.

9. Adipokines

In the context of this thesis it is important to introduce a family of agents generated from adipose tissue that play an important role in physiological homeostasis and in disease. For a long time it was believed that adipose tissues were solely depots for the storage of excess calories in the form of fats, in particular triglycerides. However, we now appreciate that adipose tissues play an important role in immunological and metabolic functions. These processes are mediated by a group of factors called adipocyte-derived cytokines, or adipokines. (Lang and Ratke, 2009). Adipokines were first described in the 1990s, and approximately 20 such agents have been recognized and classified (Housa et al., 2006). Of these, adiponection and leptin are the most studied because they play important roles in managing energy balance, as well as in connecting metabolic pathways with immune function (Lang and Ratke, 2009).

9.1. Leptin

Leptin is a polypeptide encoded by the obese gene (ob) to produce a 16 kDa hormone which is released from adipocytes (Harvey and Ashford, 2003). It appears to play an important role in processes as disparate as bone formation, angiogenesis, neuroendocrine functions and reproduction. Importantly, amongst its pleiotropic actions is the modulation of the immune responses (Lord, Matarese et al. 1998; Howard, Lord et al. 1999). For example it can regulate the activation of dendritic cells and macrophages and also causes stimulation of Th1 cytokine production from lymphocytes (Mattioli et al., 2005). Modulation of the adaptive immune response is facilitated by leptin mediated increases in T-cell survival (Lam and Lu, 2007). Indeed, many immune cells have been reported to express Leptin receptors (Guzik et al., 2006). There are also reports that leptin plays an important role in the recruitment of

monocytes by cytokine stimulated endothelial cells. (Guzik et al., 2006). Moreover, stimulation of monocytes with leptin can result in the release of inflammatory and angiogenic mediators such as TNF-α, IL-6, CC-chemokine ligand-2/CCL2 and vascular endothelial growth factor/VEGF (Tilg and Moschen, 2006). Leptin also has metabolic functions, such that it crosses the blood brain barrier (BBB). For example, it may cross the BBB by an active transport mechanism where it acts upon the hypothalamus to regulate hunger and reduce food intake (Lang and Ratke, 2009). Genetic ablation of leptin or its receptors in mice, has been found to lead to obesity and insulin resistance (Ceddia et al., 2002). The serum levels of leptin are an indicator of the energy stored in fatty tissue, i.e. its level is high in obese people and decreases after weight loss (Moschen et al., 2009).

9.2. Adiponectin

The 30 kDa adipokine, adiponectin, makes up 0.01% of total plasma proteins as it circulates at levels of approximately 3-30 µg/ml (Arita et al., 2012) and it exhibits a wide range of biological activities. Thus, adiponectin improves the sensitivity towards insulin and effects lipogenesis so that circulating lipids derived from the liver are maintained within physiological bounds (Lang and Ratke, 2009). However in obese individuals, adiponectin levels are significantly lowered which might contribute to the dislipidaemia prevalent in such people (Kern et al., 2003). Expression of adiponectin also decreases in patients with diabetic or cardiovascular diseases (Hotta, Funahashi et al. 2000; Kern, Di Gregorio et al. 2003; Matsubara, Namioka et al. 2003; Kim, Kim et al. 2006; Arita, Kihara et al. 2012). Therefore, similar to Leptin, adiponectin appears to exhibit protective roles by maintaining homeostasis (Lang and Ratke, 2009) (Figure 1-9). Broadly speaking, adiponectin appears to be anti-atherosclerotic and anti-inflammatory, and at least some part of this protection comes from its

ability to regulate the recruitment of leukocytes by endothelium.(Ouchi et al., 2003) (Ouedraogo et al., 2007) (Kelesidis et al., 2006).

T-cell activation and proliferation is also regulated by adiponectin. Moreover, adiponectin may be able to regulate (inhibit) NF-κB activation in endothelial cells (Wang et al., 2014). Adiponectin treatment of cultured macrophages caused inhibition of their phagocytic activity and reduced TNF-α synthesis in response to lipopolysaccharide in these cells (Yokota et al., 2000). There is also some evidence that it regulates NK cell function by modulating the production of cytokines such as IL-2 (Kim et al., 2006). Moreover, It is commonly known that Adiponectin regulates macrophage phenotype, therefore understanding the mechanism as to how AQ promote macrophage polirization is very important in order to improve treatment targets. Macrophage is responsible for accumulating lipids from the endothelium which leads to macrophage transformation to foam cells. Foam cells are a critical factor in early atherosclerotic lesion formation. So, it's fate could lead to either an anti-inflammatory effect by prevention or reversal of cholesterol accumulation, or it could result in a cthrough the pro-inflammatory cthrough the mediators. Recent findings show the protective effect of AQ on macrophage polarization by down regulation of the expression of scavenger receptor A (SR-A) (Ouchi et al., 2001).

There are two major receptors for Adiponectin, AdipoR1 and AdipoR2. Both of these receptors show differential affinities for various circulating types of Adiponectin (Ohashi et al., 2012). AdipoR1 is expressed in a wide variety of tissues, including skeletal muscles, breast tissue and leukocytes, whereas AdiopR2 expression is highest in the liver. Importantly, 93% of monocytes, 47% of B-cells and 21% of NK cells exhibit AdipoR2 expression (Kelesidis et al., 2006).

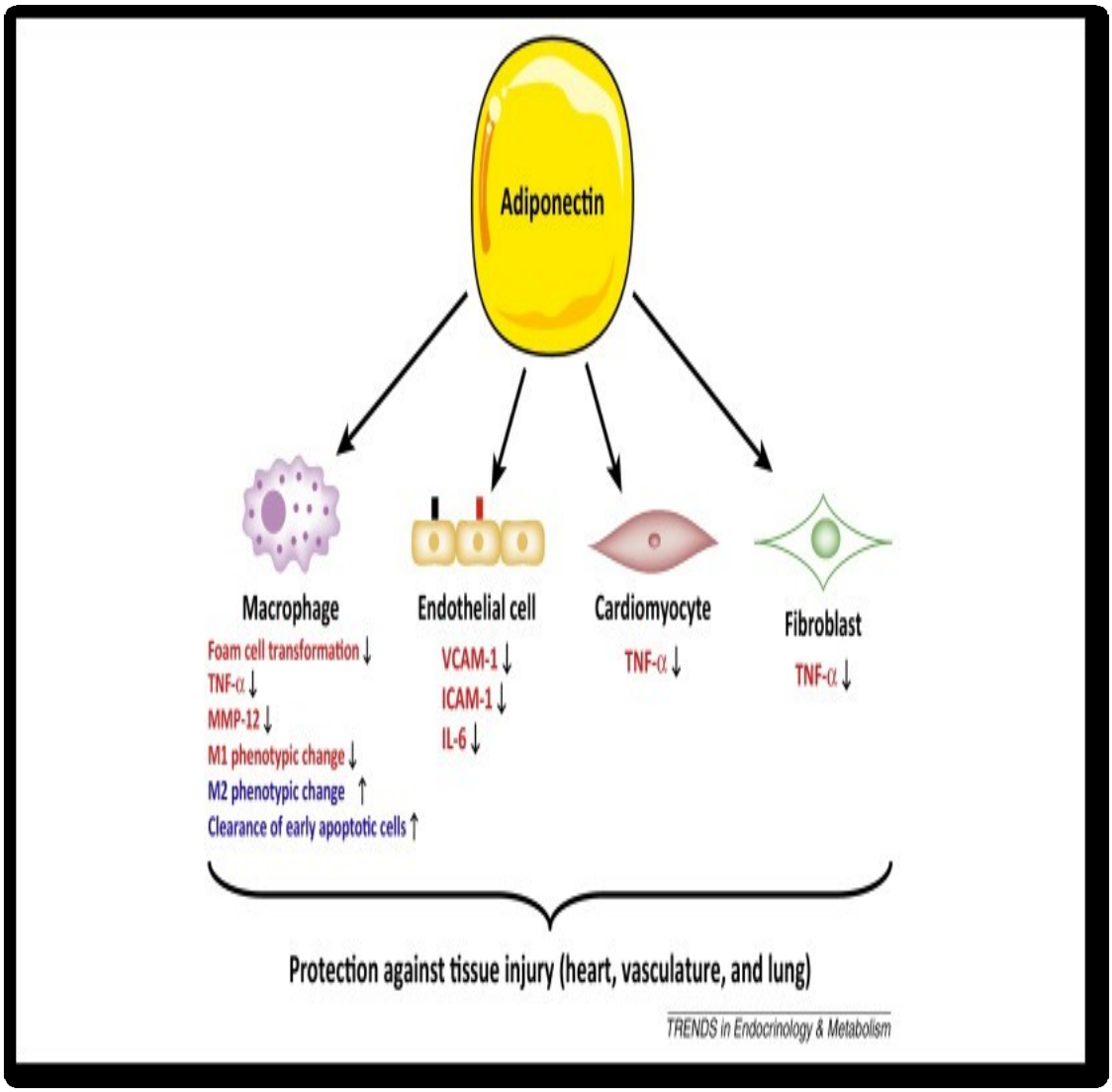


Figure 1-9:Anti-inflammatory role of Adiponectin in obesity-related diseases.

Adiponectin exhibits a wide range of biological activities. Adiponectin appears to be anti-atherosclerotic and anti-inflammatory and at least some part of this protection comes from its ability to regulate the recruitment of leukocytes by endothelium (Ohashi et al., 2014).

9.3. PEPITEM:

A novel peptide has been discovered in our lab. It is the Peptide inhibitor of trans-endothelial migration called PEPITEM and inhibits T cell trafficking by a multistep mechanism (Chimen et al., 2015). The work in our lab shows that PEPITEM is released exclusively from B cells after they are stimulated by Adiponectin (AQ). After that PEPITEM binds to EC by Cadherin-15; this binding stimulates the release of a bioactive lipid mediator called sphingosine-1-phosphate. More details in regards to this will be mentioned in chapter five.

10. Hypothesis and aims:

We recognise that the adiponectin-mediated mechanism of down regulating T-cell transmigration is deregulated in chronic inflammatory and autoimmune diseases such as type-1-diabtes and rheumatoid arthritis. We hypothesise that similar deficiencies may be present in individuals with atherosclerosis. If this is the case it may mean that this pathway forms a tractable therapeutic target for intervening in the process of atheroma formation.

The aims of this thesis are:

- To investigate the patterns of leukocyte recruitment using in vitro adhesion assays for leukocytes isolated from atherosclerosis patients and age-matched healthy controls.
- To determine whether changes in the PEPITEM pathway are evident in such cohorts.
- To determine whether deficiencies in the control of leukocyte trafficking can be rectified using exogenous adiponectin or PEPITEM.

CHAPTER 2: MATERIAL AND METHODS

1. Material

1.1. List of main reagents

Reagent	Supplier	Application	Working solution\ concentration
Histopaque 1119	Sigma-Aldrich	PBMC isolation	1X
Histopaque 1077	Sigma-Aldrich	PBMC isolation	1X
PBS (Phosphate-buffered saline)	Sigma-Aldrich	Cells Washing	1X
BSA (Bovine serum albumin)	Sigma-Aldrich	Cell washing Buffer	5%
EDTA	Sigma-Aldrich	Cell washing Buffer	200mM
M199	Gibco Invitrogen	Culture media	1X
Endothelial basal low serum media	Promocell	Culture media	1X
Fetal Calf Serum (FCS)	Gibco Invitrogen	PBMC, DMEC culture	10-20%
Penicillin/ Streptomycin	Gibco Invitrogen	Cell culture antibiotic	1:500
Gentamycin	Sigma-Aldrich	Cell culture antibiotic	35µg/ML
Amphotericin B	Sigma-Aldrich	cell culture antifungal drug	2.5µg/ML
Trypsin	Gibco Invitrogen	Cell Dissociation reagent	2.5mg/ML
TrypLE™ Express	Life technologies	Cell dissociation reagent	1X

IFN-γ	Peprotech	Endothelium activation	10 ng/ml
TNF-α	R/Dsystems	Endothelium activation	100U/ml
Adiponectin	NovoNordisk	PBL treatment	15µg\ml
Accutase	Sigma-Aldrich	Transmigrated PBL dissociation reagent.	1X
Gelatin	Sigma-Aldrich	Endothelium culture	2%
Taqman Master Mix	Applied biosystems	qPCR	1X
dNTPs	Promega	qPCR	10mM
Superscript buffer	Invitrogen	qPCR	1X
RNase out Recombinant Ribonuclease Inhibitor	Invitrogen	qPCR	10U
Superscript II reverse transcriptase	Invitrogen	qPCR	10U
Random primers	Promega	qPCR	NA
RNA easy kit	Qiagen	RNA Extraction	NA
Sphingosine-1-phosphate (S1P)	Cayman chemicals	PBL treatment	5mg

1.2. List of flow cytometry antibodies

Antibody	Supplier	Application	Optimised volume for 100 µl
CD4-FITC(Fluorescein isothiocyanate)	eBioscience	CD4+ T cells identification	2
CD3-PerCpCy5.5 (Peridinin Chlorophyll Protein Complex-Cyanine	eBioscience	T cells identification	2
CD4-APC CY^7(Allophycocyanin CY^7)	eBioscience	CD4+ T cells identification	2
CD8-PB (Pacific Blue)	eBioscience	CD8+ T cells identification	2
CD45RO-PE (Phycoerythrin)	eBioscience	Memory T cells identification	2
CD19-PECy7	eBioscience	B cells identification	2
CD56-PE	eBioscience	NK cells identification	2
CD56- APC	eBioscience	NK cells identification	2
Goat anti-rabbit AlexaFluor 488	Invitrogen	Conjugated secondary antibodies	2

IgG1-FITC	Ebioscience	Isotype control	10
Human CXCR3 FITC	R&D Systems	CXCR3 identification	10
Human CX3CR1 FITC	R&D Systems	CX3CR1 identification	10
Human CCR5 FITC	R&D Systems	CCR5 identification	10
Human CD18 FITC	Invitrogen	Integrin Beta-2 identification	10
Human CD29 FITC	Invitrogen	Integrin Beta-1 identification	10
Human Integrin α L/CD11a FITC	R&D Systems	Integrin-α L identification	10
Human Integrin α 4 (FITC)	R&D Systems	Integrin α 4 identification	10
Sphingosine-1-phosphate Receptors 1 (S1PR1)	Cayman chemicals	Sphingosine-1-phosphate Receptors 1 identification	2
Sphingosine-1-phosphate Receptors 4 (S1PR 4)	Cayman chemicals	Sphingosine-1-phosphate Receptors 4 identification	2

Target	Forward (5'-3')	Reverse (5'-3')	**Product size**
AR1	TGCCCTCCTTTCGGGCTTGC	GCCTTGACAAAGCCCTCAGCGATAG	526
AR2	GGAGCCATTCTCTGCCTTTC	ACCAGATGTCACATTTGCCA	467

Table 2-1: Conventional PCR Primers.

2. Methods:

2.1. Static Adhesion and migration assay

Dermal Microvascular Endothleial Cells (DMECs) were purchased from PromoCell. DMECs and were passaged 4 times in Endothelial Cell Growth Medium and frozen using CryoSFM freezing media (Promocell). A week before the migration assay, DMECs were cultured in 5ml of Endothelial Cell Growth Medium (Promocell) in 25cm2 flasks. Confluent DMECs were cultured again in 4 wells of a 6-wells plate. Briefly, DMECs were washed with 1 ml 0.02% EDTA (Sigma), then 2 ml of TrypLE™ (Invitrogen) was added to dissociate DMECs Trypsin was neutralised using M199 20% FCS, cells were centrifuged at 1500rpm for 7 minutes and then Plated in 6-wells plate. DMECs were then stimulated with TNF-α (100U/ml) and IFN-γ (10 ng/ml) suspended in 1 ml Endothelial basal low serum media and kept for 24 hours in incubator (37°C 5% CO2).

2.2. Peripheral blood lymphocytes (PBL) Isolation for migration assay

10 ml of venous Blood was collected in blood tubes coated with anticoagulant EDTA (1.6MG/ml, Sarstedt, Leicester, UK) from healthy adult volunteers or patients with atherosclerosis in accordance with local ethical guidelines and with approval of the South

Birmingham Local Research Ethics Committee (UK). Lymphocytes were separated using two-step density gradients of Histopaque 1119 and 1077 (Sigma, Poole, UK).

Briefly, in a10 ml round bottom tube, 2.5 ml of Histopaque 1119 was gently layered onto 2.5 ml of Histopaque 1077. Then, 5ml of EDTA blood was added gently and the mixture was centrifuged at 2500 RPM for 30 minutes. The blood was separated into 6 distinct bands: plasma, PBMC, isolation media, neutrophils, more isolation media, and the red blood cell pellet. The PBMC was carefully collected, transferred to 15 ml Falcon tube, and washed twice using the washing solution (M199+ 20% BSA). The cell suspension was transferred to a new 25cm2 flasks which contains 5 ml M199+BSA and left in the flask for 30 minutes at 37°C. After 30 minutes, the supernatant was collected and centrifuged at 1500 RPM for 7 minutes. We collected only the supernatant to remove monocyte that will adhere to the surface firmly and lymphocyte will be floating in the supernatant. The pellet was then re-suspended to the final concentration of 1x106cells/ml.

2.3. Migration assay:

PBLs were isolated as previously prescribed. Prior transmigration assay, PBL (1x106 cells per condition) were treated with(20 ng/ml)1µl of Pepitem, (20 ng/ml)1µl of Scramble peptide (0.5 mg/ml), 1µl of Adiponectin (15µg/ml). Before adding PBL, DMECs were washed twice with M199 0.15% BSA to wash off the excess cytokines. PBL suspension was added to the designated well for 6 minutes at 37°C. Then the non adherent cells were washed off and finally each well was video recorded using phase contract video-microscope. Seven random fields were recorded from each well to measure the percentage of transmigration. Recordings were analysed using Image-Pro 6.2 software. Phase dark cells were considered transmigrated cells whereas the bright phase cells were considered surface adherent cells. The total adhesion

of cells was measured in cells per mm2 by multiplying calibrated microscope dimensions to the surface area.

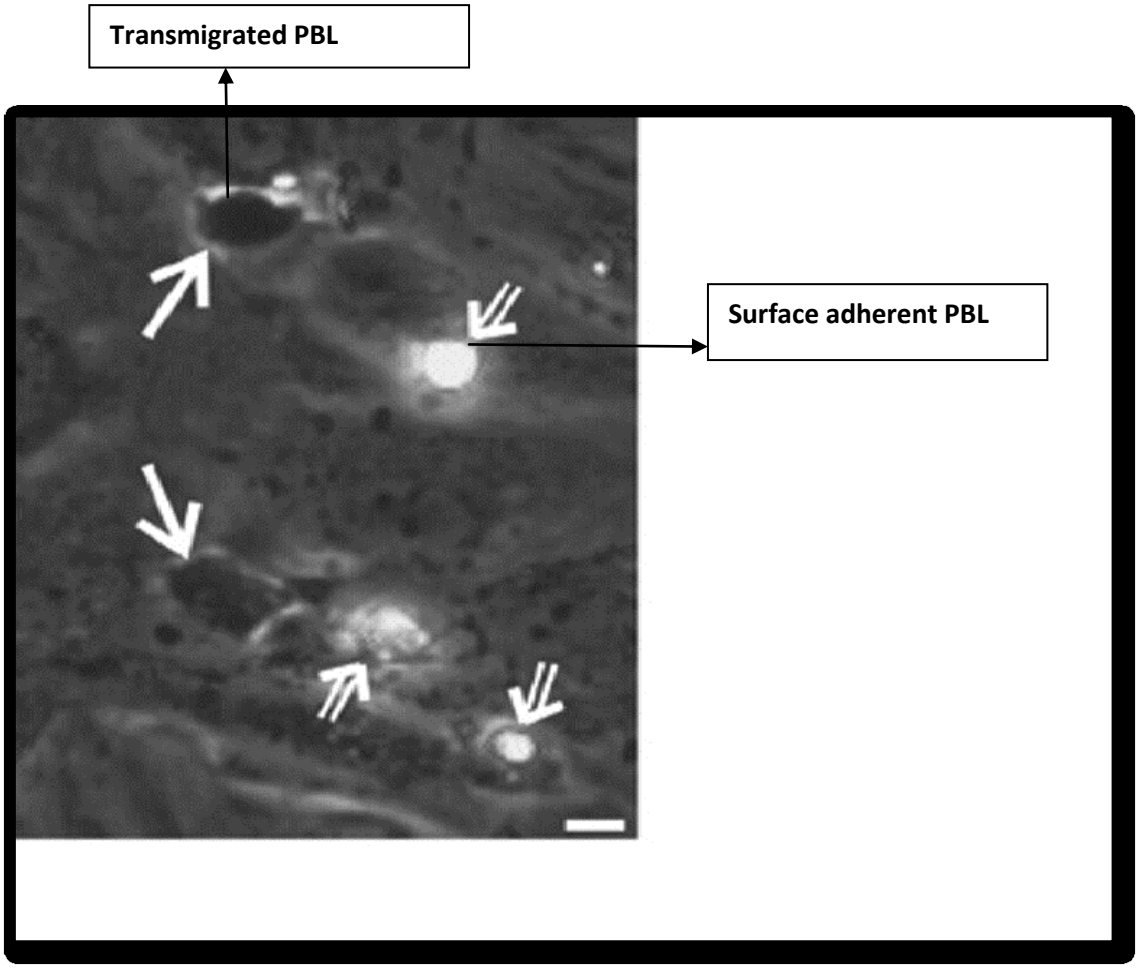


Figure 2-1: Illustration of a recorded filed instatic adhesion assay: Surface adherent PBL (Phase bright PBL) are adhered firmly on the endothelium monolayer, transmigrated PBL (Phase dark PBL) transmigrated through the endothelium.

2.4. Measurement of adiponectin receptors on PBMCs (peripheral blood mononuclear cell) using flow cytometry

2.4.1. Flow cytometry staining

$1.5x10^6$ PBMC for PBL subsets and $2.5x10^6$ PBMC for monocyte subsets were transferred to Polypropylene tubes (BD Bioscience) and the tubes were centrifuged at 1500 RPM for 7 minutes. Then, 2µl of FcR blocker (Miltenyi Biotec) was added to the tubes and PBMCs were stained with PBMCs markers (see tables for details) using recommended concentration of suppliers (see table 1 for details). Secondary antibody combinations were used to measure the expression of AR1/2, briefly; PBMCs were stained with the AR1/2 antibodies for 30 minutes at 4°C, then washed to remove unbound antibodies. After that, samples were labelled with the secondary antibody conjugated to Alexa 488 (Alexa Fluor® 488 Goat Anti-Rabbit IgG) and incubated at 4°C for 30 minutes. Following another necessary washing step,; FACS samples were then assayed using a Cyan (Dako) and the results were analyzed by FlowJo software. The number of events that were recorded per sample was 50000 events, plotted as positive cells frequency or mean fluroscence intensity (MFI). When directly conjugated antibodies were used, PBMC were incubated with the relevant antibodies for 30 minutes at 4°C and washed in PBS at 1500rpm for 7 minutes.

2.5. ELISA

Human total adiponectin level has been measured in serum of atherosclerosis patients and the healthy control group using Quantikine ELISA kit (R/D Systems) according to the blood was collected in yellow top chemistry tubes and spun at 2500rpm for 15 minutes. Then serum was separated from the blood because of the spin and collected by pasture pipette. The manufacturer's protocol was used to measure the concentration. Briefly, the serum samples

were taken from freezer and were allowed to thaw at room temperature, then diluted 100-fold. Substrate solution and adiponectin standard had been prepared and reconstituted, and finally samples were added to the ELISA kit reagents and the optical density was measured using microplate reader Synergy HT (BioTek). Adiponectin serum level was measured using calibration curve and expressed in ng/ml.

2.6. Measurement of gene expression:

mRNA was extracted from PBMCs using RNAeasy kit (Qiagen) following the supplier protocol. Briefly, frozen PBMCs were lysed using RTL lysis buffer, transferred to a column, then after three washes, RNAase free water was used to elute mRNA. Finally, the mRNA concentration and 260/280 ratio was measured using Nanodrop spectrofluorimeter (LabTech) to test the purity and the validity of samples. mRNA was then converted into cDNA. Random primers (Promega, UK) have been annealed to 1µg of mRNA using Touchgene Gradient Thermal Cycler (TECHNE, UK) for five minutes at 70°C. Then, the master mix was added to make the final volume of 30µl. The master mix is consists of 10mM dNTPs, 1X Superscript Buffer, 10U Superscript II Reverse Transcriptase, 10U RNAout RNase inhibitor (Invitrogen, UK). The reaction was done at 37°C for 60 minutes, then another 5 minutes at 95°C.

For Real-Time PCR (qPCR), Taqman Universal Mastermix (volume) (Applied Biosystems, UK), Taqman probes as well as reverse and forward primers were added with 2 ul cDNA. Each reaction was a mixture of equal quantities of both primers, 18s as endogenous controls, cDNA, and nuclease free to make up the volume to 25µl (see table 2-1 for genes and primers details). (qPCR) The qPCR was performed using Real Time PCR 7500 (Applied Biosystems, UK). The gene expression of AR1 and AR2 was calculated using the delta Ct methods using 18S to normalise the Ct values. In singleplex reactions; the relative expression

was measured by the comparison between the ΔΔCt normalised to 18S. The following equation was used to calculate the relative expression units (REU):

REU= 2 ΔCt.

2.7. Intracellular staining

A permeabilisation/fixation kit (EBioscience) has been used for the staining procedure following the manufacturer's protocol. In summary, PBMCs were isolated as previously described, and then 1 ml of Fix/Perm solution (1 ml solution concentrate + 3 ml of solution diluents) was added to the cell suspension and wrapped in foil in order to keep it in the dark for 45 minutes at 4 degrees Celsius. After that, the suspension tubes were washed using Fixation and Cell Permeabilization buffer (FIX \ PERM) 1X, then the same flow cytometry protocol which has been described before was followed. Perm/fix buffer had been used in all the washing steps during this staining.

2.8. Protocol PBL binding on ICAM-SIPR1/4 expression

A 96 well plate was coated with 10μ/ml recombinant ICAM/Fc and covered with foil at 4°C overnight. The next day, the plate was blocked by adding 2% of PBS/BSA for at least an hour at room temperature. Meanwhile, PBLs have been isolated as described above, but PBL was resuspended at $4X10^6$ PBL/ml. In one eppendorf tube, 7.5 μL of IP-10 (80NG/ml) was added to 350μL of PBLs, then 50 μl of mixture has been added to the appropriate well plate and the plate was incubated at 37°C for 30 minutes. After that, cold PBS was used to wash the wells and finally PBLs have been collected by rough pipetting with cold PBS in FACS tube and stained for flow cytometry.

2.9. Measurement of surface expression of adhesion molecules and chemokine receptors on fresh blood PBL by flow cytometry

In this experiment we measured the expression of the following adhesion molecules and integrins Alpha-L (αL), Alpha-4(α4), Beta-1 (β1), Beta-2 (β2), P-selectin glycoprotein ligand-1 (PSGL-1), CCR5, CXCR3, and CX3CR1 on different PBL subsets. We have three conditions; the first one is to measure the surface expression on freshly isolated PBL using the same PBL isolation protocol mentioned above. Regarding flow cytometry staining; conjugated antibodies have been used to stain PBL using the recommended concentration from suppliers (using recommended concentration of suppliers (see table 1 for details). The second and the third conditions are that the PBL which were used for transmigration assay, they are called surface adherent PBL and migrated PBL. The migration assay protocol was used to isolated surface adherent PBL and migrated PBL from tissue culture flask. The same steps were followed and then after 6 minutes of incubation at 37°C, cold EDTA was used to detach the surface adherent PBL (bright phase PBL) by adding 2 ml of EDTA to the flask with gentle agitation, then the supernatant was collected and spun for 7 minutes at 1500rpm. Finally, the pellet was re-suspended in the washing medium and PBL was calculated and prepared for staining. The remaining migrated PBL (dark phase PBL) and the DMEC, were collected by adding 2 ml of Trypsin which was neutralised after 2 minutes by using M199 20% FCS. The mixture was spun for 7 minutes at 1500rpm and the pellet was re-suspended in in washing medium and PBL was calculated and prepared for staining.

2.10. Gating strategy for flow cytometry analysis

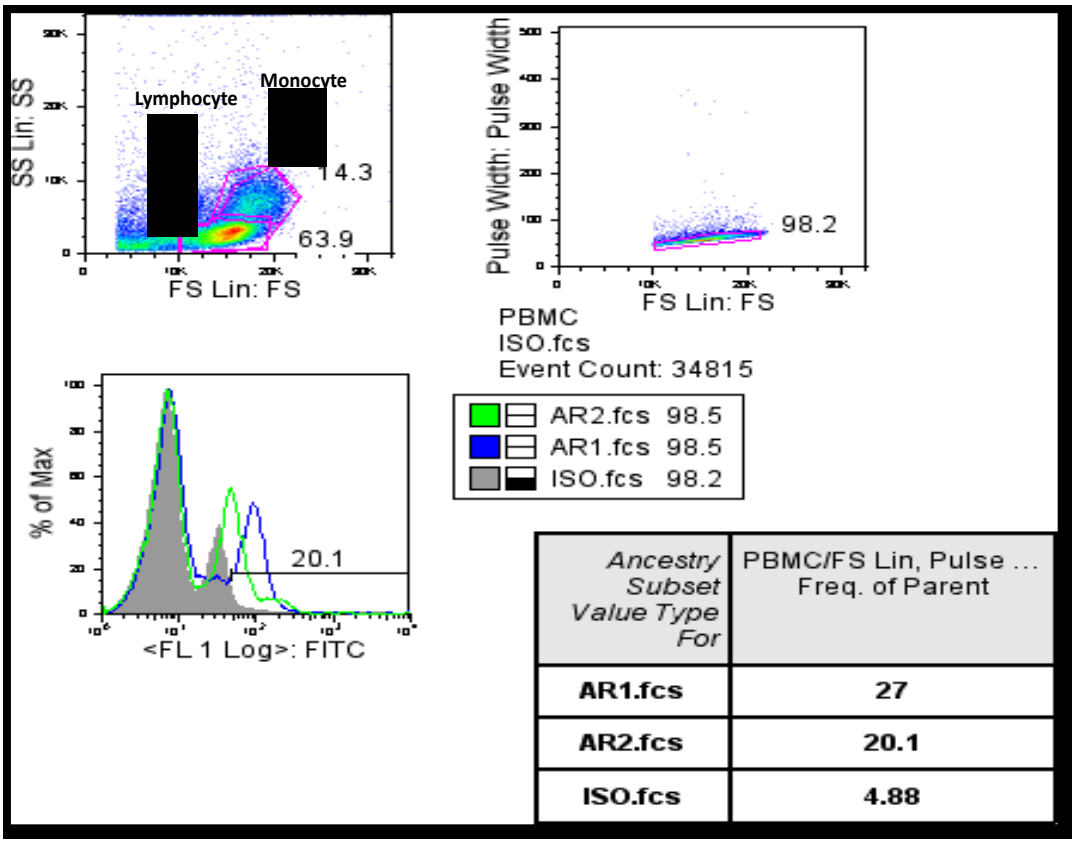


Figure 2-2: Gating strategy for Adiponectin receptors (AR1, AR2) on PBL.

Flow cytometry was used to measure the frequency of ARs; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. ARs expression was measured on the basis of the isotype control for each subset.

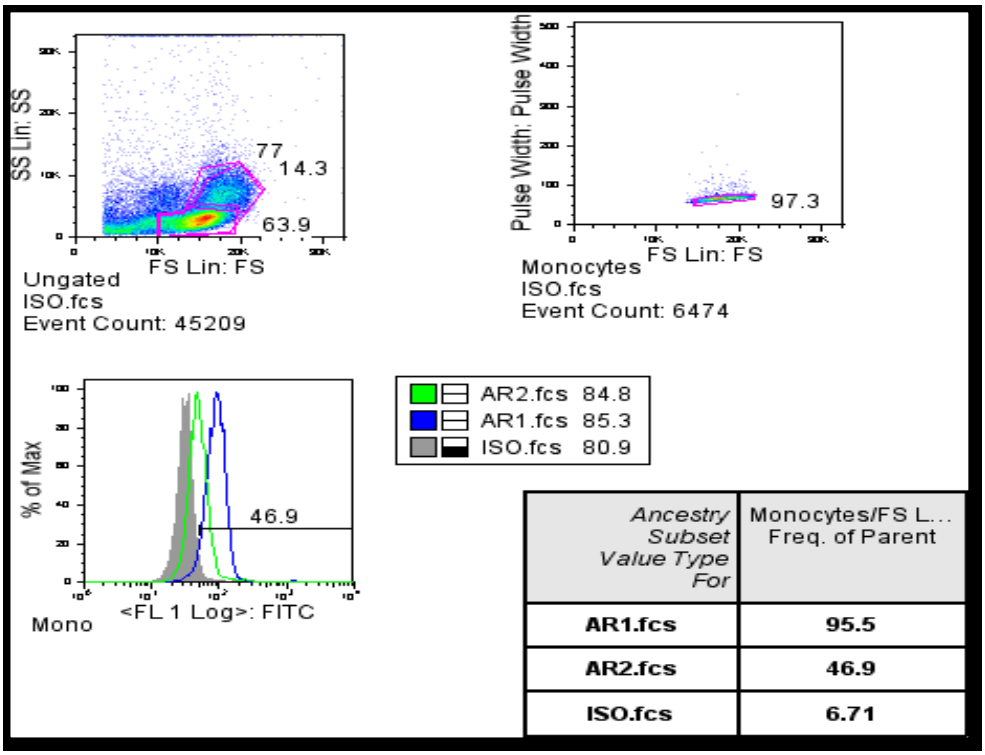


Figure 2-3: Gating strategy for Adiponectin receptors (AR1, AR2) on Monocyte.

Flow cytometry was used to measure the frequency of ARs; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. AR1 expression was measured on the basis of the isotype control for each subset

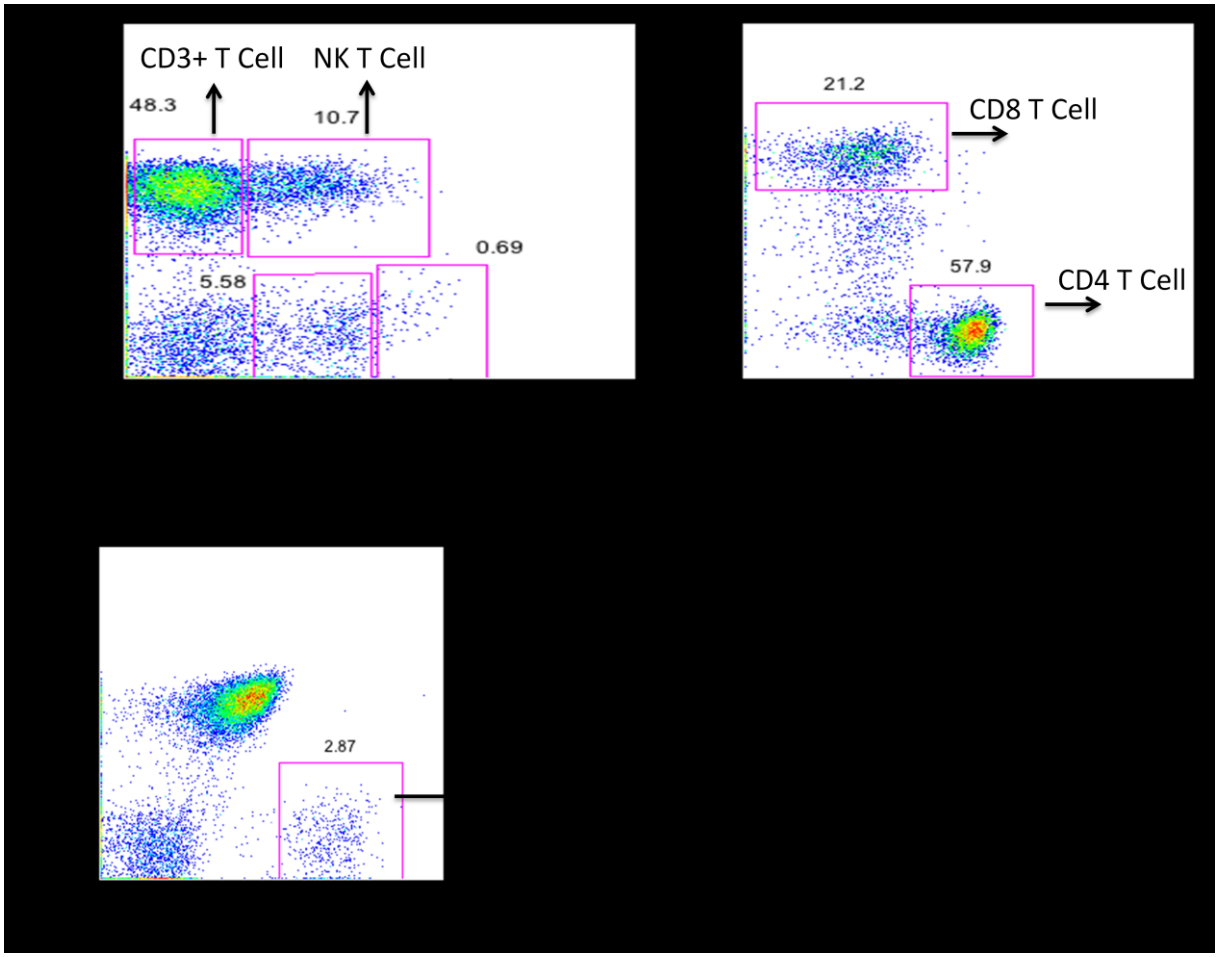


Figure 2-4: Gating strategy for Adiponectin receptors (AR1, AR2) on PBL subsets.

PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD19-PECY^{7} antibody was used to identify B cells. CD3-PerCpCy$^{5.5}$ and CD56-PE antibodies were used to identify the different population of NK Cells.

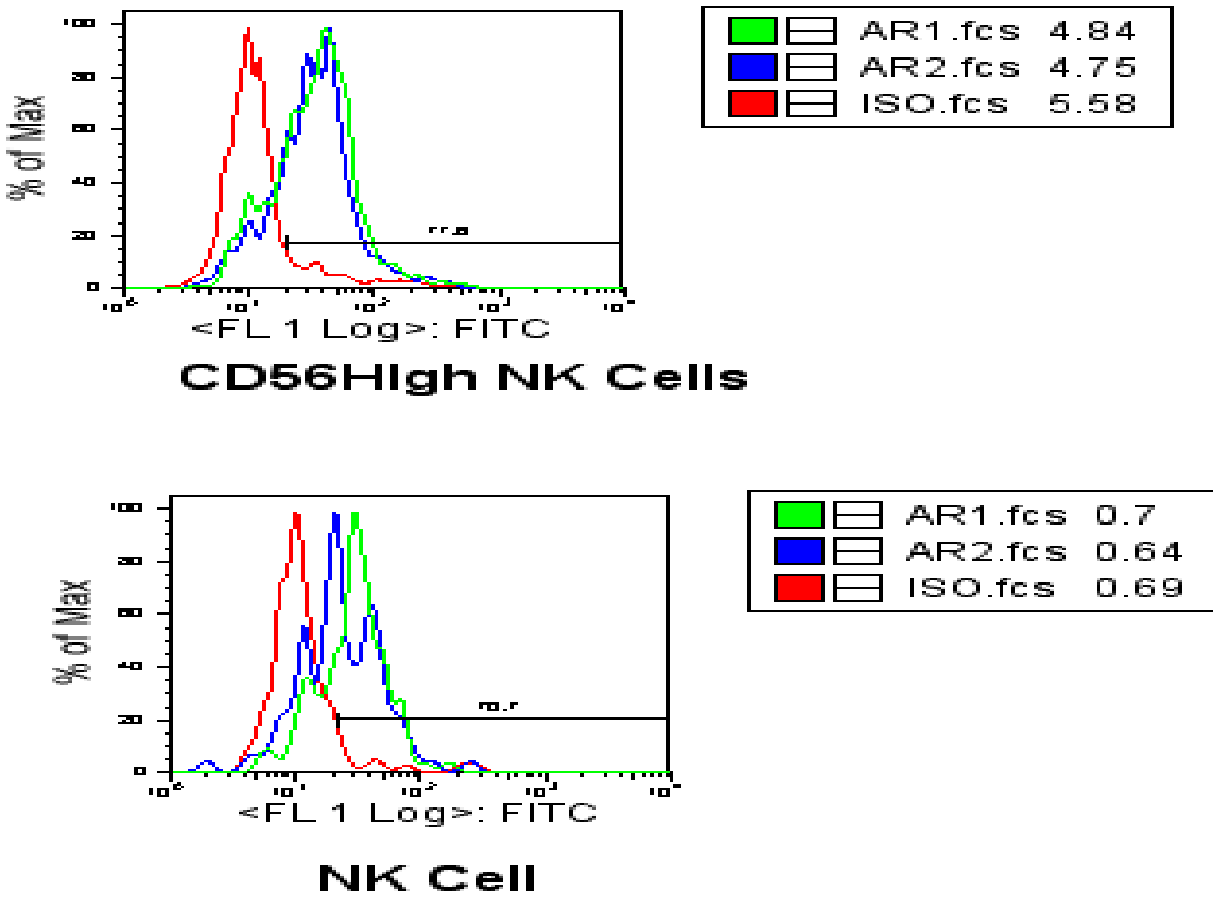


Figure 2-5: Histograms for ARs expression on CD56High NK Cell and NK Cells.

ARs expression on NK cell subsets expression was measured as shown on the above histograms. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Isotype control (Red), AR1 (Green), AR2 (Blue).

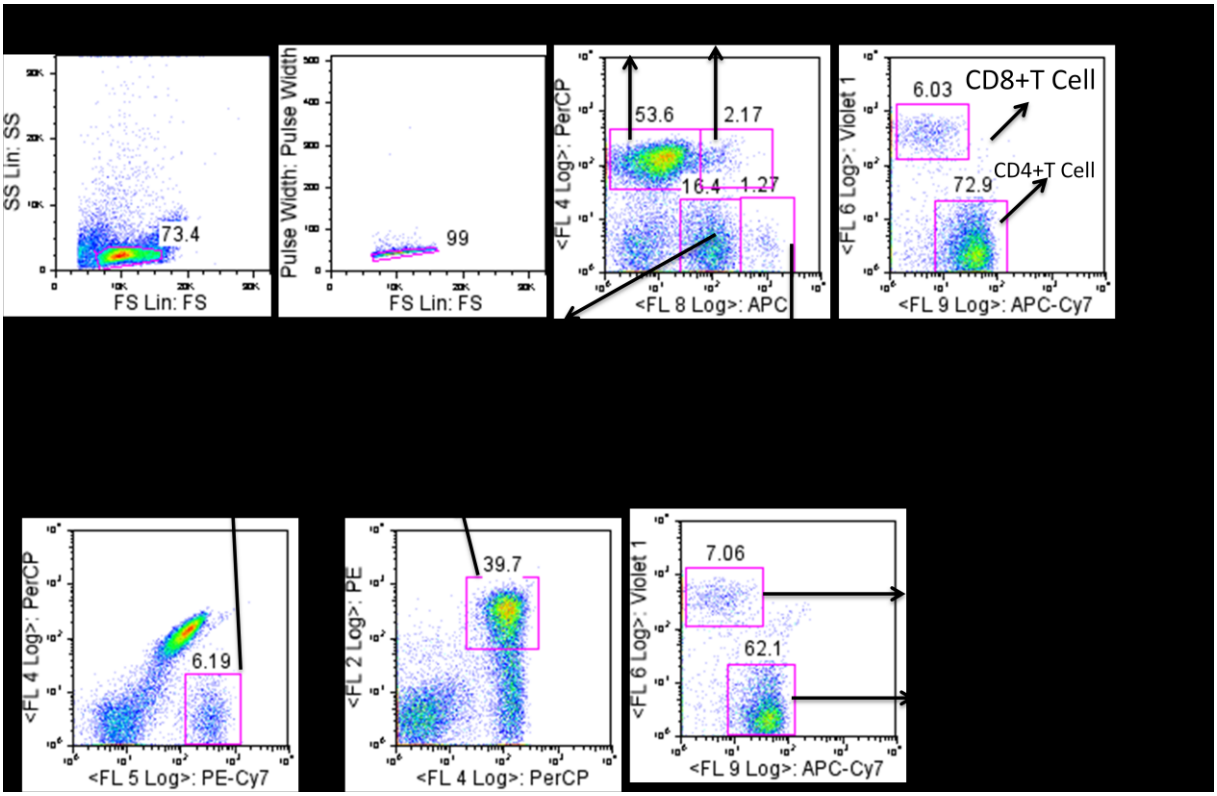


Figure 2-6: Gating strategy for PBL subsets when measuring Integrin and chemokine recptors.

PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD19-PECY^{7} antibody was used to identify B cells. CD3-PerCpCy$^{5.5}$ and CD56-APC antibodies were used to identify the different population of NK Cells.

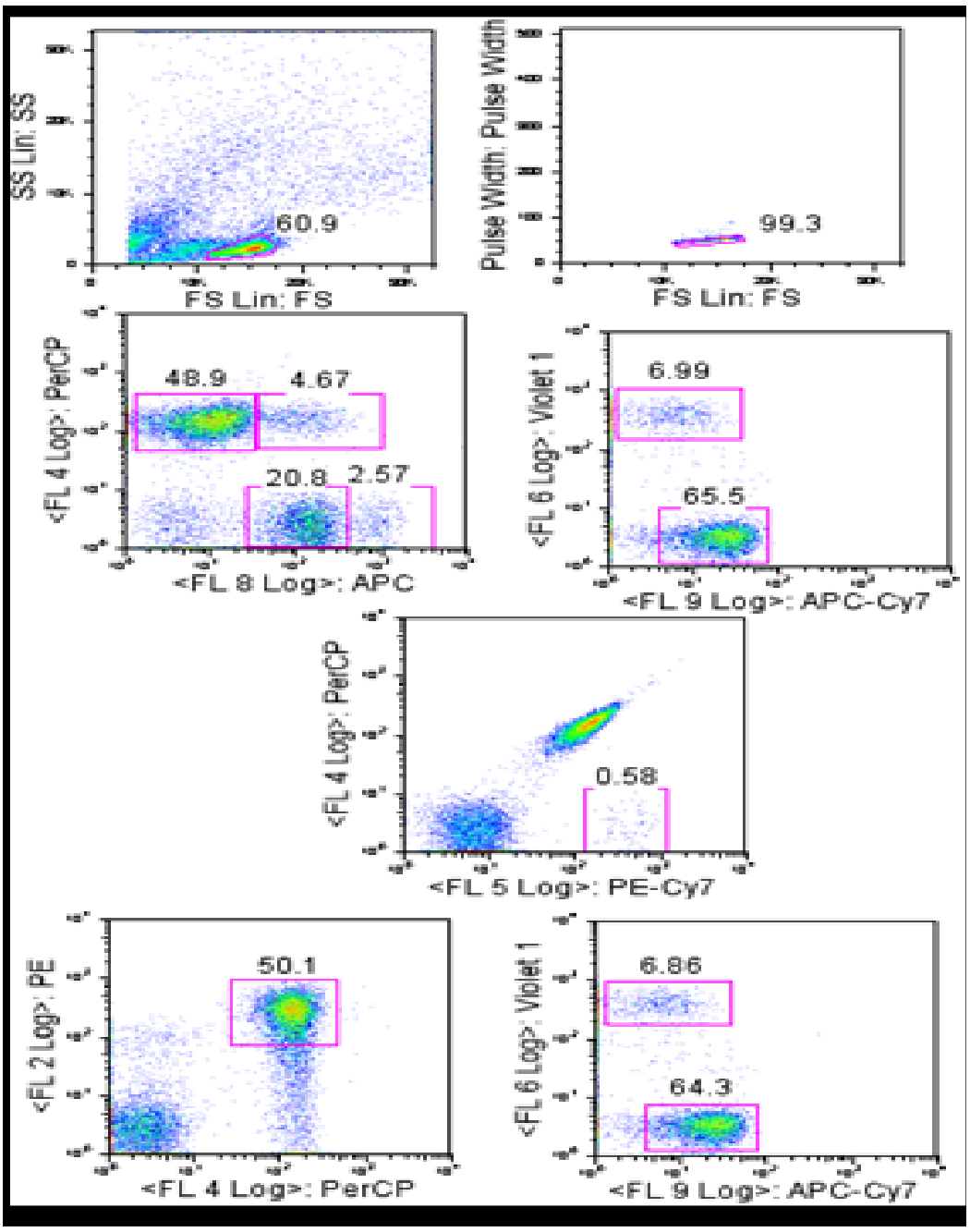


Figure 2-7: Gating strategy for Migrated PBL

PBL was collected after static adhesion assay, migrated PBL are the dark phased cell which was collected by harvesting DMECs by Trypsin treatment. Migrated PBL stained, and gated on their forward/side scatter profile. CD19 antibody was used to identify B cells. CD3 and CD56 were used to identify the different population of NK Cells

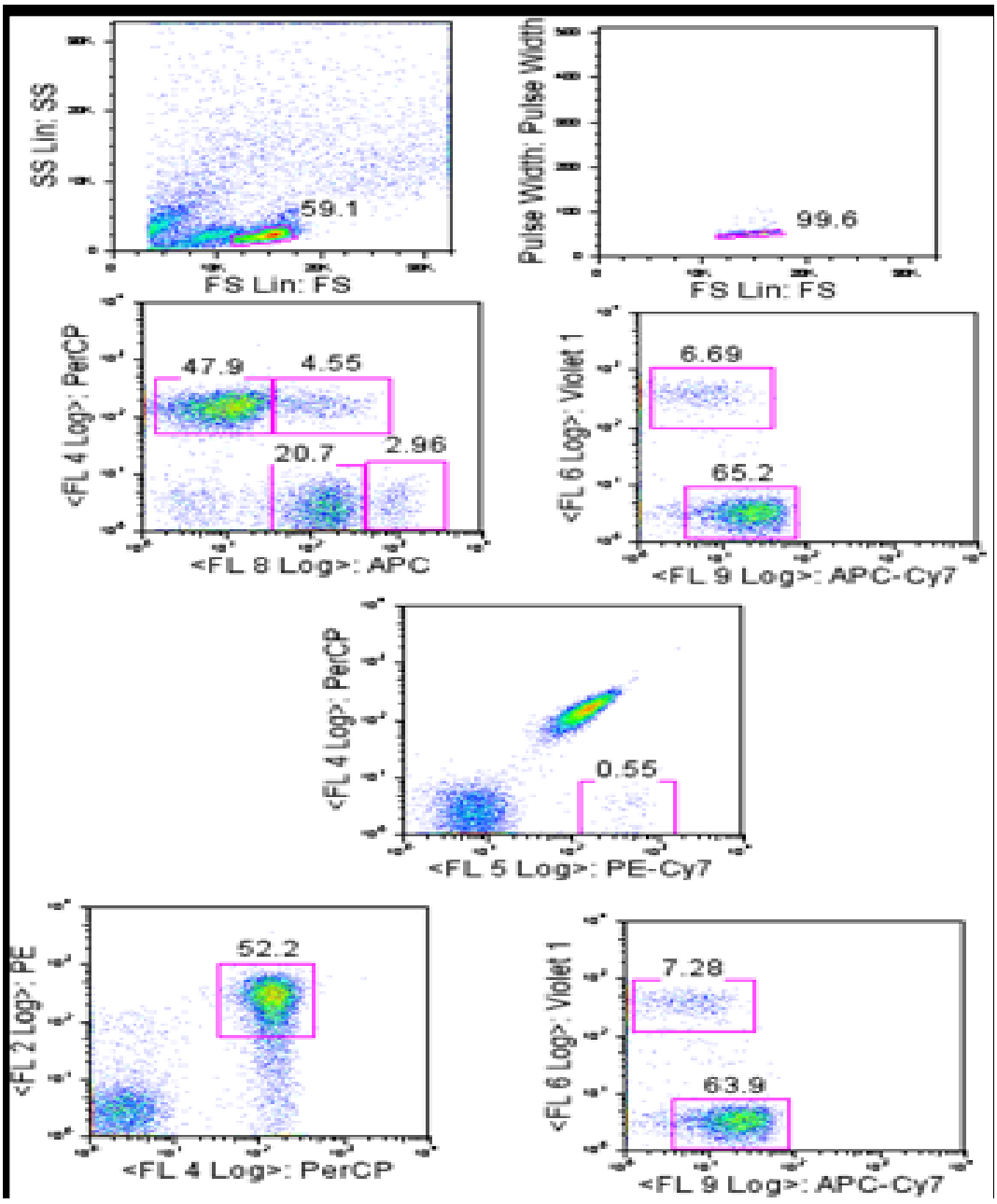
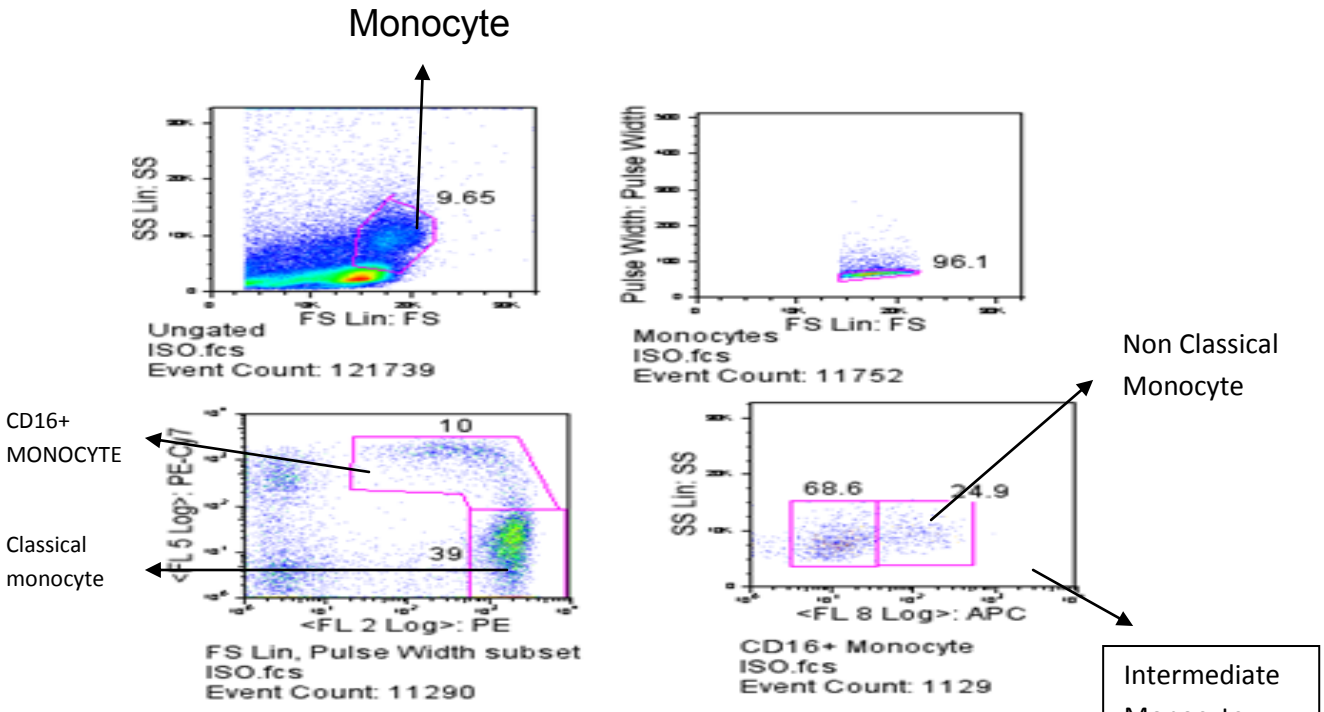


Figure 2-8: Gating strategy for surface adherent PBL

PBL was collected after static adhesion assay, surface adherent PBL are the bright phased cell which was collected by adding cold EDTA to the tissue culture flask to detach surface adherent PBL. Surface adherent PBL stained, and gated on their forward/side scatter profile. CD19 antibody was used to identify B cells. CD3 and CD56 were used to identify the different population of NK Cells.



Ancestry Subset Value Type For	Monocytes... Freq. of Pa...	Monocytes/... Freq. of Pa...	Monocytes/FS ... Freq. of Parent
AR1.fcs	99.5	100	99.3
AR2.fcs	99.6	100	99.4
ISO.fcs	4.09	8.54	6.19

Figure 2-9: Gating strategy for Adiponectin receptors (AR1, AR2) on Monocyte subsets.

Flow cytometry was used to measure the frequency of ARs; monocytes were isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD14 antibody was used to identify classical monocytes. CD16 and CCR2 antibodies were used to identify non-classical and intermediate monocytes. AR1 expression was measured on the basis of the isotype control for each subset.

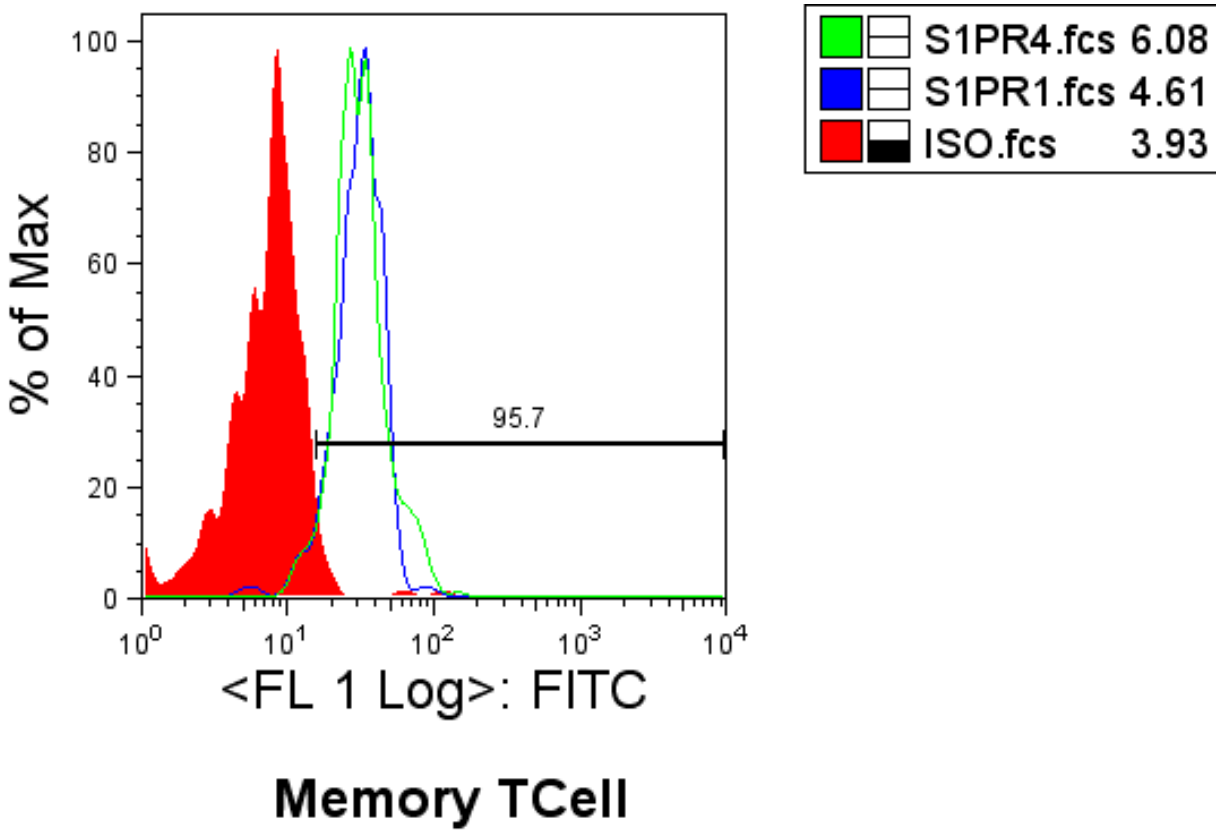


Figure 2-10: Histograms for SIPR1/4 expression on memory T cell.

Flow cytometry was used to measure the expression of SIPR1/4 on memory T cell; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD45RO-PE antibody was used to identify memory T cell. Isotype control (solid red), SIPR1 (Blue), SIPR4 (Green).

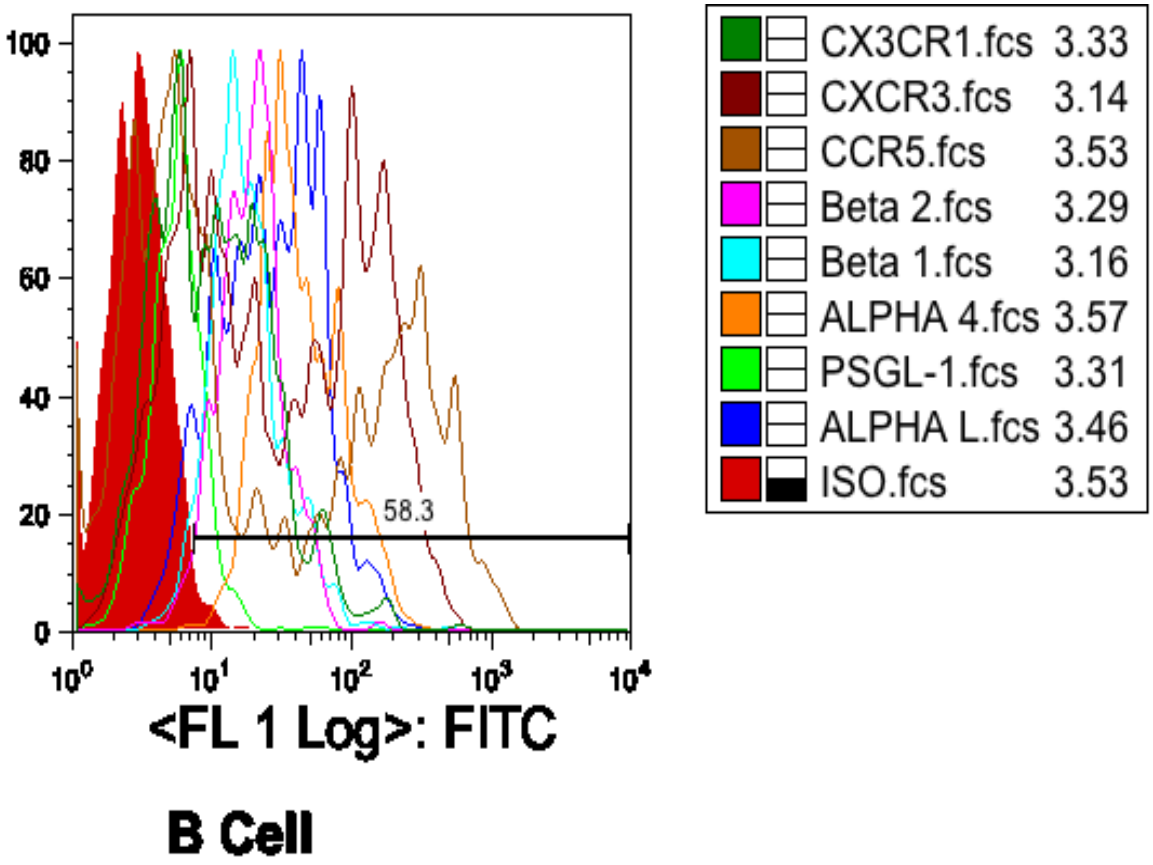


Figure 2-11 Histograms for Integrin and Chemokine receptors on B cells.

Flow cytometry was used to measure the expression of the above Integrin and Chemokine receptors on B cell; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Isotype control (solid red).

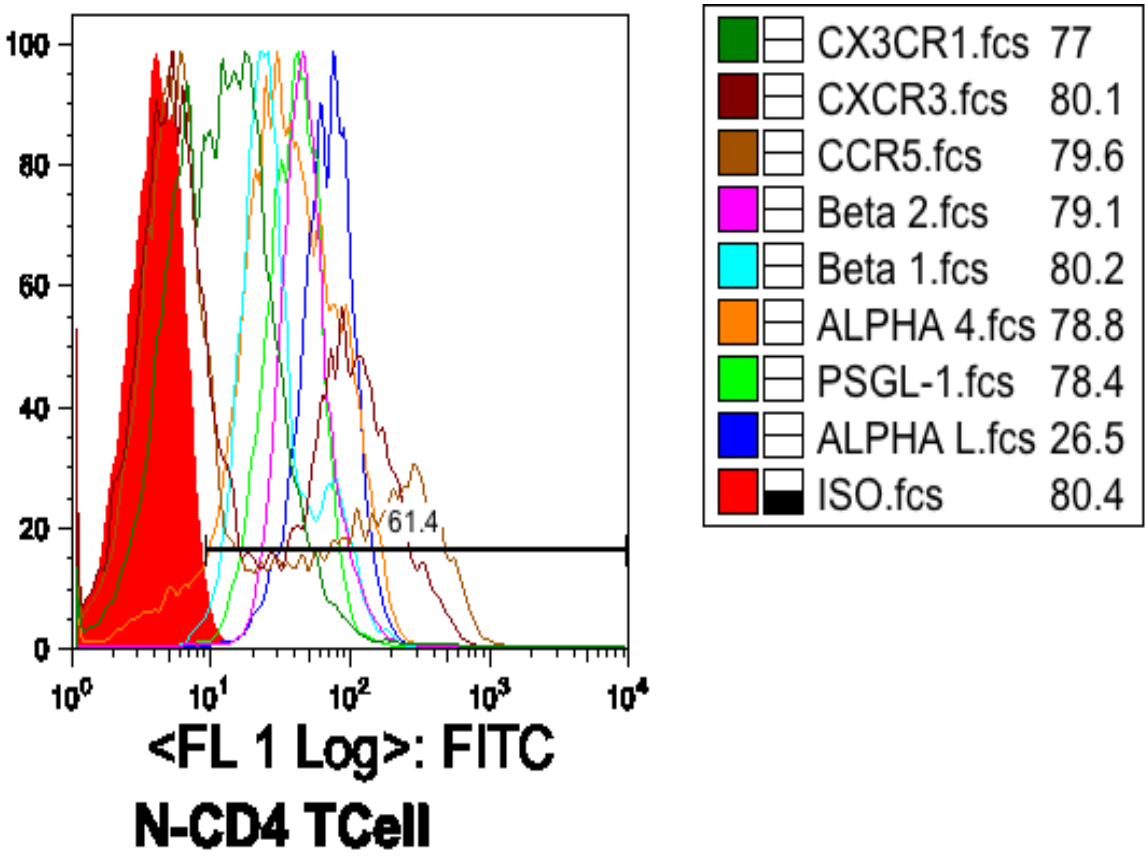


Figure 2-12: Histograms for Integrin and Chemokine receptors on N-CD4 T cells.

Flow cytometry was used to measure the expression of the above Integrin and Chemokine receptors on N-CD4 T cell; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Isotype control (solid red).

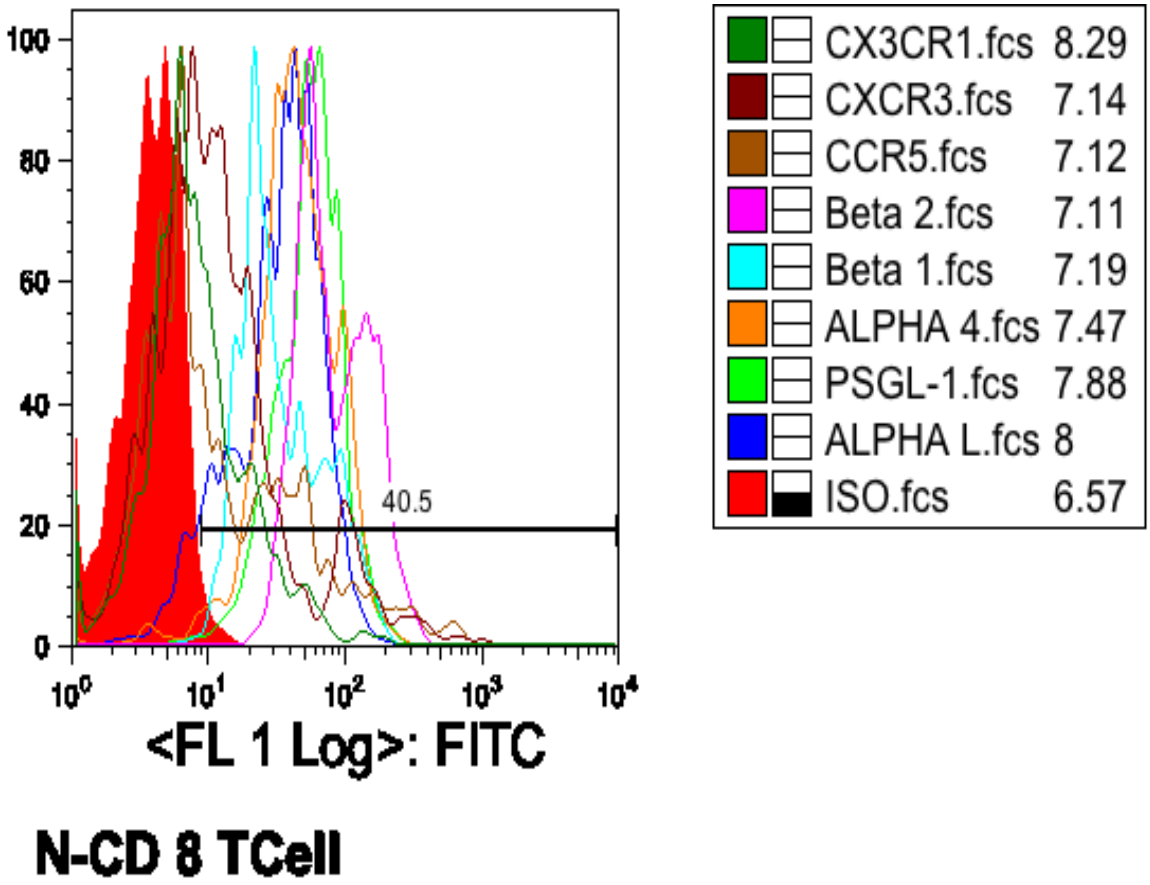


Figure 2-13: Histograms for Integrin and Chemokine receptors on N-CD8 T cells.

Flow cytometry was used to measure the expression of the above Integrin and Chemokine receptors on N-CD8 T cell; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Isotype control (solid red).

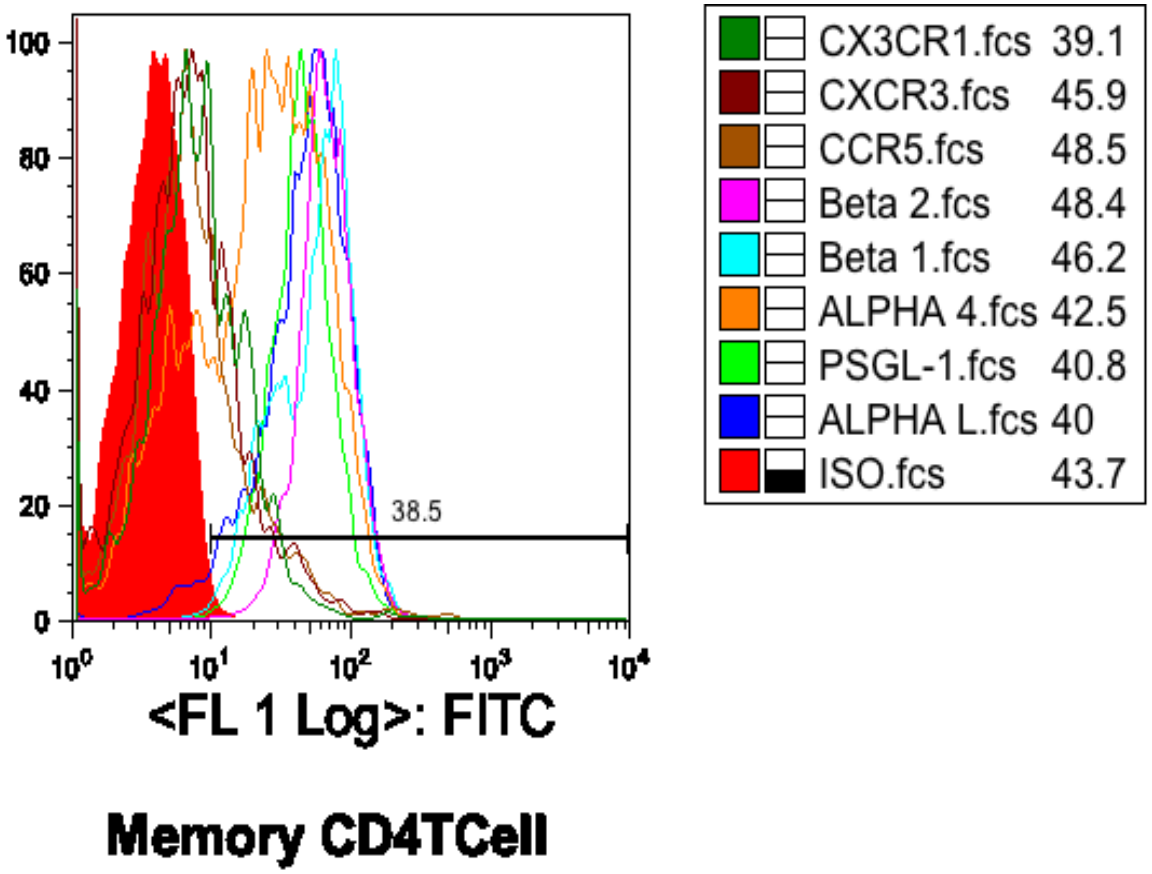


Figure 2-14: Histograms for Integrin and Chemokine receptors on Memory-CD4 T cells.

Flow cytometry was used to measure the expression of the above Integrin and Chemokine receptors on memory-CD4T cell; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Isotype control (solid red).

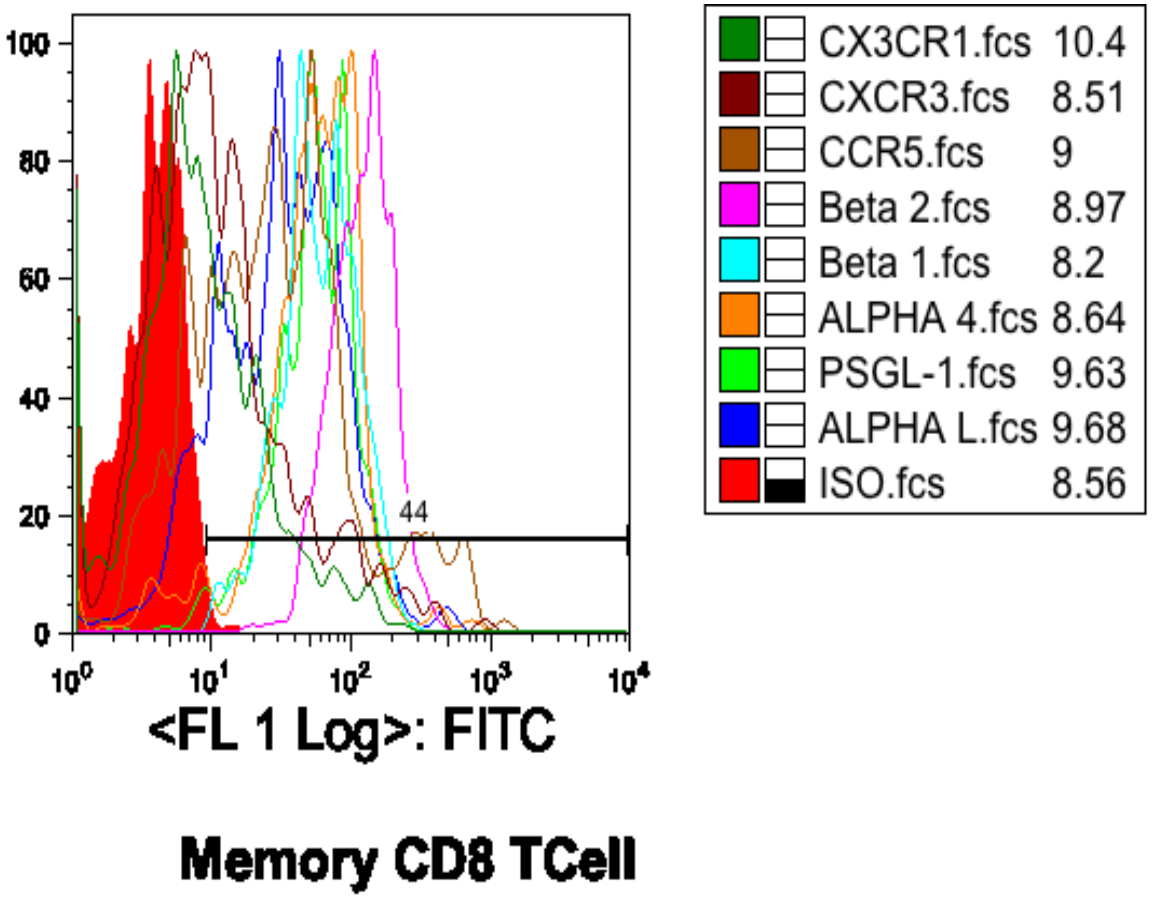


Figure 2-15: Histograms for Integrin and Chemokine receptors on Memory-CD8 T cells.

Flow cytometry was used to measure the expression of the above Integrin and Chemokine receptors on memory-CD8T cell; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Isotype control (solid red).

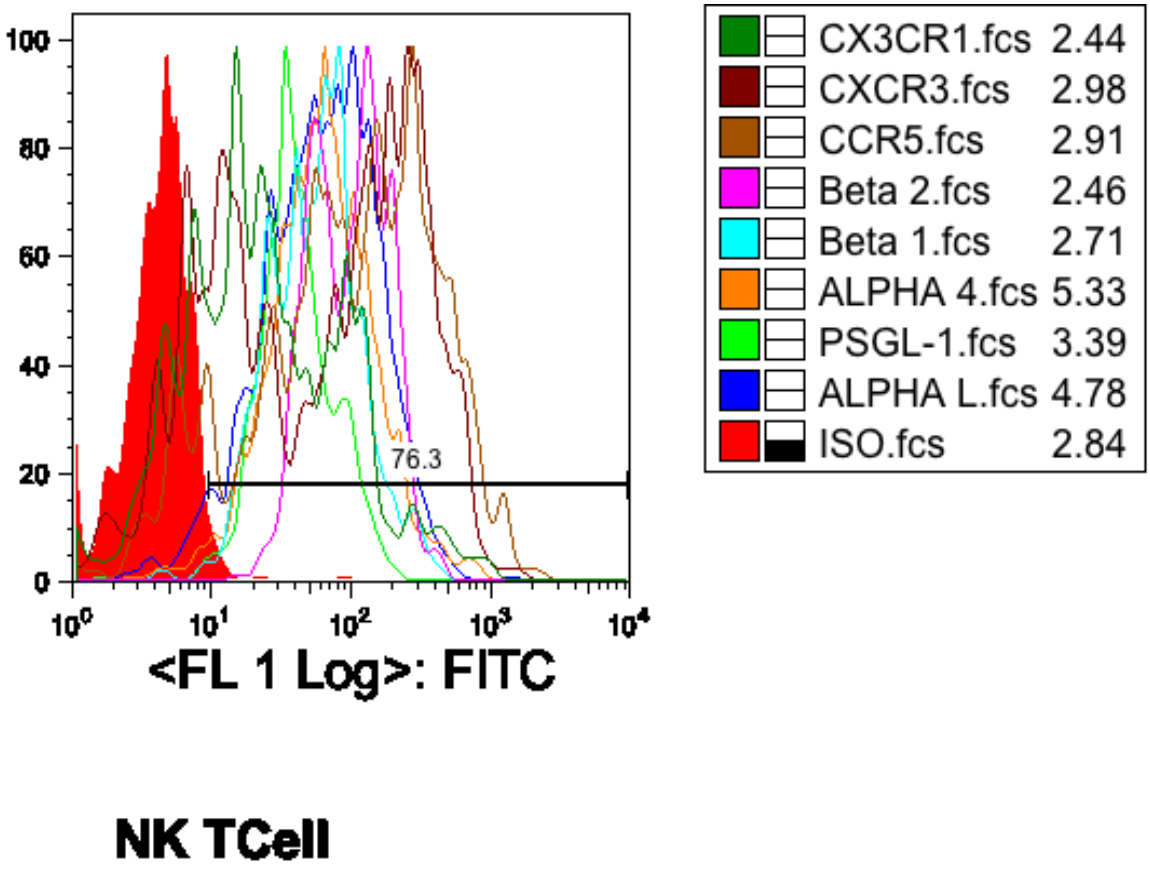


Figure 2-16: Histograms for Integrin and Chemokine receptors on NK T cell.

Flow cytometry was used to measure the expression of the above Integrin and Chemokine receptors on NK T cell; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Isotype control (solid red).

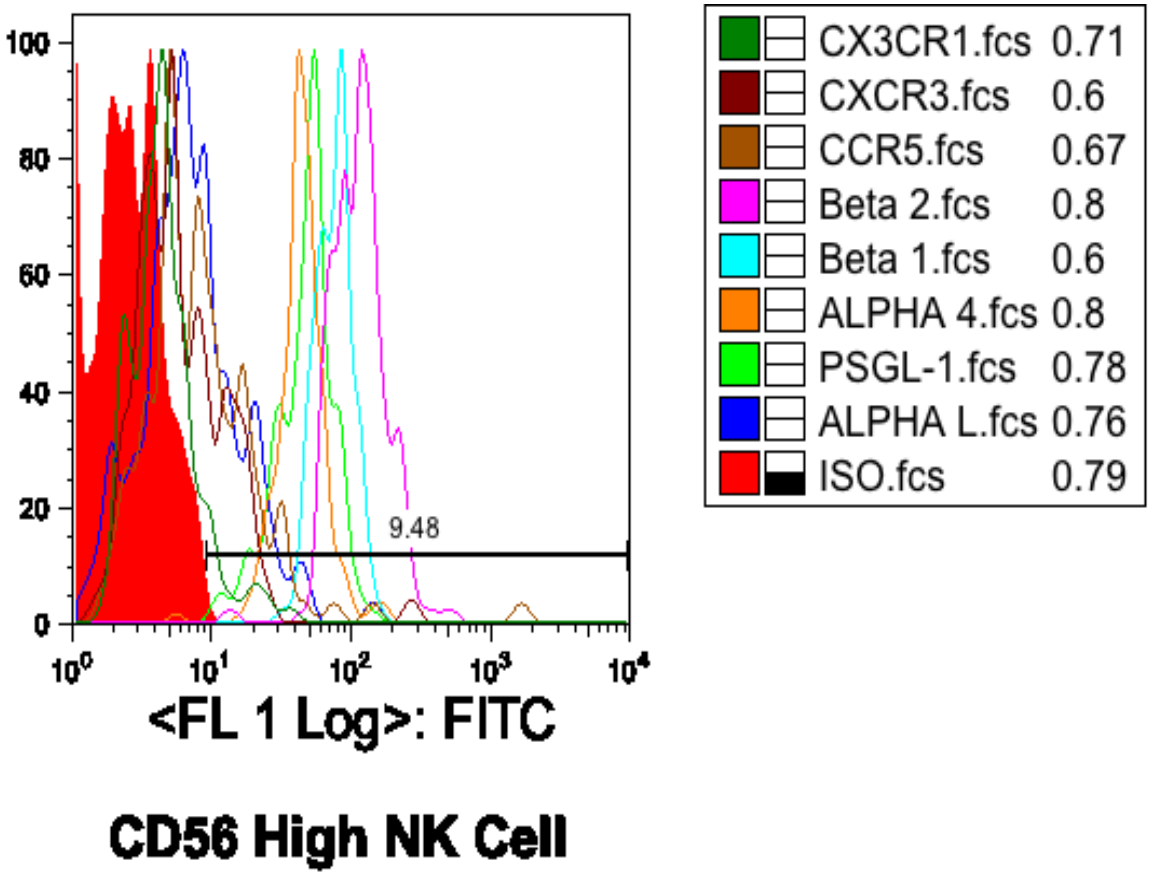


Figure 2-17: Histograms for Integrin and Chemokine receptors on CD56 High NK cell.

Flow cytometry was used to measure the expression of the above Integrin and Chemokine receptors on CD56 High NK cell; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Isotype control (solid red).

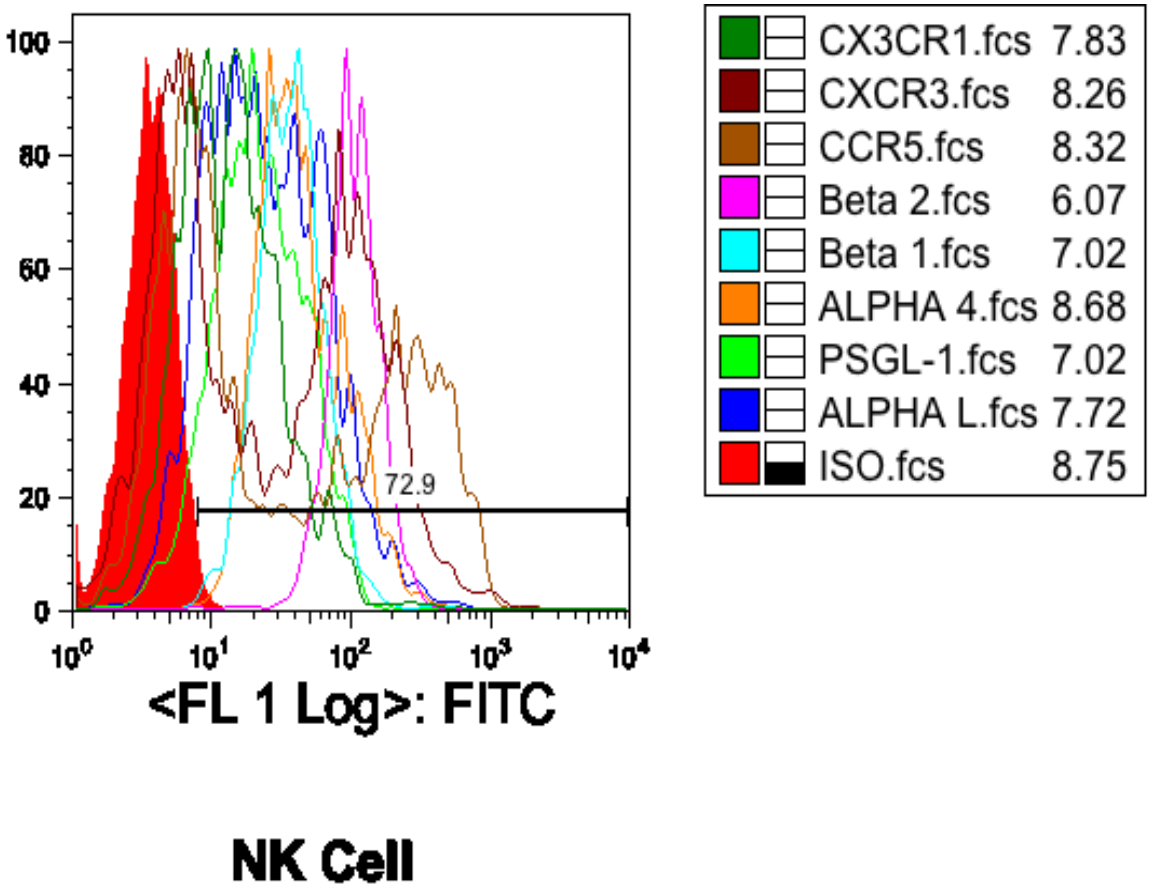


Figure 2-18: Histograms for Integrin and Chemokine receptors on NK cell.

Flow cytometry was used to measure the expression of the above Integrin and Chemokine receptors on NK cell; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Isotype control (solid red).

3. Statistics:

The data is mean ± standard error of the mean (SEM) and was analysed using a one-way ANOVA, with a Bonferroni post hoc test. P-value less than 0.05 were considered significant.

CHAPTER 3: STUDYING THE EFFECT OF AGE ON HUMAN ADIPONECTIN SERUM LEVEL AND ON THE SURFACE EXPRESSION OF ADIPONECTIN RECEPTORS AR1 AND AR2 ON PBL SUBSETS.

1. Introduction

An imbalance or dysfunction of adipose tissue can play a part in obesity-induced chronic inflammation which subsequently leads to metabolic diseases like insulin resistance, type 2 diabetes and cardiovascular disease. Adipose tissue can be considered to be an immune organ, and adipose tissue macrophages are the most significant source of inflammatory mediators, including adopkines, in adipose tissue. (Kadowaki and Yamauchi, 2005).

The adipokine, adiponectin, circulates as either a full-length form, or as a smaller globular fragment in human plasma (Cheng et al., 2012). In humans it was found by Lodish's group that only a small fraction of circulating adiponectin is in the globular form. (Folco et al., 2009). There is wide expression of adiponectin receptors, AR1 and AR2, in organs and in cells of the immune system, e.g. human peripheral monocytes, as well as in macrophages obtained from monocyte (Nielsen and Jensen, 1997) (Spiegelman et al., 1993). In these cells adiponectin regulates inflammatory activity and plays a critical role in restricting their transformation into foam cells, which is a characteristic of atherosclerosis (Zhang et al., 1995) (Maeda et al., 2012). Adiponectin particularly stimulates the release of the anti-inflammatory cytokine IL-10 in human monocytes and macrophages (Maeda et al., 2012) (Arita et al., 2012) and reduces the LPS-stimulated release of IL-6 in porcine macrophages (Shimomura et al., 1996). In addition, there is a decrease in adiponectin-induced secretion of IL-6 and IL-8 in monocytes from patients that have type 1 or type 2 diabetes (Spiegelman et al., 1993) (Okamoto et al., 2000). Importantly, plasma levels of adiponectin were considerably lower in obese/diabetic mice and in humans (Lawlor et al., 2005) (Mandal et al., 2011) (Hansson, 2005). Adiponectin also reduced the tumour necrosis factor-α-induced expression of adhesion molecules on vascular endothelial cells (Eckel et al., 1992) (Skurk et al., 2005). These aspects

of adiponectin biology may have substantial consequences with respect to the atherosclerotic processes, because a critical role is performed by these cells in the pathogenesis of atherosclerosis (Steinberg et al., 1989). It is recognized that adiponectin levels are lower in patients who are suffering from type 2 diabetes, obesity and coronary artery disease (CAD) (Ross, 1993) (Kodama et al., 1990). However, it has been shown that adiponectin was associated with a negative outcome in patients suffering from CAD, which challenged the emerging concept of anti-inflammatory and protective role (Schnabel, 2008). This may indicate that the increase in adiponectin serum level is a form of counter-regulatory mechanism in order to resist pro-atherosclerotic processes but it fails due to the of burden and the severity of the atherosclerotic changes. The same concept could be used in case of obesity, even though AQ is released from adipocytes, obesity is associated with low level of AQ serum level as mentioned above.

In several clinical studies, it has been found that hypoadiponectinemia (AQ less than 4ug/ml) is related to insulin resistance and type 2 diabetes, and also to dyslipidemia, cardiovascular disease and hypertension (Dellinger et al., 2008). There is a decrease in plasma adiponectin levels in patients suffering from coronary artery disease, including myocardial infarction (Eckel et al., 1992). Levels of plasma adiponectin are also related to the severity of atherosclerosis (Ouchi et al., 1999) (Kumada et al., 2003) (Otsuka et al., 2006). In addition, the level of circulating adiponectin had a significant and negative correlation with the risk of coronary artery disease in Caucasian men (Ai et al., 2011). Furthermore, when there was a low concentration of plasma adiponectin (below 4.0 μg/ml) it served as an independent risk factor for coronary artery disease in Japanese men after adjustment for other risk factors (Kumada et al., 2003) . It was also shown that hypoadiponectinemia is related to the complexity of coronary lesions in patients, who had stable coronary artery disease and those

who had acute coronary syndrome (Wang XY1, 2007). However, it is notable that in other studies, there is no relationship between plasma adiponectin levels and coronary artery disease (Kern et al., 2003) (Kumada et al., 2003) (Lawlor et al., 2013). It was also demonstrated that the levels of adiponectin did not determine the risk of coronary heart disease in British women (Laughlin et al., 2007).

The biological basis for this difference is poorly understood, with some studies suggesting that estorgen influences AQ productions but this evidence is limited and contradictory. One study reported that menopausal women have higher AQ production than premenopausal women (Sieminska et al., 2005), but another study refutes this claim and shows that it has no effect (Cnop et al., 2003).

Similarly, it was found that adiponectin levels were not related to coronary artery disease in American Indians in a case controlled study from Strong Heart Study (Lindsay et al., 2005). But a meta-analysis of seven studies showed that there was a weak but significant link between adiponectin levels and coronary heart disease (Sattar et al., 2006).

The positive, protective effects of adiponectin in the vasculature are also well documented. It has been found that adiponectin affects endothelial activity in several ways. A clinical study showed that there was a significant relationship between the levels of plasma adiponectin and vaso-relaxation in reaction to reactive hyperaemia (Ohashi et al., 2009). Adiponectin regulates blood pressure of APN-KO mice through an eNOS-dependent system (Ohashi et al., 2009). When cultured endothelial cells are treated with adiponectin, there is greater differentiation with the development of capillary-like structures and migration, while there is a decrease in apoptosis upon serum starvation, in part through AMP activated protein kinase (AMPK)- eNOS signalling pathways (Ouchi et al., 2004). In addition, adiponectin

increases revascularization in a mouse model of hind limb ischemia through an AMPK-dependent system (Shibata et al., 2004). It was reported that adiponectin encourages angiogenic responses in vivo through its capacity to increase expression of cyclooxygenase-2 (COX-2) (Ohashi et al., 2010).

It seems that adiponectin also has an impact on the differentiation and migration of vascular smooth muscle cells, which are critical players in vascular remodelling. Adiponectin stops the expansion and spread of vascular smooth muscle cells stimulated by platelet derived growth factor-BB (PDGF-BB), platelet-derived growth factor-AA, and heparin binding epidermal growth factor (HB-EGF) by inhibiting extracellular signal-regulated kinase (ERK) activation (Arita et al., 2012). Adiponectin binds to PDFG-BB, basic fibroblast growth factor (b-FGF) and HB-EGF and stops their proliferative functions in smooth muscle cells (Wang et al., 2005).

It has been found in several studies that adiponectin regulates the activity and phenotypes of macrophages. The expression of class A scavenger receptor (SR-A) is reduced in human macrophages by adiponectin, which also curtails the conversion of macrophages into foam cells (Ouchi et al., 2001). The tissue inhibitor of metalloproteinase-1 is also increased by adiponectin upon expression of interleukin-10 (IL-10) in human macrophages (Kumada et al., 2004). This result suggests that adiponectin may impede foam cell formation and prevent plaque rupture in atherosclerotic lesions. Moreover, it stops the infiltration of $CD4^+$ T lymphocytes in atherosclerotic lesions by decreasing the expression of T lymphocyte chemo-attractant in macrophages (e.g. MDC, I-309, PARC)(Okamoto et al., 2008). It is also apparent from recent findings that there is a link between the macrophage differentiation and chronic inflammatory states within adipose tissue (Lumeng et al., 2007). There are essentially two kinds of macrophages in adipose tissue, a classically activated macrophage (MI) and an

alternatively activated macrophage (M2). M1 macrophage polarization in rodent adipose tissue is a result of obesity, which plays a role in inflammatory reactions and tissue damage (Xu et al., 2003). It is indicated that M2 macrophage polarization also plays a part in wound repair and vascular security with anti-inflammatory features (Gordon, 2003). It has been found in various studies that adiponectin can encourage the phenotypes of macrophages to turn to the anti-inflammatory states. There is an increase in concentrations of M1-related genes expressed, such as interferon-γ in peritoneal macrophages (Okamoto et al., 2008) and stromal vascular fractions (SVFs) separated from APN-KO mice in comparison to wide-type mice (Ohashi et al., 2010). Consistent with the in vivo outcomes, when mouse peritoneal macrophages and SVF are treated with adiponectin, there is greater expression of M2 markers and lower expression of M1 markers (Ohashi et al., 2010). These results imply that adiponectin works to adjust the macrophage polarization from pro-inflammatory states to anti-inflammatory phenotypes, which ultimately reduces chronic inflammation.

2. Results

In this chapter we wanted to investigate the implication of old age on the expression of AR1, AR2. This was done by measuring the surface expression of both receptors on PBMCs subsets using flow cytometry and by measuring the gene expression of the receptors on mixed PBMCs. In addition, we planned to measure the circulating levels of AQ by commercial kit. We were particularly interested in the B cell due to PEPITEM being released exclusively from B cells after they are stimulated by Adiponectin (AQ) (Chimen et al., 2015).

To conduct this experiment, we recruited both young and old subjects, placing them into two different groups (n=6). The young subjects were aged between 20-35 years old and the old subjects aged between 60-75 years old. All subjects were matched for sex, ethnicity,

BMI, physical activities, illness, and statin drugs usage. See clinical parameters of the study on Table 3-1.

To investigate the expression of the receptors of our panel, we used designated markers to differentiate PBL subsets. B-cells (CD19+), NK T cell (CD56+ CD3+), Memory (CD4+\CD8+ CD45RO+) T-cells, Naïve (CD4+\CD8+ CD45RO-) T cell, NK cells (CD56+ CD3-), and CD56 high NK cell (CD56high CD3-). It's important to note that in Adiponectin measurement experiments, we used CD56+ PE antibody to identify CD3+ T cell population. This was necessary because in Integrin and Chemokine receptors chapters, CD56+ APC antibodies were utilised in order to identify CD3+ T cell population.

In case of monocytes, we studied 3 populations: classical monocytes (CD14+CD16-), non-classical monocytes (CD14+CD16++) and intermediate monocytes (CD14++CD16+ CCR2+). The gating strategy for flow cytometry, which was used, is discussed in the methodology section.

	young subjects	old subjects
N	6	6
AGE	25	67.3
BMI	24.05	26.79

Table 3-1: Clinical parameters of study subjects
Data figures are represented as a median (n=6); subjects were divided into two groups: young and old, and subjects in both groups were matched for sex and ethnicity. BMI was higher in the old subjects.

2.1. Surface expression of AR1, AR2 on PBMCs cell isolated from young or old controls.

By accruing the percentage frequency and MFI for each subset, we were able to study the surface expression of AR1 and AR2. By measuring the positive cells of AR1 on PBL subsets (Figure 3-1), we did not see a significant difference in both groups. When we look at MFI graphs (Figure 3-2), we saw a significant increase of AR1 expression on B-cells in young subjects when compared to old subjects. In cases of NK cells and CD56 high NK cells did not show any significant disparities between the groups. In the case of T cell subsets, the expression of AR1 was very low or absent which was expected. In case of AR2, the MFI of B cells was significantly higher in young subjects compared to old ones. Similarly, the expression of AR2 on other T-cell subsets was very low, or absent (Figures 3-3, 3-4).

Following this, we focussed on the surface expression of AR1 and AR2 in monocyte subsets, as mentioned above; we studied three distinct populations of monocyte: classical monocytes, non-classical monocytes, and intermediate monocytes. Consistent with the

literature, the percentage of frequency of AR1 and AR2 on monocyte subsets was very high in both groups (Figure 3-5 and 3-7). However MFI graphs of AR1 (Figure 3-6) show marginal differences between young and old subjects, no significant difference was noticed. On the other hand, we noticed a substantial difference in AR1 expression between classical and non-classical monocytes within young subjects (Figure 3- 6). The same observation was seen in old subjects (Figure 3-8).

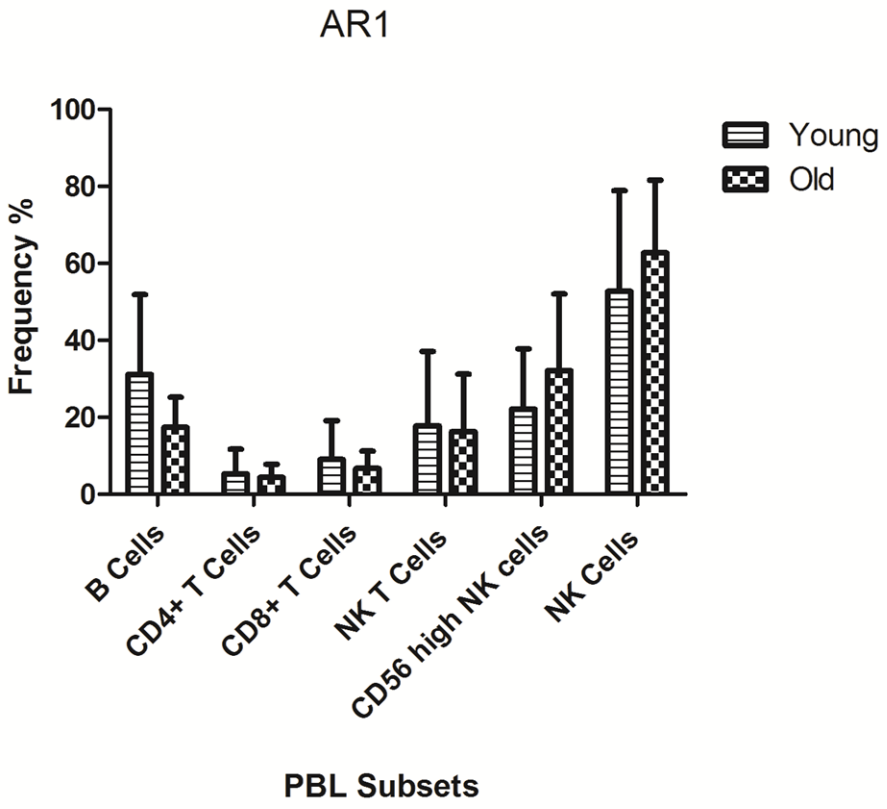


Figure 3-1:AR1 frequency on PBL subsets.

Flow cytometry was used to measure the frequency of AR1; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD19 antibody was used to identify B-cells. CD3 and CD56 were used to identify the different population of NK Cells. AR1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

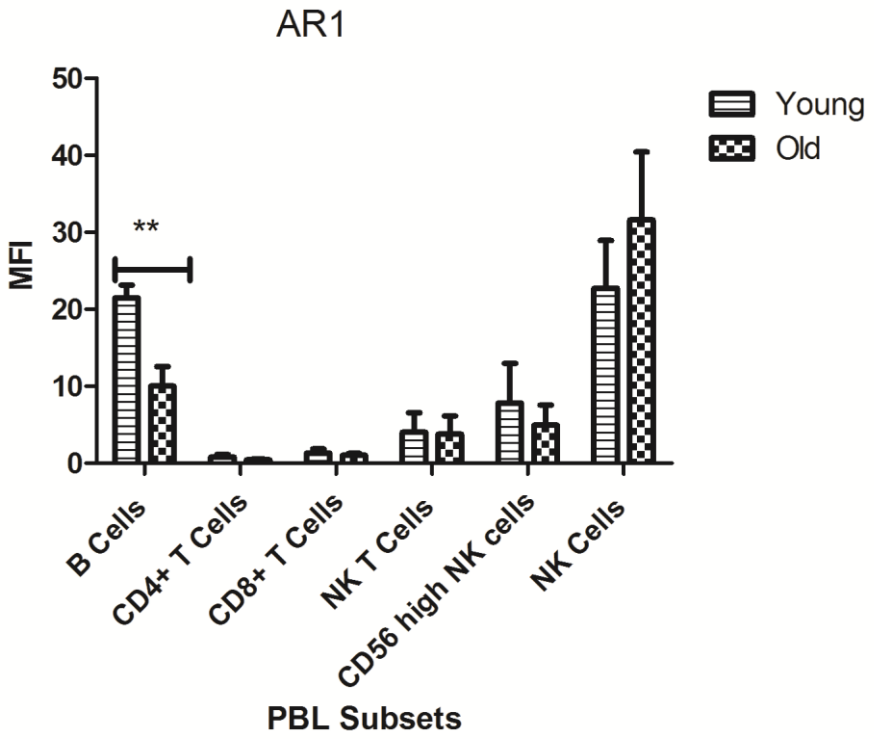


Figure 3-2:AR1 mean fluorescent intensity on PBL subsets

Flow cytometry was used to measure the fluorescent intensity of AR1; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD19 antibody was used to identify B-cells. CD3 and CD56 were used to identify the different populations of NK cells. AR1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

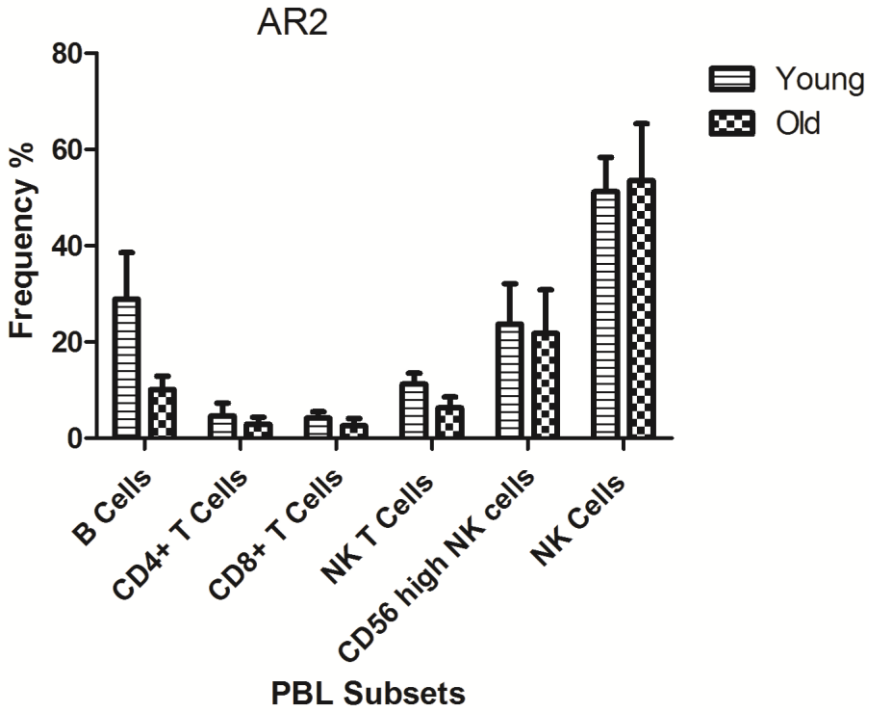


Figure 3-3: AR2 frequency on PBL subsets

Flow cytometry was used to measure the frequency of AR2; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD19 antibody was used to identify B-cells. CD3 and CD56 were used to identify the different populations of NK Cells. AR1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

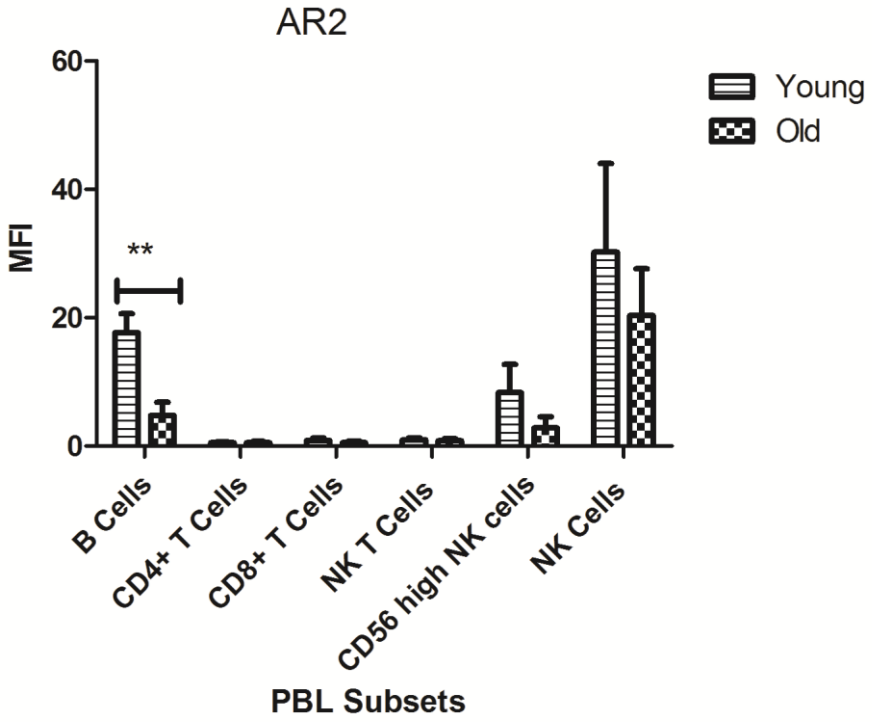


Figure 3-4:AR2 mean fluorescent intensity on PBL subsets

Flow cytometry was used to measure the fluorescent intensity of AR2; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD19 antibody was used to identify B-cells. CD3 and CD56 were used to identify the different populations of NK Cells. AR1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

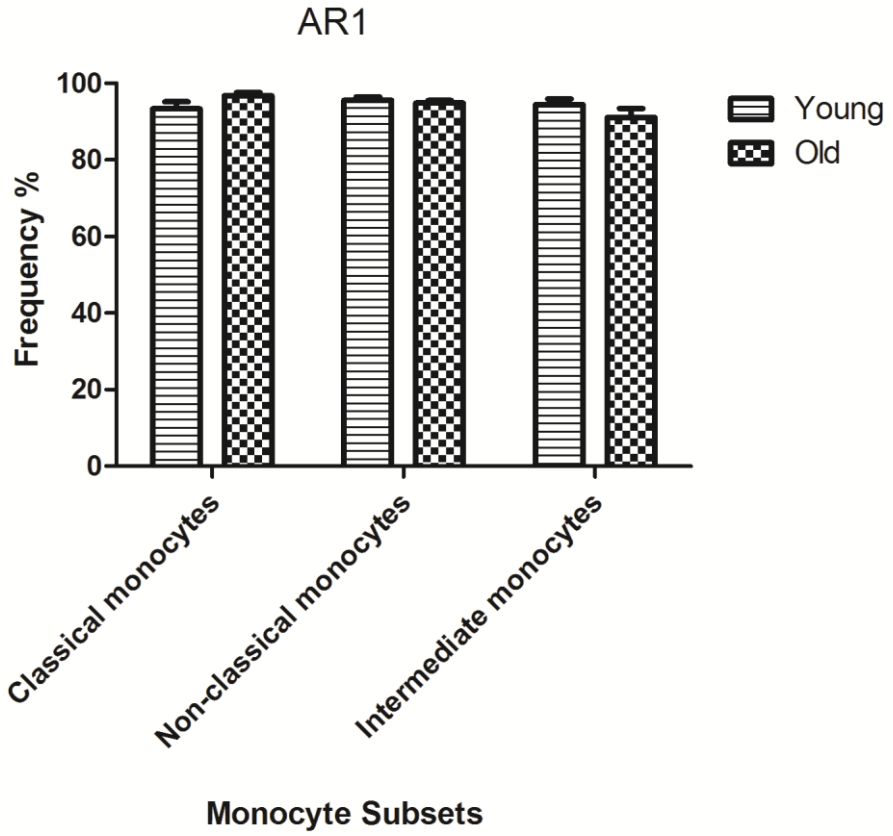


Figure 3-5: AR1 frequency on monocytes subsets

Flow cytometry was used to measure the frequency of AR1; monocytes were isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD14 antibody was used to identify classical monocytes. CD16 and CCR2 antibodies were used to identify non-classical and intermediate monocytes. AR1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

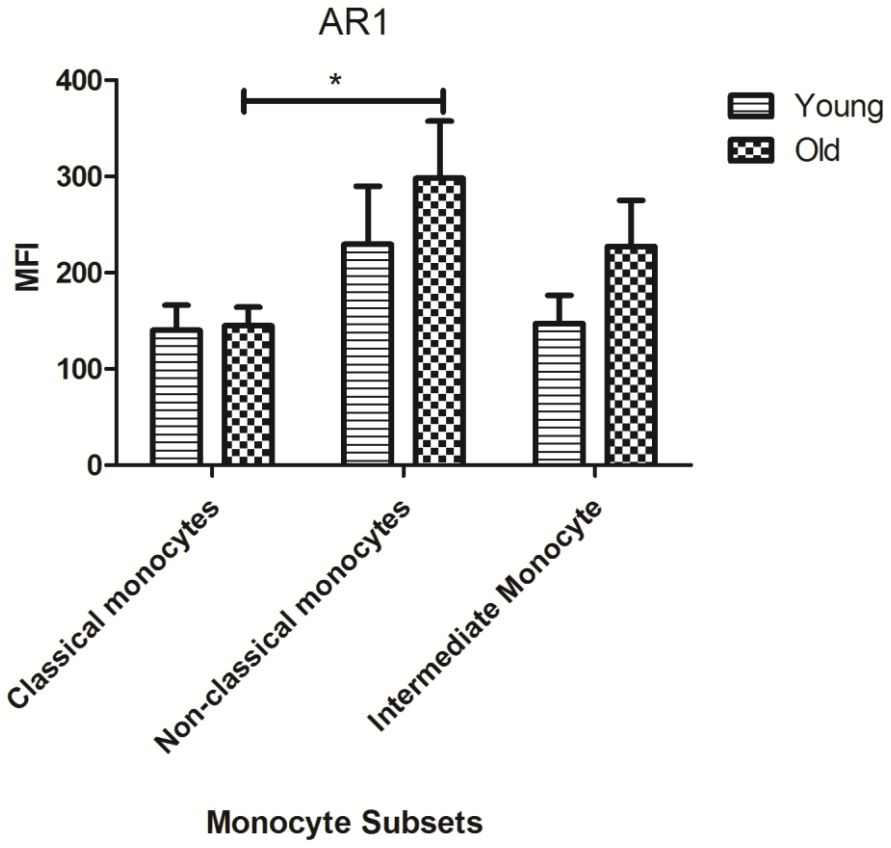


Figure 3-6: AR1 mean fluorescent intensity on monocytes subsets

Flow cytometry was used to measure the fluorescent intensity of AR1; monocytes were isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD14 antibody was used to identify classical monocytes. CD16 and CCR2 antibodies were used to identify non-classical and intermediate monocytes. AR1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

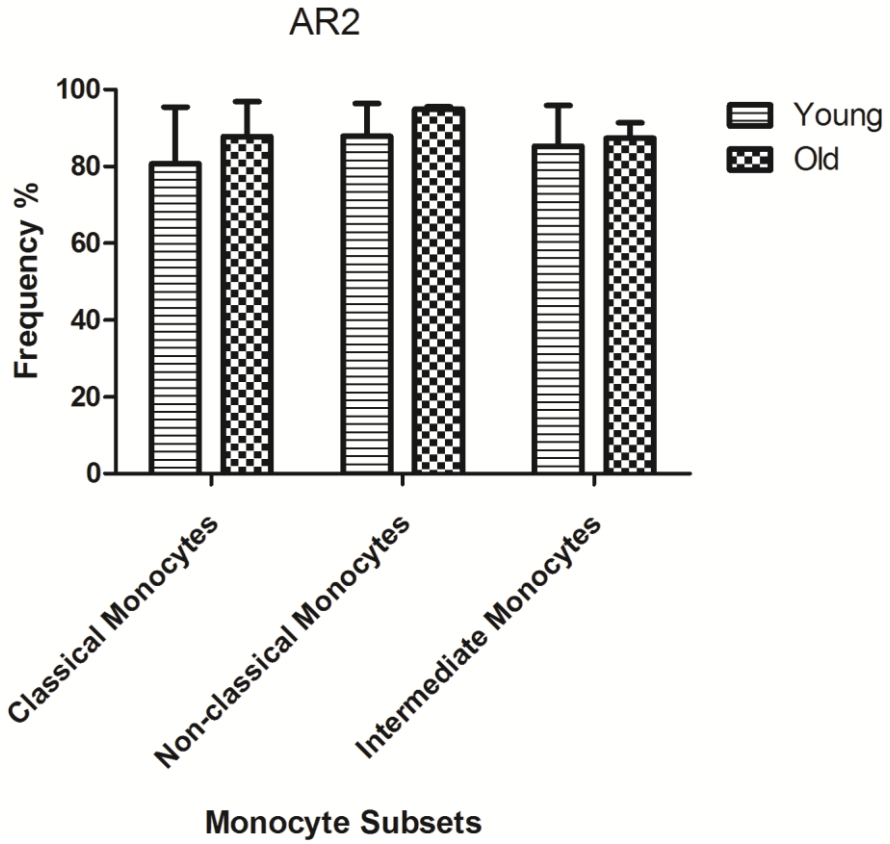


Figure 3-7: AR2 frequency on monocyte subsets

Flow cytometry was used to measure the frequency of AR1; monocytes was were isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD14 antibody was used to identify classical monocytes. CD16 and CCR2 antibodies were used to identify non-classical and intermediate monocytes. AR1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

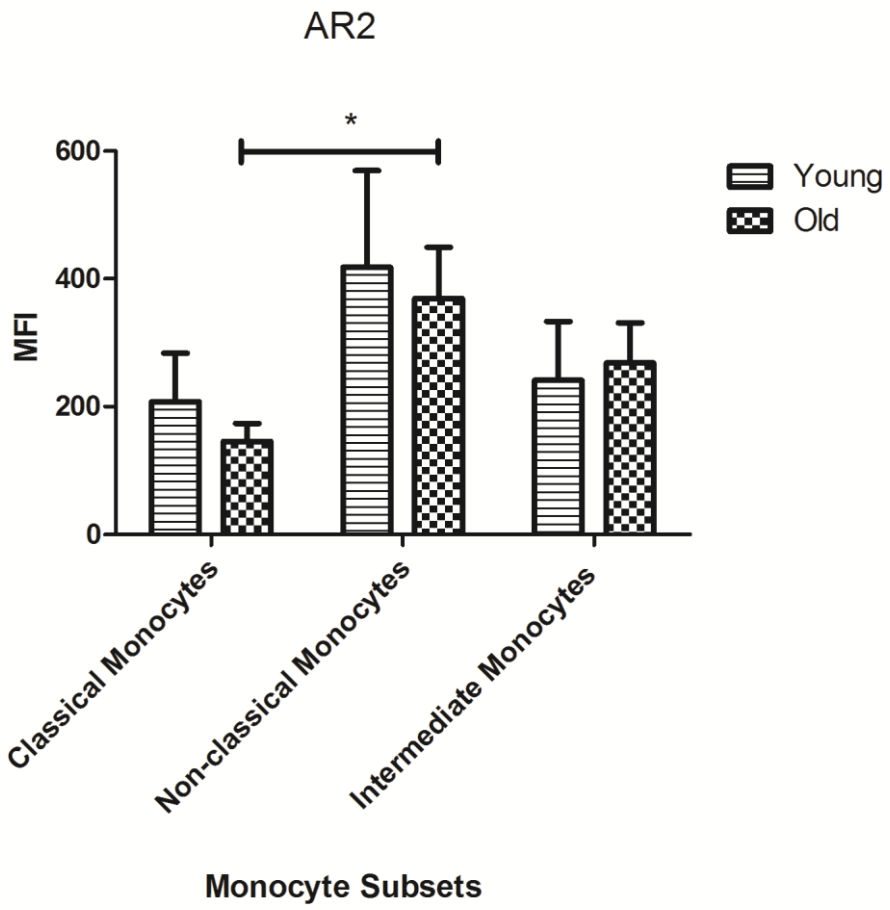


Figure 3-8: AR2 mean fluorescent intensity on monocytes subsets

Flow cytometry was used to measure the fluorescent intensity of AR2; monocytes were isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. CD14 antibody was used to identify classical monocytes. CD16 and CCR2 antibodies were used to identify non-classical and intermediate monocytes. AR1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.2. The levels of gene expression for AR1 and AR2 on PBMCs in young and old controls.

To correlate surface expression of AR1 and AR2 with the gene expression of both receptors on the same young and old groups, we measured the gene expression on PBMCs. However, we found no significant difference in AR1 or AR2 gene expression between young and old subjects (Figure 3-9).

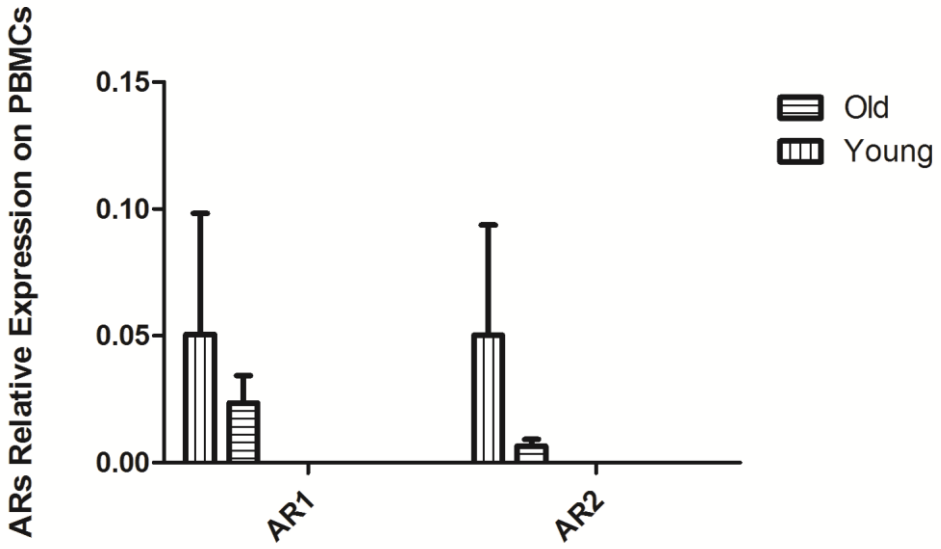


Figure 3-9:AR1/2 gene expression on PBMCs in young and old subjects.

PBMCs were isolated from fresh blood samples and gene expression was measured and quantified using qPCR. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.3. The levels of adiponectin in young and old controls.

Since AQ is an important factor in leukocytes trafficking and the PEPITEM pathway, it is very useful to measure the AQ serum level in both groups. Hence, we collected serum samples from the same young and old subjects and then used Quantikine ELISA Kits (R&D SYSTEMS). This assay revealed significant increase in AQ level in the serum of old subjects compared to that found in young subjects (Figure 3-10).

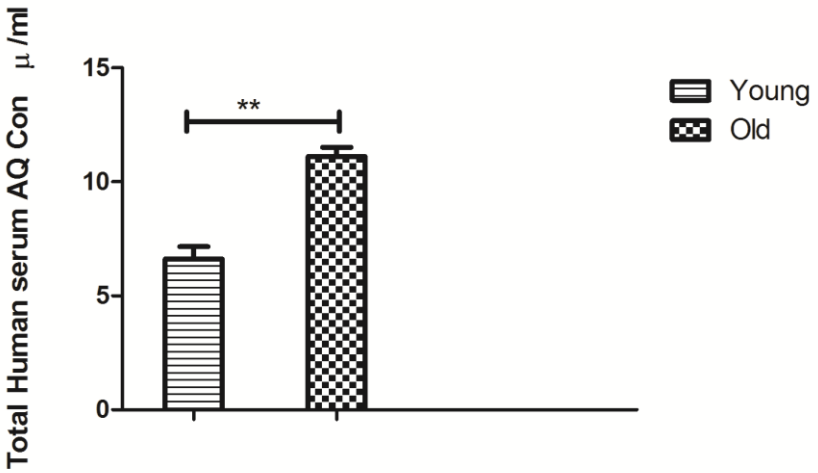


Figure 3-10: Circulating AQ levels in young and old subjects.

Circulating AQ levels were measured using ELISA kits and the numeric values were calculated using a standard curve, as described in the manufacturer's protocol. Data is mean ± SEM (N=6), and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (N=6). (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

3. Discussion

We argue that old age has a negative correlation on the expression of Adiponectin receptors 1 and 2 (AR1, AR2) on B cell, which may contribute to the increased susceptibility of elderly people developing cardiovascular disease. It is well established that Adiponectin (AQ) has a protective function against cardiovascular diseases (Kato et al., 2006), therefore from the data we showed that the expression of Adiponectin receptors (ARs) decreases with old age. This may inhibit AQ protective action, and subsequently increases the chances of developing cardiovascular disease. Other findings demonstrated the distribution of adiponectin receptors on PBL. The literature has shown that the frequency of ARs on B cell is the highest among PBL; by 47% then NK cell with 21% (Pang and Narendran, 2008), however our data shows that NK cells express more ARs than B-cells. This variation could be due to technical issues, such as the fluorescence difference between analysers or due to employing a different protocol for sample preparation. Moreover, there are some variations in the protocol of creating antibodies, for example the incubation time, which could affect the intensity of the fluorescence.

The immune-regulatory effect of Adiponectin is focused on myeloid-monocytic lineage cells (Tilg and Moschen, 2006). Therefore, the fact that B and NK cells express more adipoectin receptors suggests that those subsets have potential capabilities to regulate the immune and inflammatory systems.

Regarding monocytes expression, our data is consistent with the literature that suggests that monocytes have the highest expression of ARs among PBMCs. There was no significant variation in the frequency or MFI of both receptors on the three subsets. It was expected that there would be a significant decrease of the expression ARs on monocytes subsets because of

the hypothesis which states that monocytes differentiate into M1 macrophages (proatherogenic) more than into anti-inflammatory M2 macrophages (Lovren et al., 2009). We postulated that due to the fact that older subjects have lower expression of ARs on B and NK cells, this could mean that they are less sensitive to Adiponectin. Based on the hypothesis of Lovren et al. that states that Adiponectin primes monocytes to differentiate into the anti-inflammatory macrophages (M2), we expected low ARs expression on monocytes subsets.

During the analysis, interesting differences between the expressions of AR1 and AR2 were noticed on monocyte subsets within the groups, with non-classical monocytes higher in expression in both groups compared to classical monocytes. It is well known that classical monocytes are considered as scavenger cells (Ziegler-Heitbrock, 2015), whereas non-classical monocytes are regarded as pro-inflammatory monocytes. In fact, non-classical monocytes have been proven to be involved in the progression of atherosclerosis (Stansfield and Ingram, 2015). We could conclude from this that the increase of AR1 and AR2 expression on non-classical monocytes in old subjects, compared to that on classical monocytes in the same group, could be an early indicator of inflammation.

The results from AR1 and AR2 gene expression are not conclusive or significant due to variability within groups. From the literature we know that the surface expression of ARs on PBMCs are very sensitive to lifestyle because studies have shown that that aerobic exercises modulate the gene expression of AR1 and AR2 on PBMCs (Lee et al., 2015). Therefore, we can speculate that our young subjects may have been more physically active during their participation in the study and this may have confounded our observation. So, it could be suggested that the optimum level of AQ in healthy young subjects aged between 20 and 35 years is 7.0 ± 0.7 μg/ml (Adamandia D. Kriketos, 2004), whereas the AQ level of old subjects was 8.9±4.2 μg/ml in a study done by Isobe et al. (2005) where there were 372 subjects aged

60.3± 12,5 years (Isobe et al., 2005). However, in our study the level is higher than this in healthy, older subjects. We speculate that the increase is a compensation mechanism to overcome the reduction in ARs that occurs on some leukocytes with age. This would maximise signalling through those receptors.

4. Conclusion

Old age has a negative correlation on the surface expression of AR1 and AR2 on PBL. This negative impact presented as a significant decrease on the surface expression on AR1 and AR2 on B-cells in old subjects, no significant change was seen on monocytes however. AQ serum level is higher in old subjects when compared to young ones something which may be a compensating mechanism to ensure efficient AQ function.

CHAPTER 4: STUDYING THE EFFECT OF AGE ON CHEMOKINE AND INTEGRIN RECEPTOR EXPRESSION ON PBL SUBSETS.

1. Introduction

With the progress in age, cells of the smooth muscles and vascular endothelium become dysfunctional and complex functional and structural transformations are induced by aging which is an important factor for developing cardiovascular diseases (Herrera et al., 2010) (Lundberg and Crow, 1999). The pro-inflammatory proteins i.e. the adhesion molecules, actively take part in inflammation process and immune response, and are crucially involved in cell-matrix/cell-cell interactions (Zou et al., 2003). The majority of cardiovascular disorders such as coronary artery disease (Heinz et al., 2003) and peripheral arterial disease (Ridker et al., 1998), and other aging related commonly occurring conditions (Espinosa-Heidmann et al., 2002), plasma levels of soluble adhesion molecules are used as risk factors and biomarkers, and are also used as indicators of disease prognosis and severity (Ridker et al., 1998).

Limited research with inconsistent data is available for reporting the aging related alterations in soluble adhesion molecules expression. For example, an increase in soluble ICAM-1 and VCAM-1 in humans, caused by old age, has been reported by the study of Miles et al. (Miles et al., 2001). It has been shown by the study of Morisaki et al. (Morisaki et al., 1997) that positive correlation with age is associated only with soluble VCAM-1, and not with soluble ICAM-1. However, a decrease in the levels of VCAM-1, ICAM-1 and E-selection has been reported in another study (Nash et al., 1996).

There is no doubt that there is a poor understanding of aging mechanisms at present, but the conjecture suggested that the responsibility of aging consequences is due to the oxidative stress hypothesis and according to it the distinctive dysfunctions of aged subjects and abnormality in cellular functions are attributed to an imbalance in cellular redox system (Yu, 1996) (Harman, 1956). It was concluded by the authors that increase in oxidative stress is

responsible for altered expressions soluble adhesion molecules with advanced age (Herrera et al., 2010).

It has been observed that cell trafficking is reduced towards T-cells in older mice for $CD4^+$ T-cells (through down-regulation of CD31 which contributes in the activation and adhesion of T-cells) (Turner and Orme, 2002) and for mononuclear cells of peripheral blood in older rats (Schmucker et al., 2002).

1.1. Integrins

Integrins are a significant family of cell surface-adhesion receptors that are manifested in all metazoans. They are heterodimers of non-covalently linked α and β subunits (Harburger and Calderwood, 2009). It is important for them to have particular attachment to extracellular matrix (ECM) proteins, or in certain situations, to counter-receptors on adjacent cells, as it allows for cell adhesion and brings about embryonic development, tissue maintenance and repair, host defence and haemostasis (Harburger and Calderwood, 2009). The processes are dependent on the relationship of integrins to the intracellular cytoskeleton through the normally short integrin cytoplasmic tails; these relationships allow for the bi-directional movement of force over the plasma membrane (Calderwood et al., 2000) (Evans and Calderwood, 2007). Apart from their mechanical functions in respect of anchorage, integrins transfer chemical signals inside the cell (outside-in signalling), providing information regarding its location, domestic setting, adhesive state and adjacent matrix (Hynes, 2002) (Miranti and Brugge, 2002). Many cellular reactions are based on these signals, and include survival, migration and differentiation. Apart from outside-in signalling, integrins can manage their affinity for extracellular ligands. This is done by making conformational adjustments to the extracellular domains that take place as a reaction to signals that modify the integrin

cytoplasmic tails – a process known as inside-out signalling or activation (Calderwood et al., 2000). The best-characterized methods for examining integrin activation are for platelet and leukocyte integrins; however, integrin activation is a broad phenomenon that is critical for several kinds of cells where it manages matrix remodelling, tissue generation, angiogenesis and cell migration (Evans and Calderwood, 2007).

1.1.1. Integrin signalling

It is important to manage integrin activity since it is unsuitable for integrin activation in blood platelets, as it may for example contribute to thrombosis (Hynes, 2002). It appears that the α subunit of integrins is more important for the extracellular ligand binding specificity as the smaller cytoplasmic tail of the β subunit is the area that interacts with regulatory proteins in the inside-out process (Hynes, 2002). When the integrins are in a state of rest, α and β cytoplasmic tails are close to each other which restricts the integrin in a low-affinity constitution. Conformational modifications are brought about following the dissociation of the two tails by signals inside the cell, which then activates the integrins (Luo et al., 2007).

- **Alpha-L**

Significant roles are performed by integrin αLβ2 (LFA-1; CD11aCD18) in leukocyte biology that comprises of leukocyte adhesion, movement, and immune synapse generation (Lu et al., 2001). Ligands of αLβ2 include the ICAMS and the junctional adhesion molecules (Staunton et al., 1990) (Defougerolles and Springer, 1992).

- **Alpha-4**

The a4 integrin subunits take part in leukocyte cell-cell and cell-matrix interactions and migration (Elices et al., 1990). The ligands of these cells are domains 1 and 4 of vascular cell adhesion molecule-1 (VCAM-1, CD106) (Defougerolles and Springer, 1992).

- **Beta-1**

There is extensive distribution of the β1 integrins (very late antigen, VLA) within several T cell subsets (Kubes et al., 1995). It was asserted in the past, there was barely any expression of β1 integrins on neutrophils so there could be no functional implication (Neeley et al., 1993). However, it has been indicated in a variety of recent reports that VLA-4 (α4β1), VLA-5 (α5β1) and VLA-9, (α9β1) demonstrate an expression on human and rat neutrophils and contribute to both adhesion and migration (Kubes et al., 1995).

- **Beta-2**

There is high expression of β2 integrins (CD18) on neutrophils and other leukocytes and this is important for adhesion to endothelial cells, particularly of CD11b/ CD18 (αMβ2, Mac-1, CR3), which is a flexible molecule that attaches to numerous different ligands like ICAM-1 (van den Berg et al., 2001). However, the most significant integrins on T lymphocytes are α4β1 (VLA-4, CD49d/CD29) and αLβ2 (LFA-1, CD11a/CD18) which attach to VCAM-1 (CD106) and ICAM-1 (CD54), respectively (Elices et al., 1990) (Makgoba et al., 1988). It is possible for α4 and β2 integrins to facilitate stable adhesion, leukocyte proliferation, and migration, while only α4 integrins can facilitate preliminary rolling interactions (Alon et al., 1995). The ligand binding ability of α4β1 and αLβ2 integrins that are constitutively expressed on circulating leukocytes is low; however, at areas of emigration during inflammation or trafficking, these integrins are “activated” in a chemokine regulated way (Chan et al., 2000).

1.2. Chemokines

Chemokines are chemotactic cytokines that activate particular G-protein-coupled 7-transmembrane receptors (Figure 4-1). They have been classified into C, CC, CXC, and CX3C families based on the position of conserved cysteine residues. CX3CL1 is the only recognized CX3C chemokine. This is also called fractalkine (Zlotnik and Yoshie, 2000) and is expressed by the activated vascular endothelial cells (Bazan et al., 1997), neurons (Harrison et al., 1998), epithelial cells (Lucas et al., 2001), smooth muscle cells (Ludwig et al., 2002), dendritic cells (DCs) (Papadopoulos et al., 1999) and macrophages (Greaves et al., 2001). The only recognized CX3CL1 receptor, CX3CR1, has expression on T-cell and natural killer (NK) cell subsets (Imai et al., 1997), DC subsets, brain microglia (Harrison et al., 1998) and also on blood monocytes. Tight, integrin-independent adhesion of CX3CR1-expressing leukocytes is encouraged by CX3CL1. It has been found that human CX3CR1 gene polymorphisms are genetic risk factors for both coronary artery diseases and atherosclerosis (Moatti et al., 2001). It was also found that mice that were deficient in client CX3CR1 or CX3CL1 demonstrated relative resistance to atherosclerosis formation in the relevant murine disease models (Combadiere et al., 2003). However, there are still arguments on the mechanistic justifications for these phenotypes.

- **CXCR3**

There has been particular focus on the chemokine system, with concern given to its contribution to several biological processes (Bonacchi et al., 2001). CXCR3 has been found on various cells of hematopoietic family, including T and B lymphocytes, and natural killer cells (Luster, 1998). Various chronic inflammatory diseases have shown expression of the CXCR3-targeting chemokines, including ulcerative colitis (Cole et al., 1998) and

arthrosclerosis (Pupilli et al., 1999). There is limited information on the signal transduction pathways that CXCR3 activates and the way they are possibly linked to biological actions shown by its agonists.

- **CCR5**

CCR5 is a functional receptor for several inflammatory CC-chemokines and has expression on macrophages, migroglia, memory T cells and dendrite cells. It has been found that CCR5 regulates chemotaxis, proliferation and immune responses (Gosling et al., 1997). There are two subsets of CD4+ lymphocytes. Th1 lymphocytes produce interferon gamma (IFNg) and lymphotoxin, which encourage cell-mediated immunity to intracellular pathogens (Abbas et al., 1996). Th2 lymphocytes produce interleukins 4 and 5 (IL-4 and IL-5), and these play a part in allergy and humoral immunity to parasites (Abbas et al., 1996). It was found that there was expression of high concentrations of CCR5 in Th1; however, it was almost non-existent in Th2 lymphocytes. Furthermore, after T lymphocytes are recovered from the synovial fluid present in rheumatoid joints, demonstrate a Th1 phenotype, express CCR5 and CXCR3 in high concentrations and CCR3 in low concentrations (Baggiolini et al., 1997). This study provides further evidence that chemokine receptors are expressed on T lymphocytes on the basis of their state of activation and differentiation. Those chemokines that are often generated in inflammation do not bring about any response in naïve and memory T lymphocytes however. CCR1, CCR2, CCR5 and CXCR3 expression and chemotactic migration determines activation, especially with IL-2 (Wu et al., 1997).

1.2.1. Chemokin receptors

These are a subgroup of heterotrimeric GTP-coupled proteins (G-proteins), a large seven transmembrane domain receptor family (Murphy et al., 2000) (Figure 4-1).

Designation of chemokine receptors is based on the chemokine (s) mediators they couple (CC, CX3C, CXC, XC), followed by R for receptor and a digit or numeric denoting the discovery order (Moser et al., 2004). G-proteins of G_i-type are required by these receptors for signal transduction as described by experiment on toxin of Bordetella pertussis (Murphy et al., 2000, Suresh and Wanchu, 2006). Molecular responses of cells to chemokines are usually temporary in duration and rapid in onset, the cells regain retrieve responsiveness to a certain chemokine after a brief period of culturing in medium lack chemokine by a transient cellular desensitization phenomenon which is a negative control mechanism evoked signal transduction of chemokine receptors (Moser et al., 2004). Directed cell migration and polarized chemokine sensitivity are maintained along a chemokine gradient by persistent chemokine receptor redistribution on leucocyte surfaces induced by rapid internalization (Moser and Willimann, 2004). It is not mandatory that chemokine coupling proteins would elicit cell migration, as described by CXCR3-B (Lasagni et al., 2003). A high indiscrimination exists in chemokine selection by several chemokine receptors, and vice versa; one receptor, for example, can bind to more than one chemokines (Zlotnik and Yoshie, 2000, Murphy, 2002, Moser et al., 2004) (Figure 4-2). Inflammation is also caused by such redundancy, in contrast to numerous monogamous chemokine systems having activity in homeostatic leucocyte migration and development process; certain natural chemokines having combinatorial agonistic-antagonistic ability, accentuating their importance as controllers of leucocyte navigation, have been reported to exist. For CCR3, the antagonists are CXCL11, CXCL10 and CXCL9 (Moser et al., 2004).

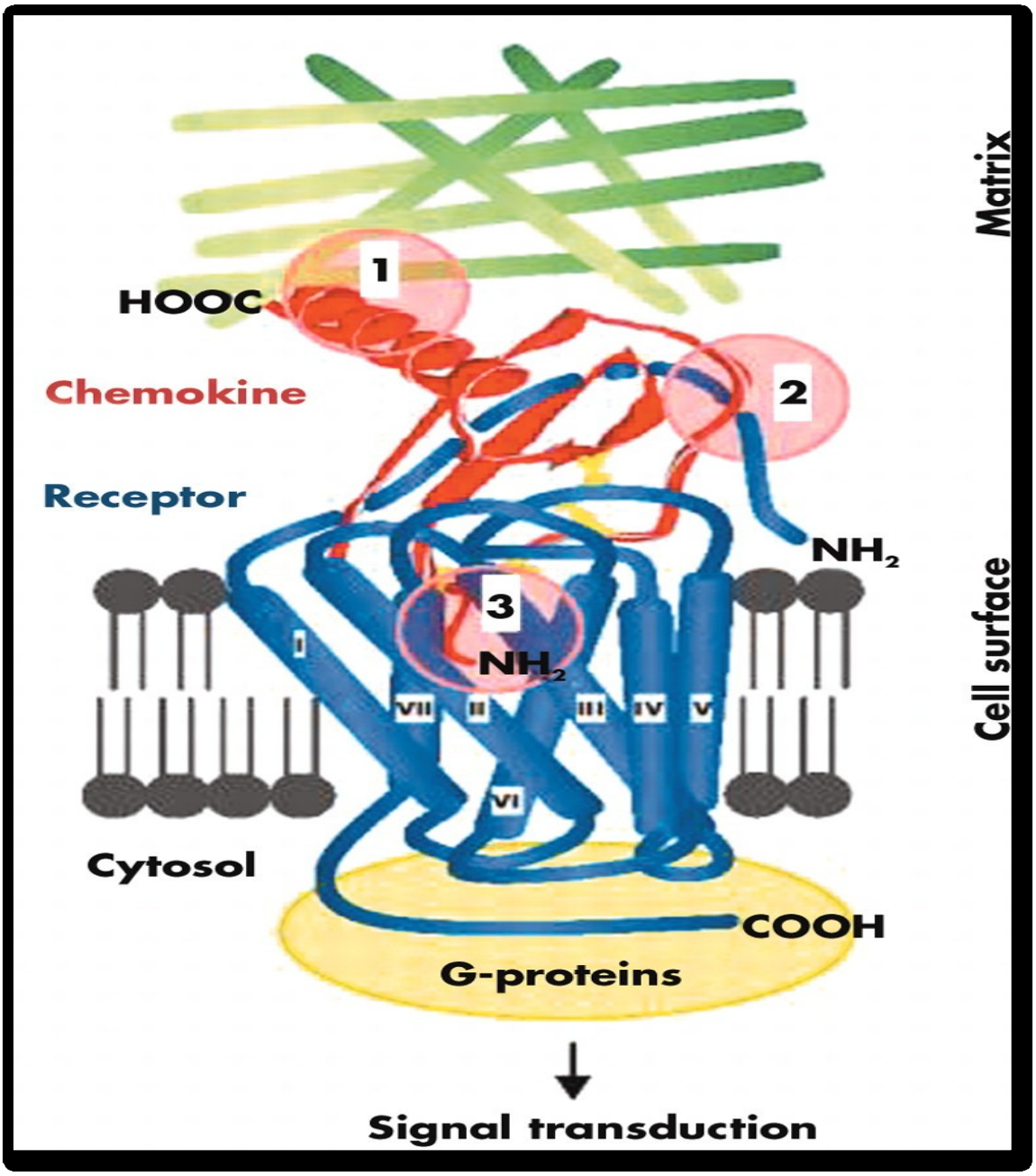


Figure 4-1: Transmembrane domains of chemokine receptors

Seven transmembrane domains tend the chemokine receptors to be imbedded into membrane. (1) NH2-terminus, which reacts with chemokine coupling pocket created by transmembrane domains of chemokine receptors, (2) N loop terminus, that enables starting receptor contact, and (3) matrix fixation site in COOH terminal α-helix or core structure, are the three functional sites of chemokines, Three intracellular loop sites and COOH terminus contribute in signal transduction mediated by G-protein whereas chemokine coupling is coordinated by three extracellular loops sites and NH2-terminus. (Israel F. Charo, 2006).

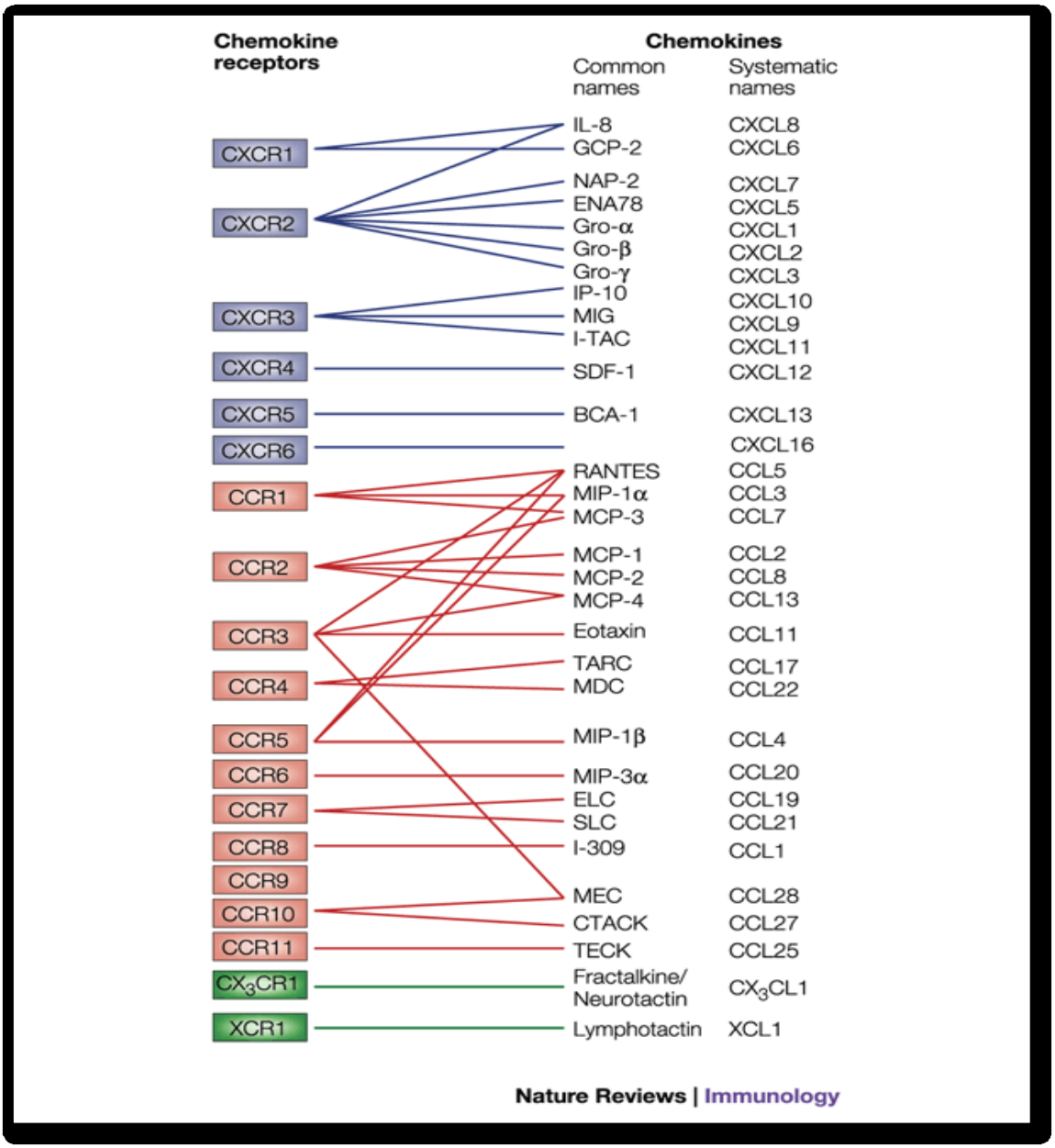


Figure 4-2: Chemokine receptors and their ligands (Proudfoot, 2002)

Chemokines are classified into subclasses based on the spacing of the N-terminal cysteine residues. Blue colour indicates the receptors for the α(or CXC) whereas the receptors for the β(or CC) subclass in red. Green colour indicates and the receptors for the minor subclasses (C and CX3C).

2. Results

2.1. Characterisation of surface expression of adhesion molecules and chemokine receptors on fresh blood PBL, migrated PBL, and surface adherent cells.

We began our experiment by comparing the expression levels of adhesion molecules and chemokine receptors on fresh blood PBL, migrated PBL, and surface adherent PBL. Our subjects were male Caucasians aged between 20-75 years of age. The PBL was isolated directly from fresh whole blood to measure the surface expression of certain adhesion molecules and chemokine receptors on circulating populations of lymphocytes. Our adhesion molecules and chemokine receptor panel included the receptors of αL, α4, β1, β2, PSGL-1, CCR5, CXCR3, and CX3CR1. From the same donors we isolated PBL from transmigration assays which enabled us to study the effect of recruitment by EC on the expression of these molecules. During this part of the experiment, The PBL was briefly placed on stimulated EC for 7 minutes, and then surface adherent PBL (Phase bright PBL) were detached from EC using cold EDTA and re-suspended and kept ready for staining. The transmigrated PBL (phase dark PBL) were collected from EC by adding EDTA for a longer time and shaking the plate with the cells that were collected and kept for staining. To investigate the expression of these receptors on different lymphocyte populations we used a panel of cell specific markers; B cells (CD19+), NK T cell (CD56+ CD3+), Memory (CD4+\CD8+ CD45RO+) T cells, naïve (CD4+\CD8+ CD45RO-) T cells, NK cells (CD56+ CD3-), and CD56 high NK cells (CD56high CD3-). Interestingly we found that fresh blood PBL have a high expression of CCR5, CXCR3, and CX3CR1 receptors on all PBL subsets but the expression is lost after migration as seen on Figures 4-3, 4-4, and 4-5. We observed very clear and significant differences between circulating cells and cells recruited by EC. Interestingly, transmigration

was not a requirement for down regulation of these receptors, as even the surface adherent population has low expression.

Furthermoe, we measured the expression of adhesion molecules β-1, β-2, α-L, α-4, PSGL-1; however, we did not notice the same pattern of a sharp loss of the surface expression after adhesion and transmigration. Figure 4-6 shows some variation on the surface expression of Alpha-L integrin on NK T cells, CD56 High NK cells, and NK cells. In the case of Alpha-4 integrin, as seen in figure 4-7, the surface expression did not change after adhesion and transmigration, except for some modest differences after transmigration on NK T cells, CD56 High NK cells, and NK cells. Regarding the surface expression of β1 integrin in figure 4-8, the graph shows expression of β1 is unchanged after transmigration on all subsets. Similar patterns of surface expression after transmigration were seen in the case of β2 and PSGL-1 in all PBL subsets except B cells as seen on figure 4-9 and 4-10. Following this, we pooled the data for surface adherent and migrated PBL and compared their surface expression with circulating PBL, the surface expression of β2 on N-CD4, N-CD8. We found that M-CD4 increased significantly the after transmigration (Figure 4-14), the expression of the remaining molecules remained unchanged after transmigration as seen on figure (4-11, 4-12, 4- 13, 4-14, 4-15).

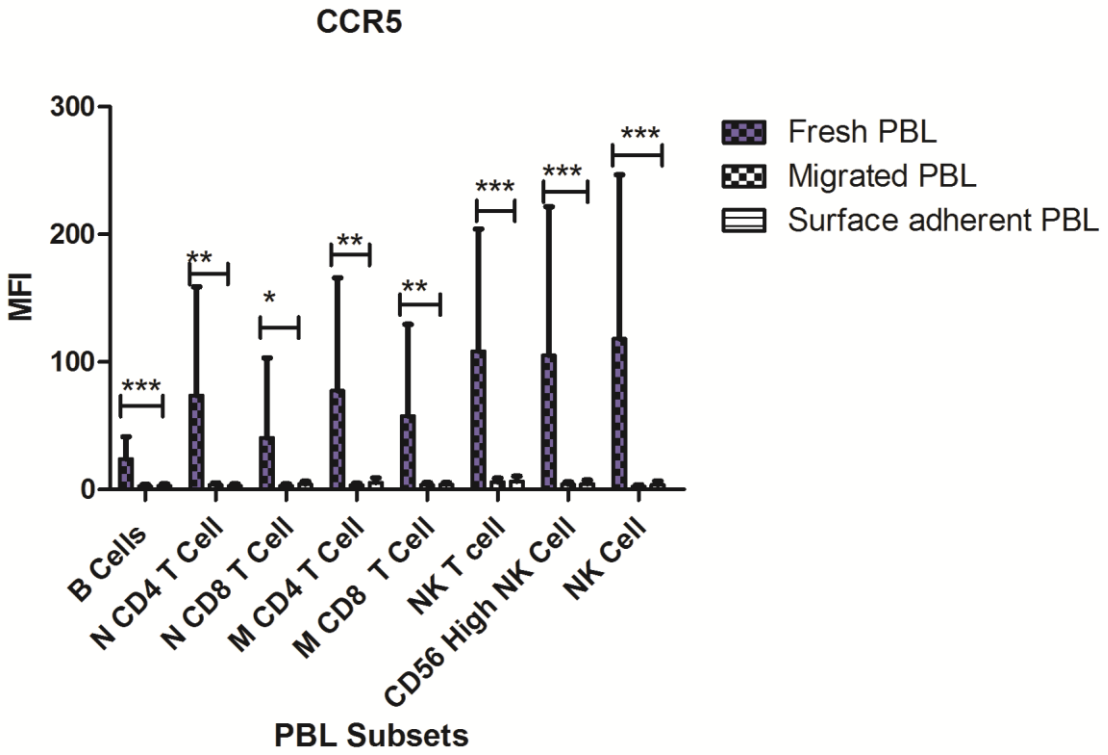


Figure 4-3: MFI of CCR5 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI of CCR5 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. Finaly, The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

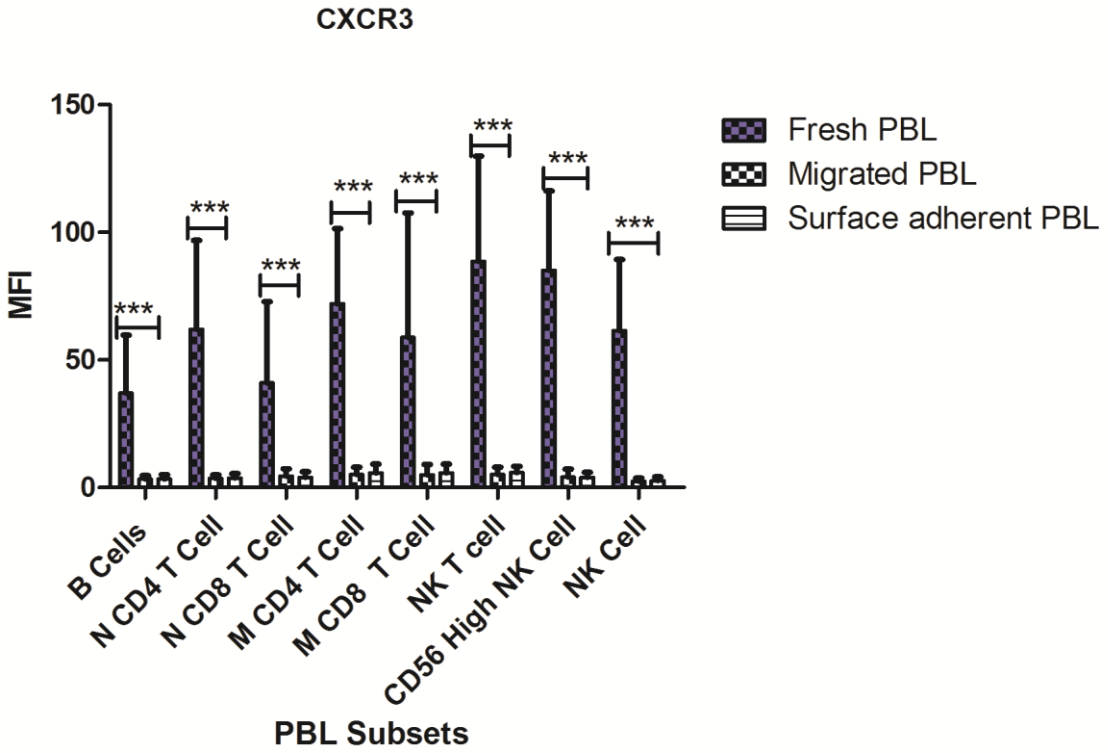


Figure 4-4: MFI of CXCR3 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI of CXCR3 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

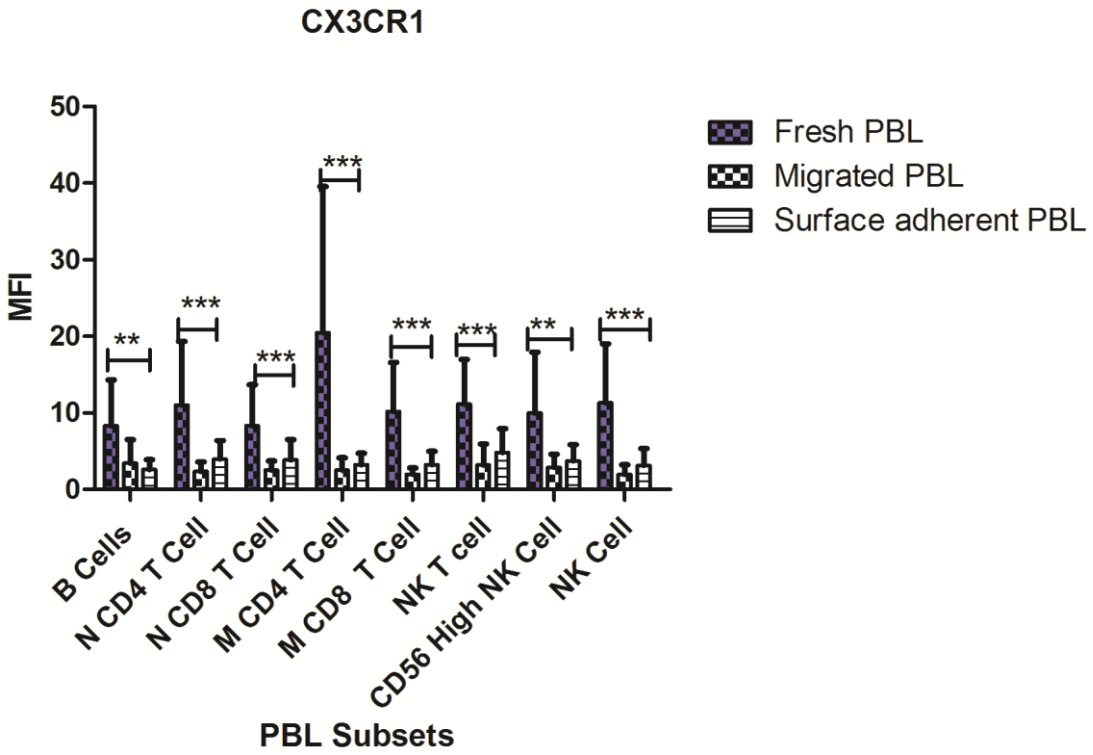


Figure 4-5: MFI of CX3CR1 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI of CX3CR1 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, the PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

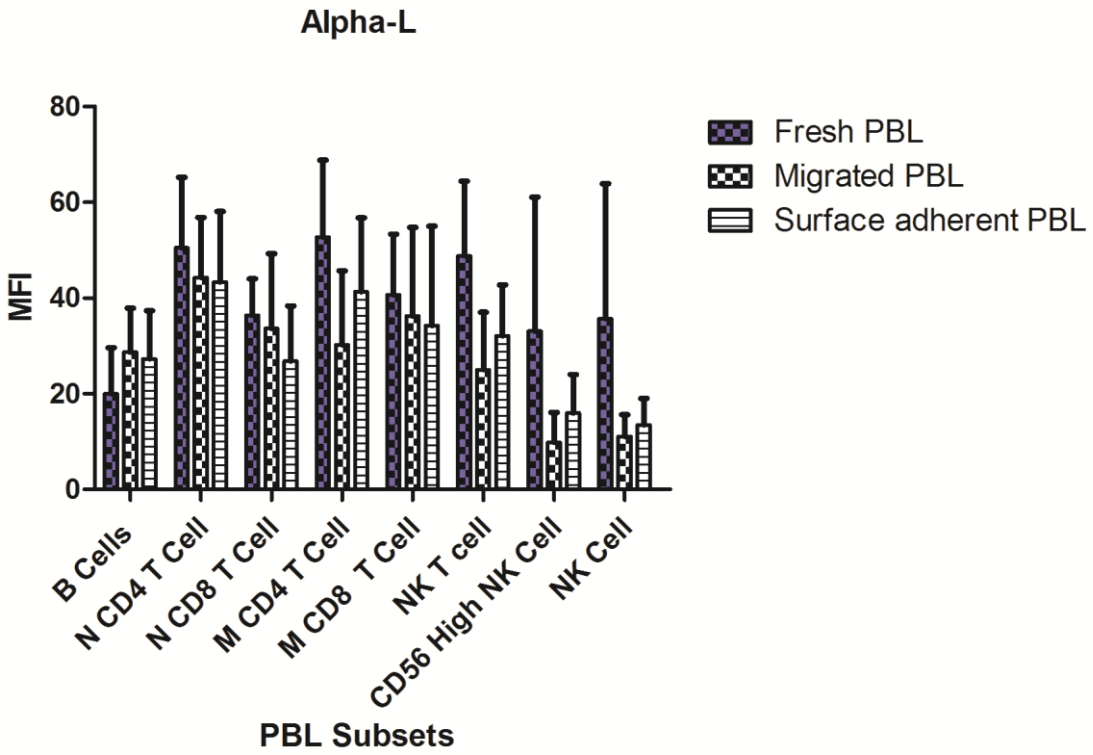


Figure 4-6: MFI of Alpha-L receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI of Alpha-L receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, the PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. Alpha-L receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

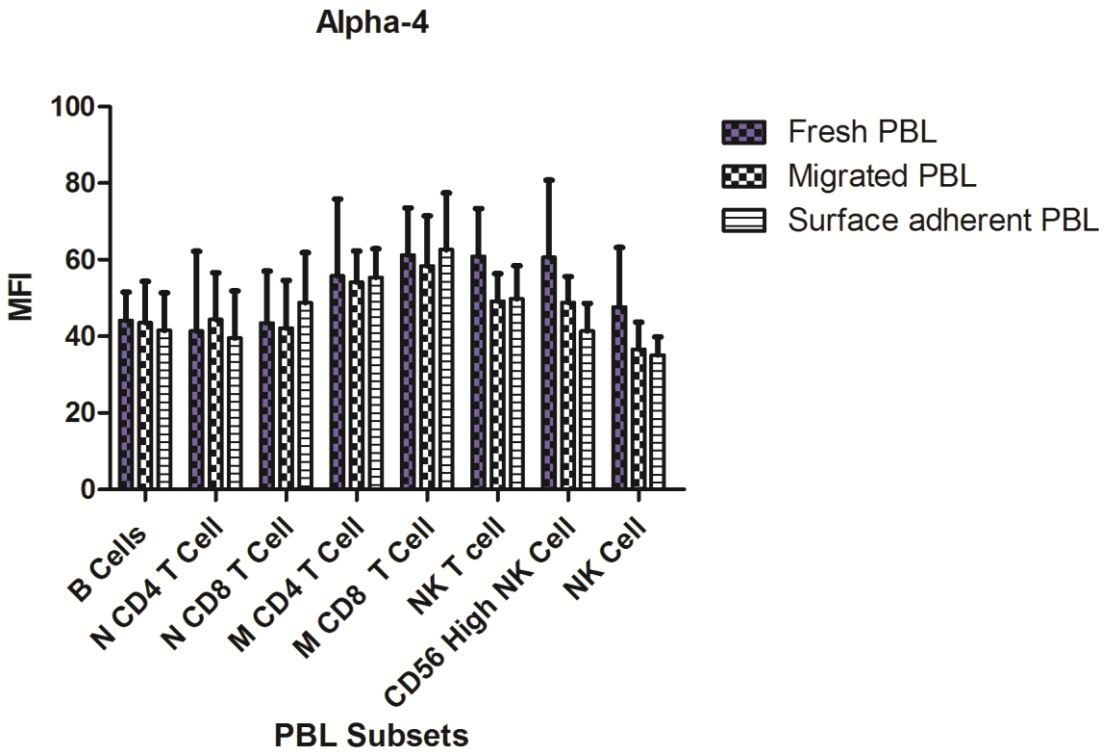


Figure 4-7: MFI of Alpha-4 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI of Alpha-4 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, the PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. Alpha-4 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

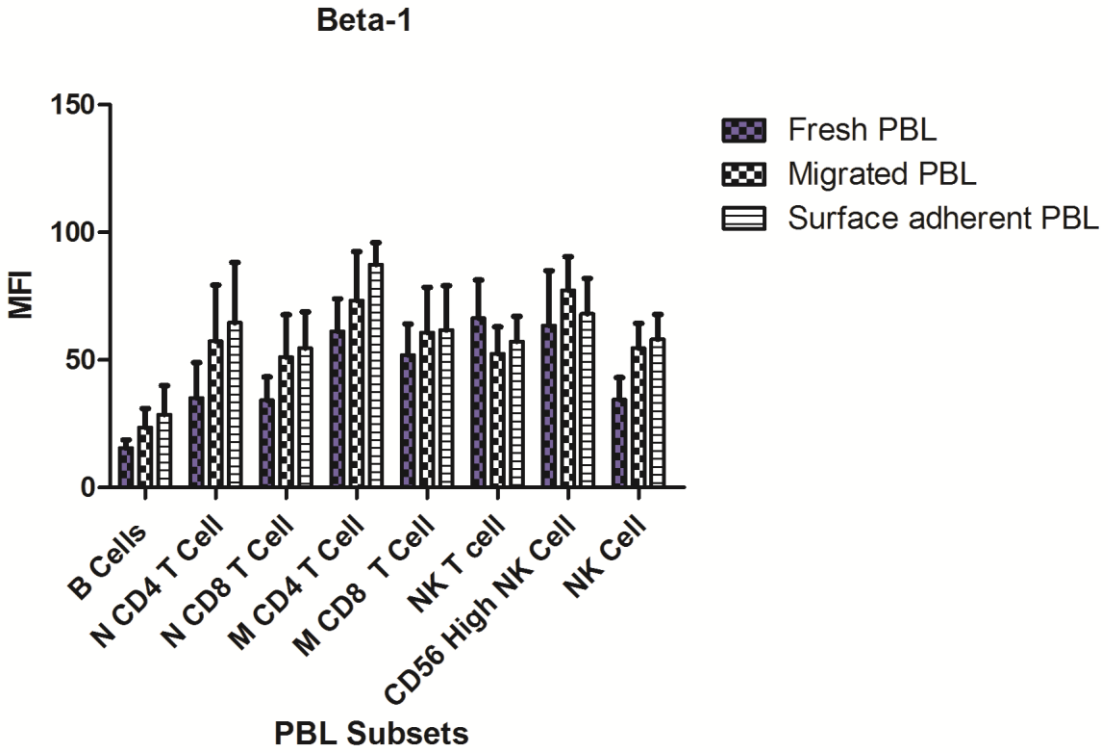


Figure 4-8: MFI of Beta-1 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI of Beta-1 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, the PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

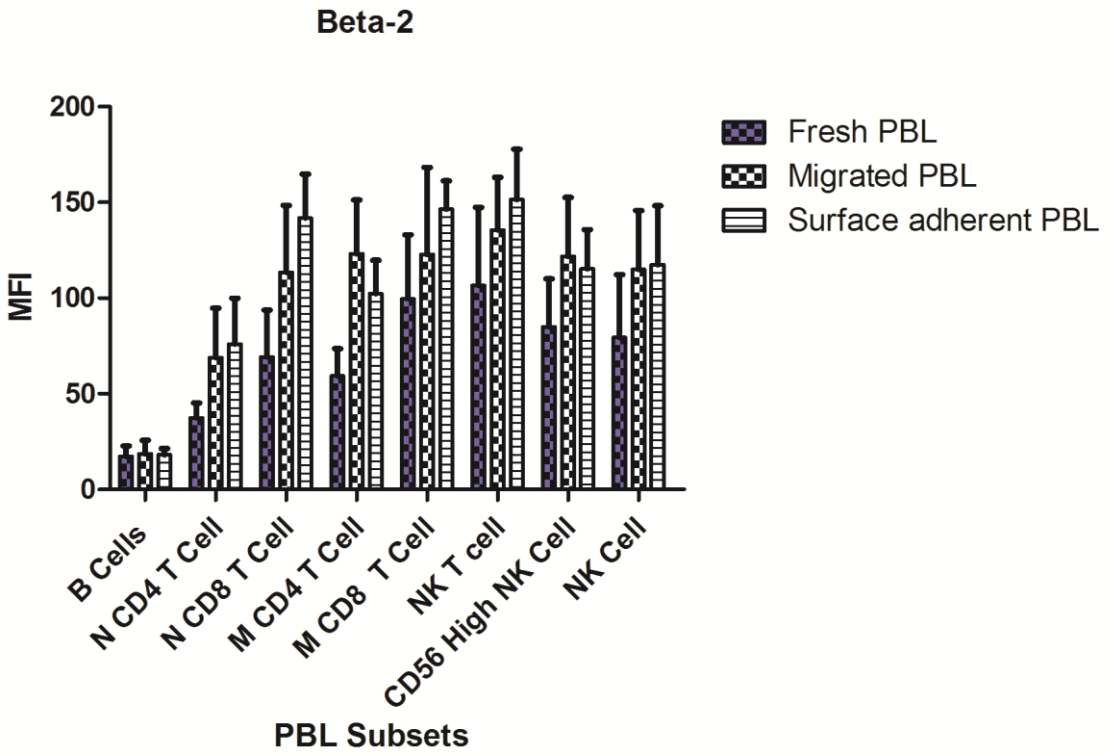


Figure 4-9: MFI of Beta-2 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI of Beta-2 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. Beta-2 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

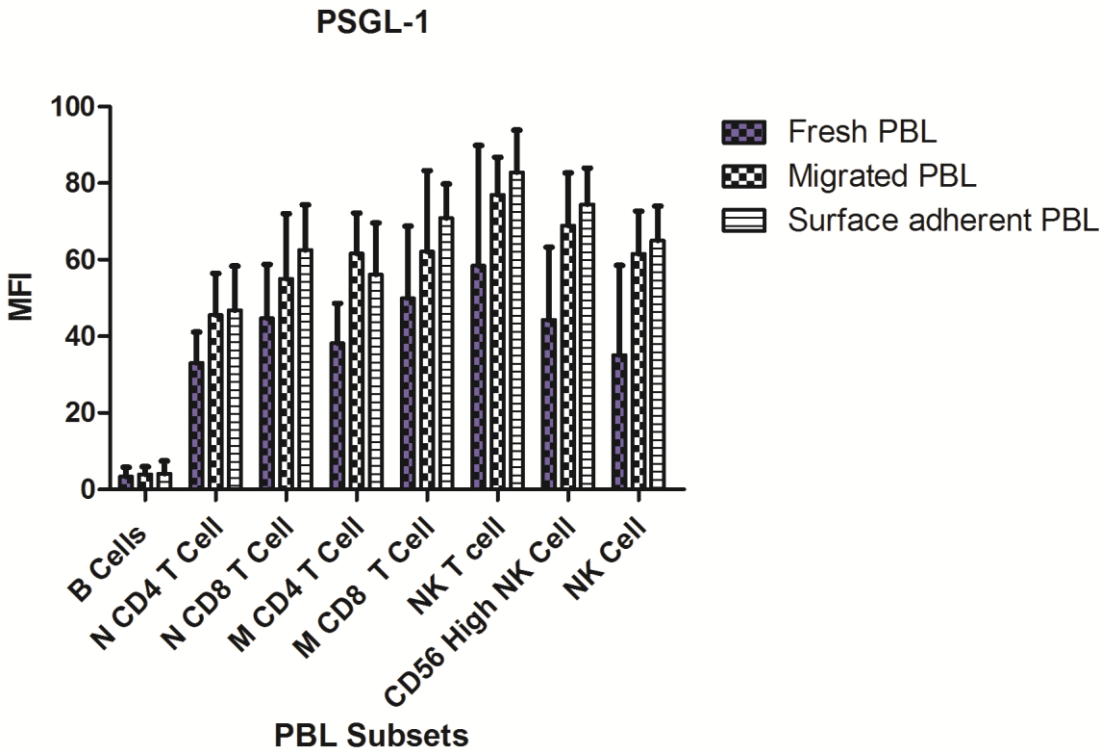


Figure 4-10: MFI of PSGL-1 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI of PSGL-1 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. PSGL-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

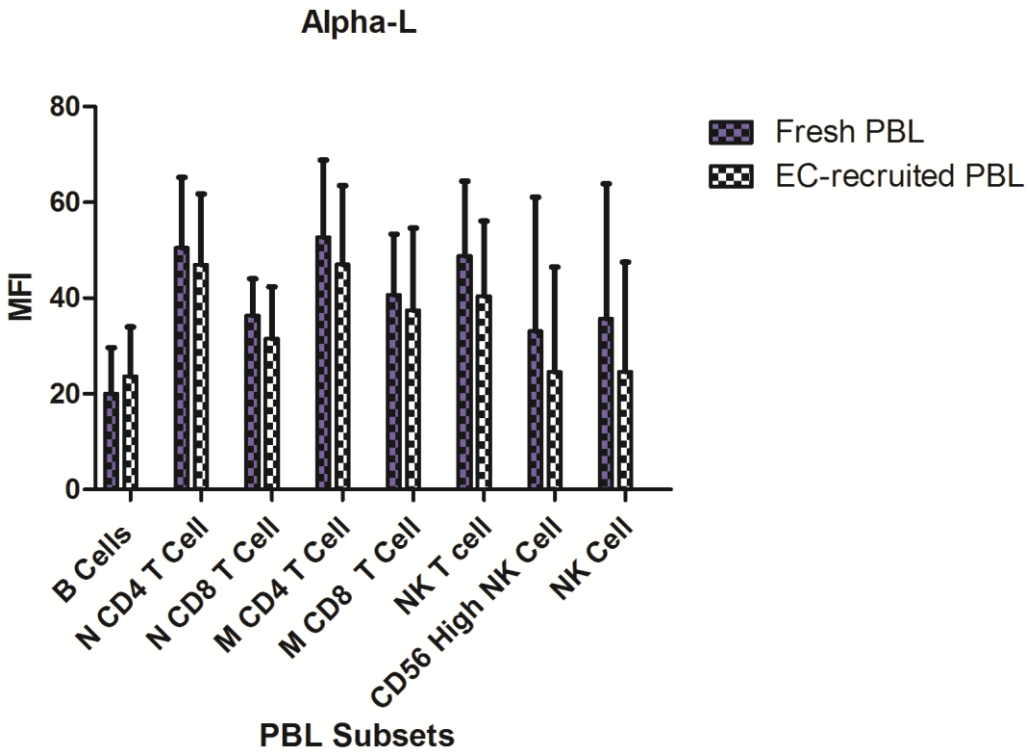


Figure 4-11: MFI of Alpha-L receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI Alphal-L; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. EC- recruited is the pool data for surface adherent PBL and migrated PBL. Direct conjugated antibodies were used for staining. Alphal-L expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

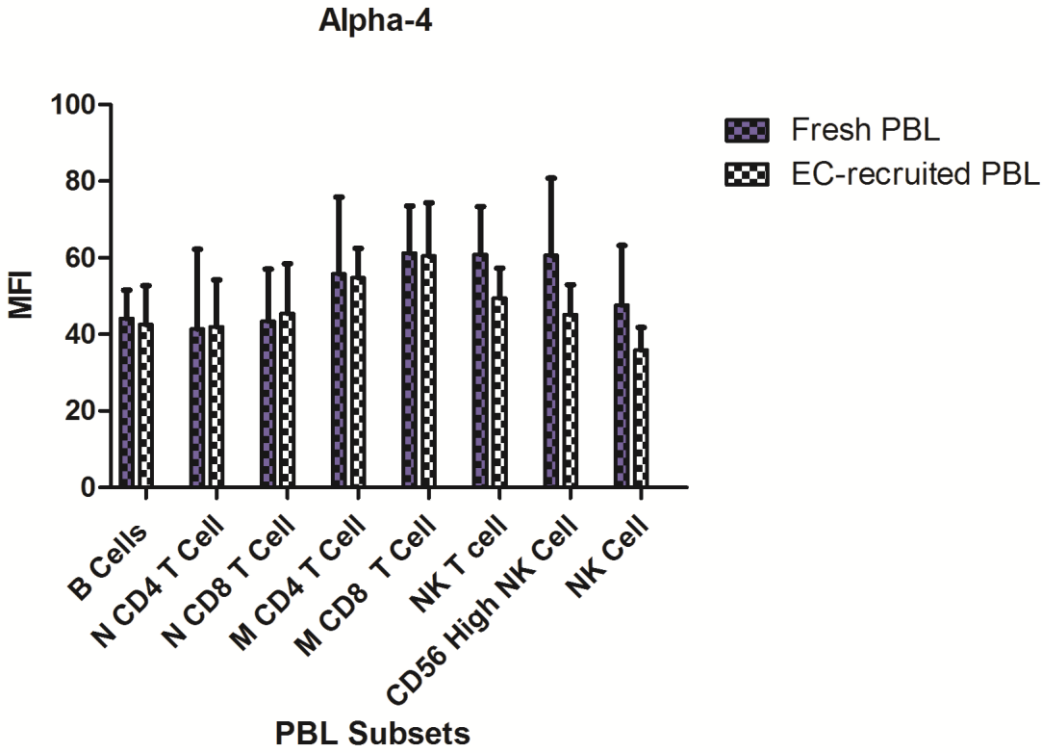


Figure 4-12: MFI of Alpha-4 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI Alphal-4; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. EC- recruited is the pool data for surface adherent PBL and migrated PBL. Direct conjugated antibodies were used for staining. Alphal-4 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

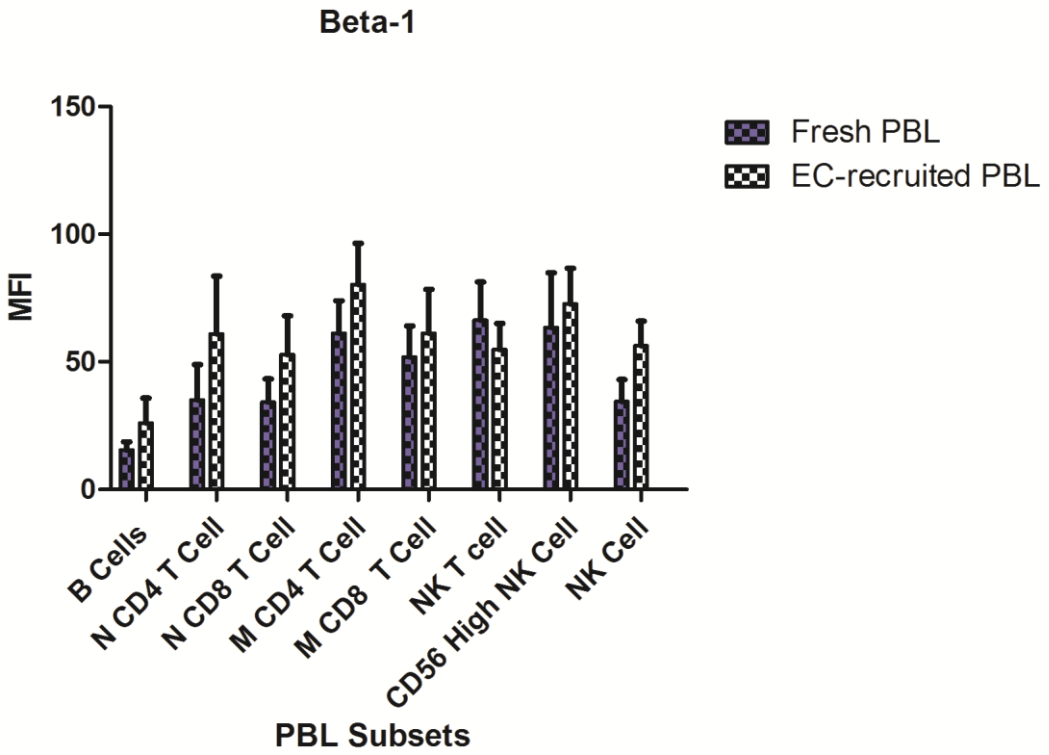


Figure 4-13: MFI of Beta-1 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI Beta-1; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. EC- recruited is the pool data for surface adherent PBL and migrated PBL. Direct conjugated antibodies were used for staining. Beta-1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

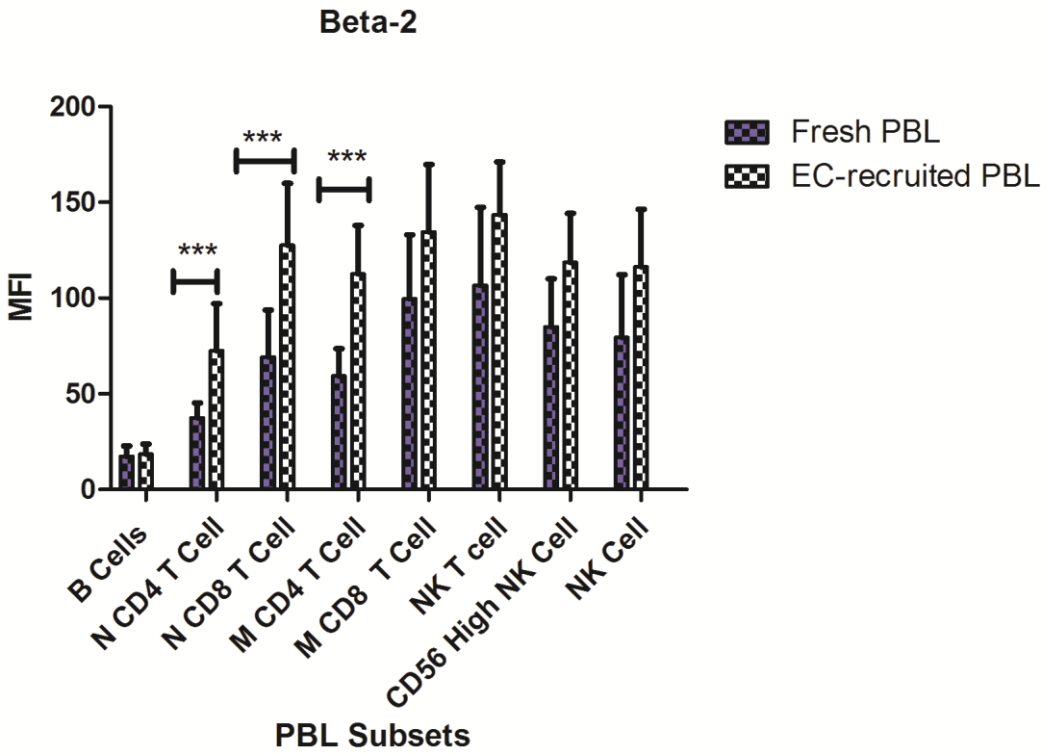


Figure 4-14: MFI of Beta-2 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI Beta-2; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. EC- recruited is the pool data for surface adherent PBL and migrated PBL. Direct conjugated antibodies were used for staining. Beta-2 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

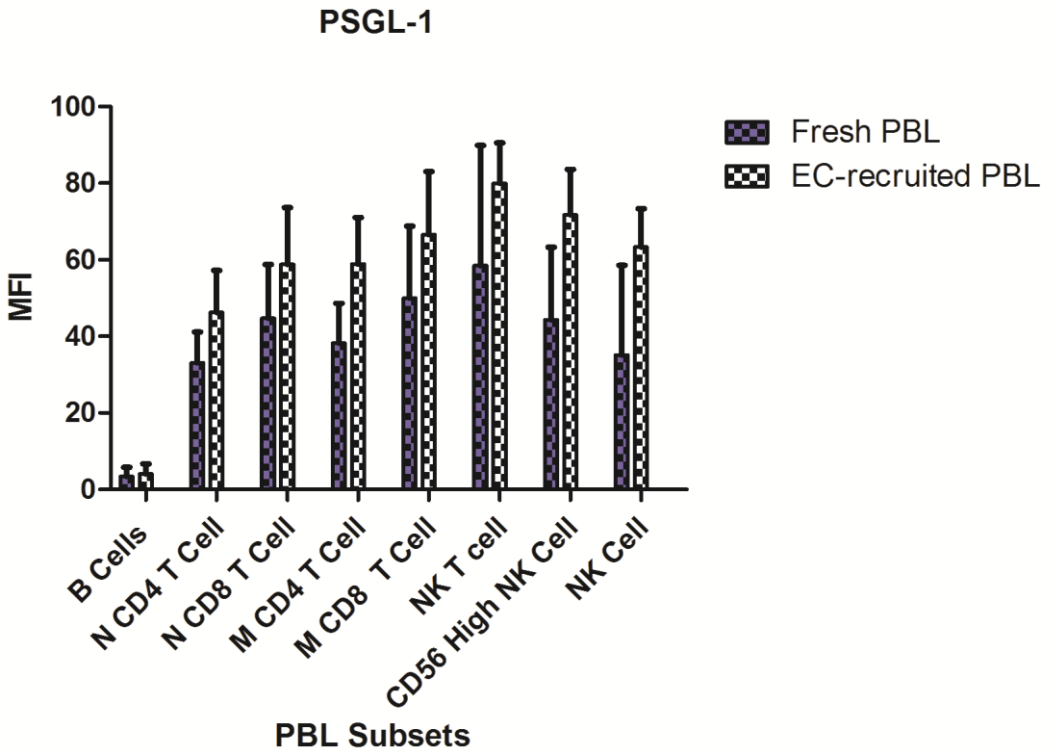


Figure 4-15: MFI of PSGL-1 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).

Flow cytometry was used to measure the MFI PSGL-1; PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. EC- recruited is the pool data for surface adherent PBL and migrated PBL. Direct conjugated antibodies were used for staining. PSGL-1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.2. The effect of age on adhesion molecule and chemokine receptors expression on fresh blood PBL

2.2.1. Surface expression of Adhesion molecules receptors on fresh blood PBL from young or old controls

The above data was of donors who were unstratified on the basis of age. Here, we did a more detailed analysis by recruiting young and old subjects; the young group were aged between 20-35 years of age, whereas old group participants were between 60-75 years of age. The clinical parameters of the study subjects are described below in table 1. Both groups were matched for sex, ethnicity, and body mass index (BMI) and were also healthy to the best of our knowledge. We measured the surface expression of the same set of adhesion molecules and chemokine receptors on the same PBL subsets used above. In the first instance we compared expression levels in circulating blood.

	young subjects	old subjects
N	6	6
AGE	26.33	66.66
BMI	24.1	28.9

Table 4-1: Clinical parameters of study subjects: Data is represented as a median (n=6); subjects were divided into two groups: young and old, and subjects in both groups were matched for sex and ethnicity. BMI was higher in the old subjects.

The data showed there was an increase in the expression of Alpha-L and Alpha-4 receptors in old subjects compared to young ones (Figure 4-16, 4-17), although this was dependent upon the lymphocyte subset and was not consistent between markers. The increases were particularly marked and statistically significant on CD56 high NK cells and

NK cells for both receptors. The expression of alpha-4 also increased significantly on naïve T cells. Regarding Beta-1 and Beta-2 receptors expression, (Figures 4-18 and 4-19) indicate different patterns of expression. Generally, with the exception of CD56 high NK cells and NK cells these receptors did not vary with age. Concerning PGGL-1, Figure 4-20 shows a decrease in the expression of PSGL-1 in old subjects when compared to young ones across nearly all lymphocyte populations with the exception of B cells which showed very low levels of expression nonetheless.

With regard to the surface expression of both adhesion molecules and chemokines receptors on surface adherent PBL, we did not deem it necessary to mention the difference from the results for transmigrated PBL as these were essentially the same as the expression patterns on migrated cells and this data is included in the appendix. Here, we have shown MFI as a measure of expression. The percentage of positive cells for each receptor measured can also be found in the appendix section.

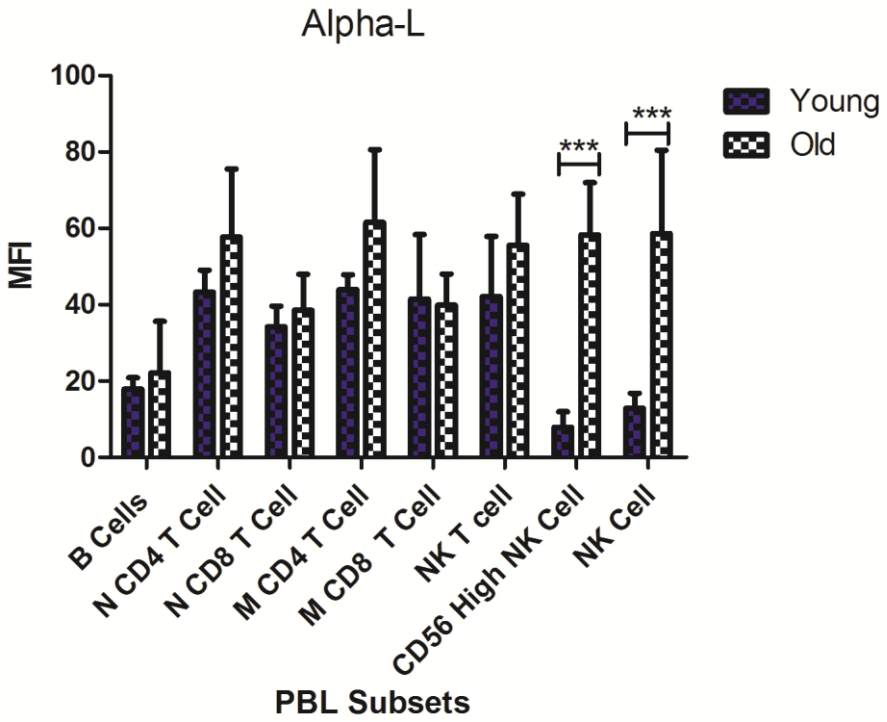


Figure 4-16: MFI of Alpha-L receptor on different subsets of PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of Alpha-L receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha L expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

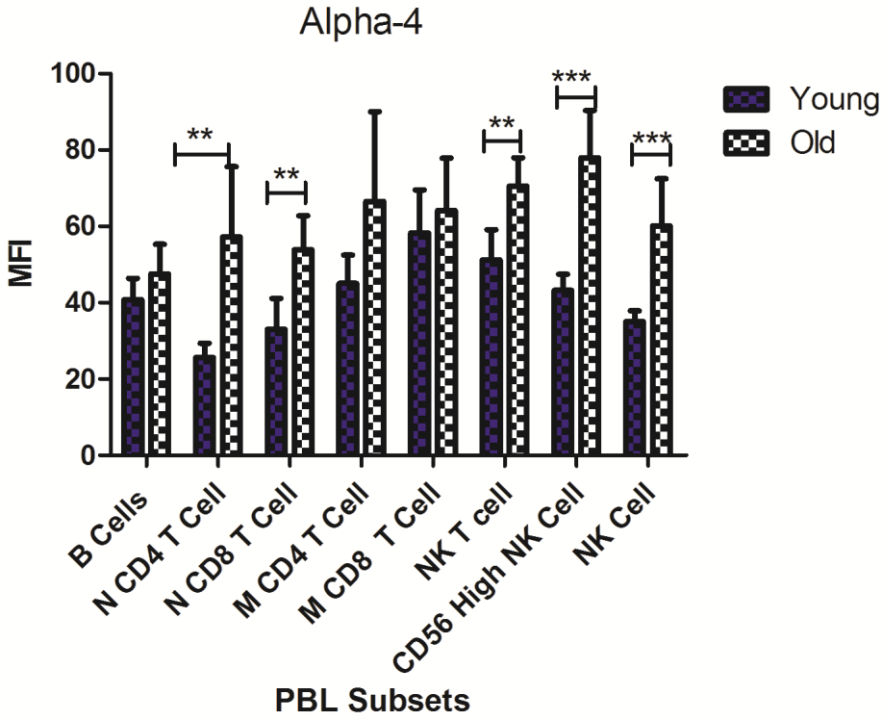


Figure 4-17: MFI of Alpha-4 receptor on different subsets of PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of Alpha-4 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-4 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

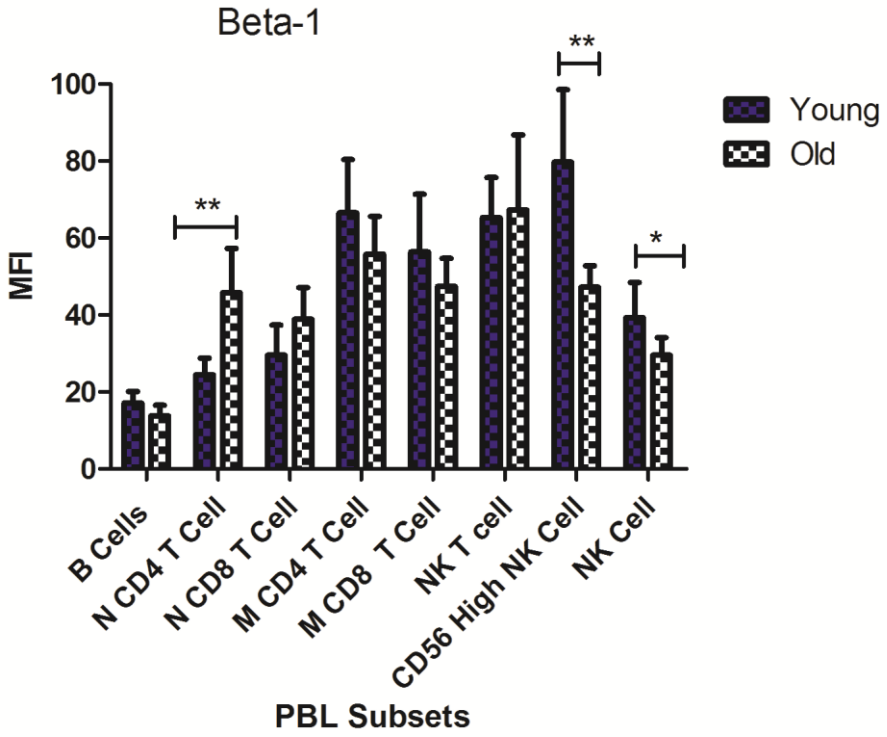


Figure 4-18: MFI of Beta-1 receptor on different subsets of PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of Beta-1 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha L expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

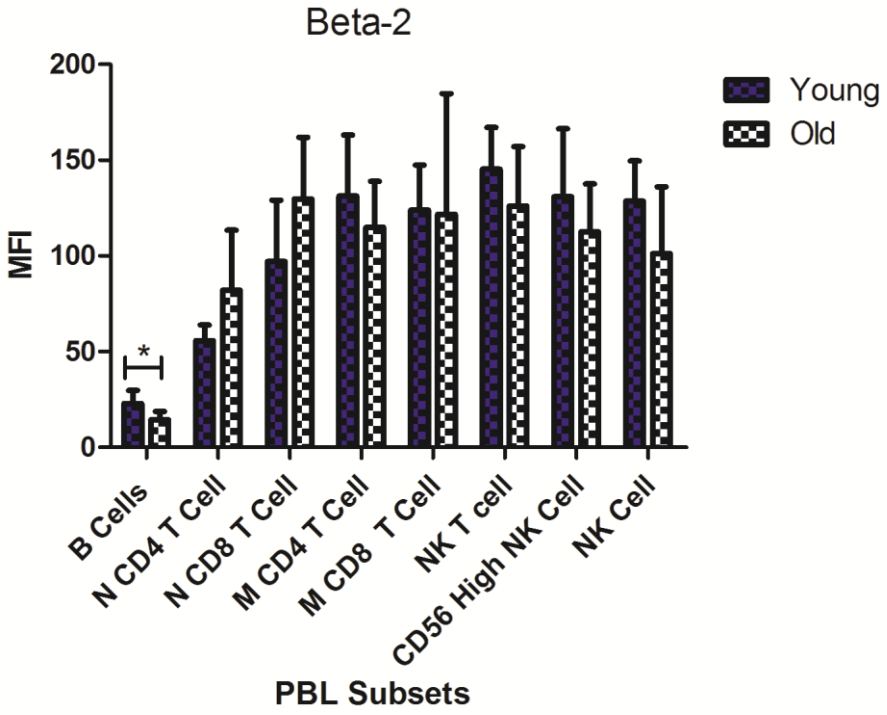


Figure 4-19: MFI of Beta-2 receptor on different subsets of PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of Beta-2 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-2 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

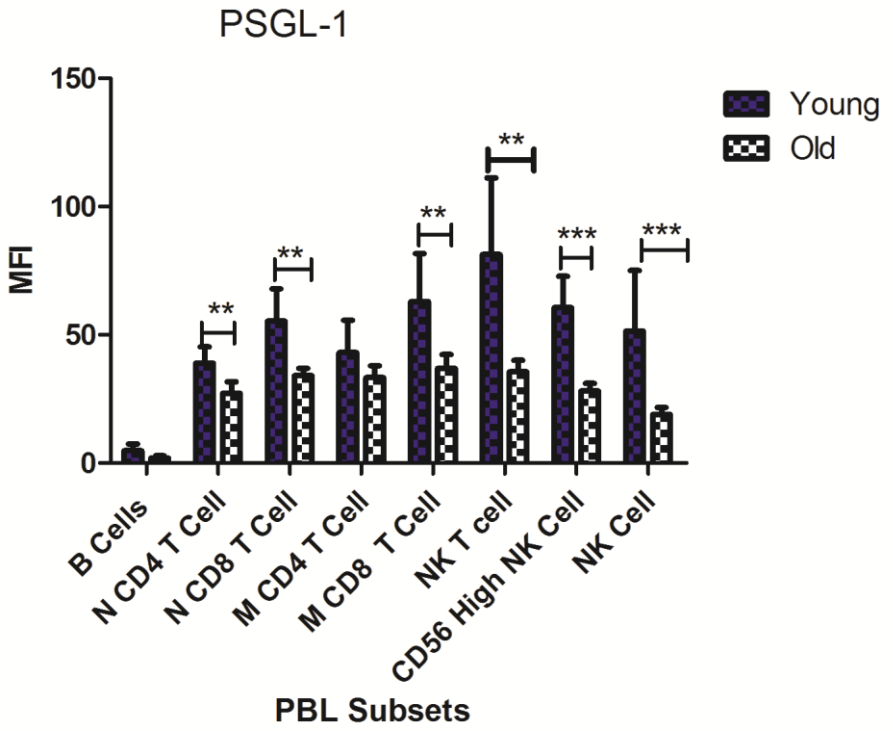


Figure 4-20: MFI of PSGL-1 receptor on different subsets of PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of PSGL-1 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. PSGL-1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.2.2. Surface expression of chemokine receptors on fresh blood PBL from young or old controls

With relation to the chemokine receptors surface expression, Figures 4-21, 4-22, 4-23 showed the expression of chemokine receptors on PBL subsets in both young and old groups. Figure 4-21 shows an increase in the expression of CCR5 receptors in the older group when compared with the younger group, and this increase is significant on all subsets except memory CD8+ T cells and NK T cells. Figure 4-22 shows that old subjects have a higher surface expression of CXCR3 when compared to young subjects on all subsets except MCD8+ T cells. CX3CR1 receptors are highly expressed on all PBL subsets in old subjects compared to young subjects. It is also worth mentioning that in the case of CCR5 the increase in expression was over threefold.

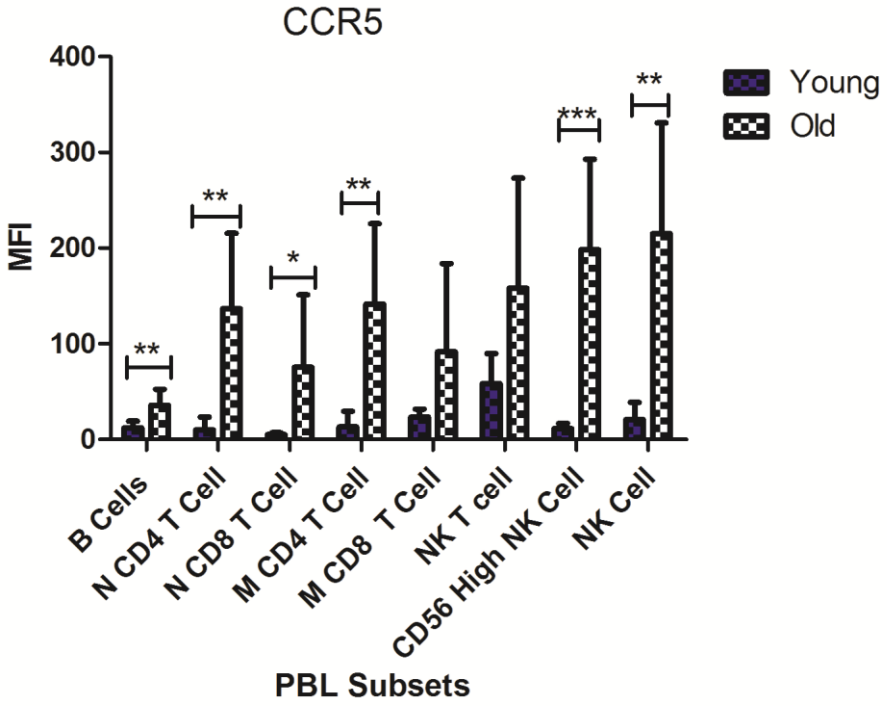


Figure 4-21: MFI of CCR5 receptor on different subsets of PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of CCR5 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

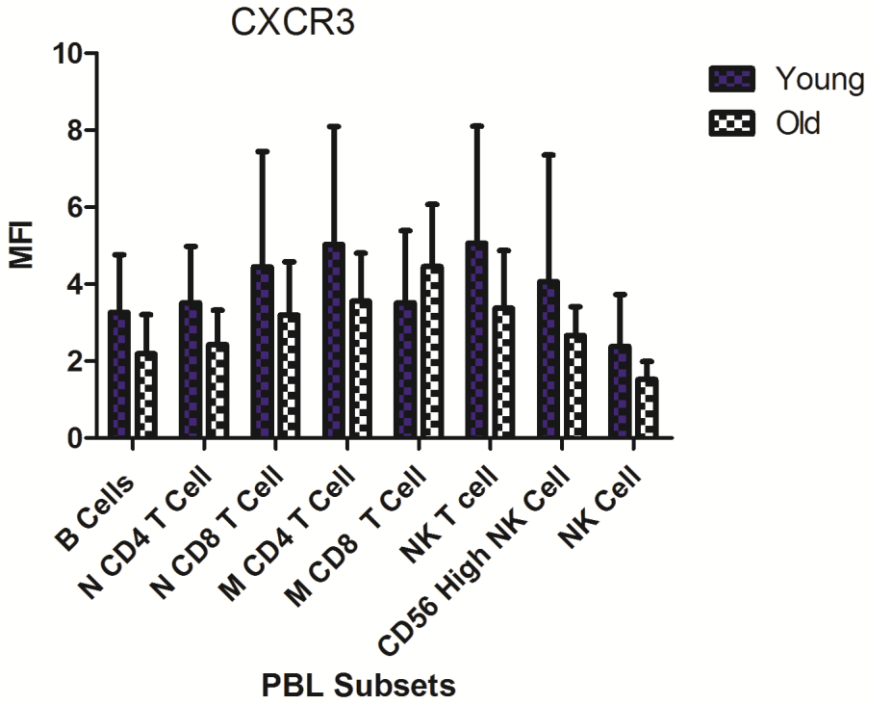


Figure 4-22: MFI of CXCR3 receptor on different subsets of PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of CXCR3 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

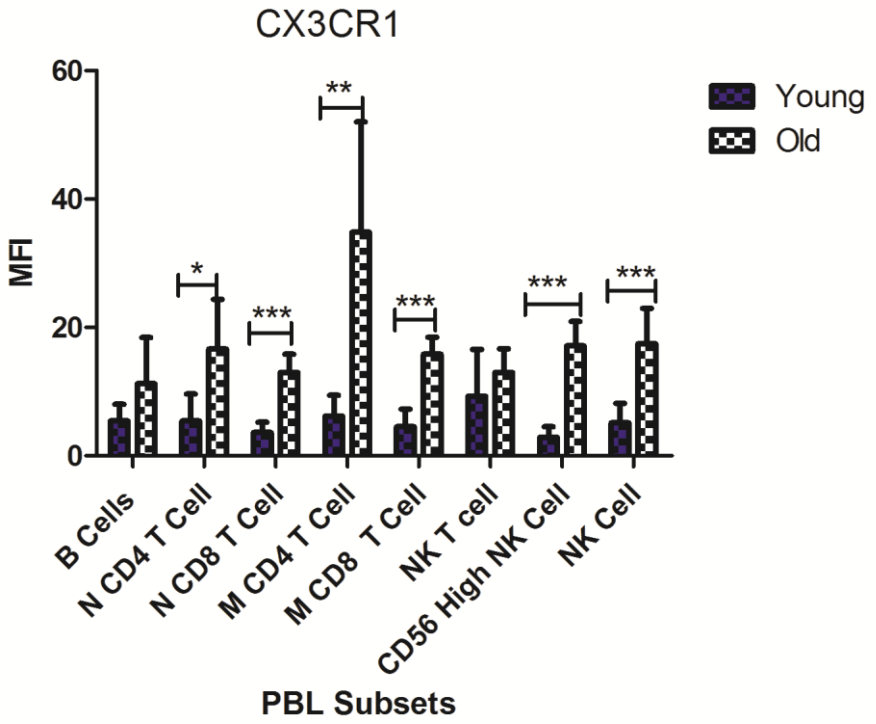


Figure 4-23: MFI of CX3CR1 receptor on different subsets of PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of CX3CR1 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.3. The effect of age on adhesion molecule and chemokine receptors expression on migrated PBL

2.3.1. Surface expression of adhesion molecules on migrated PBL from young or old controls

Our aim in this section was to investigate the effect of the process of leukocyte adhesion and transmigration on the same set of adhesion molecules and chemokine receptors on PBL subsets. Here the PBL was not freshly isolated from the donor and stained, but were collected after in vitro transmigration assay across cytokine stimulated DMEC.

After transmigration, it appears that the surface expression of Alpha-L receptors has was dramatically different in young and old donors with the exception of B cells and CD8+ memory T cells (Figure 4-24).. Interestingly figure 4-25 shows a slightly higher level in the surface expression of Alpha-4 receptors on the B cells and CD8 T cells of the older subjects in comparison to the younger subjects. It seems, therefore, that age might have some minor effects on activation dependent changes of Alpha-4 and Alpha-L expression on some lymphocyte subsets Similarly to Alpha-4, there was a difference in the surface expression of Beta-1 receptors on N CD4+ T cells, N CD8+ T and on M CD4+ T cells as illustrated on figure 4-26. The Beta-2 receptors in figure 4-27 show a higher level of expression in younger subjects in comparison to the older subjects. However in the case of PSGL-1, in figure 4-28 shows marginal variations of expression. These experiments show there may be some differential in the activation dependent regulation of chemokine receptors and adhesion receptors with age. An analysis of this by calculating the percentage difference between blood borne and EC activated lymphocytes shows no difference on the surface expression as seen on figure 4-29 and 4-30.

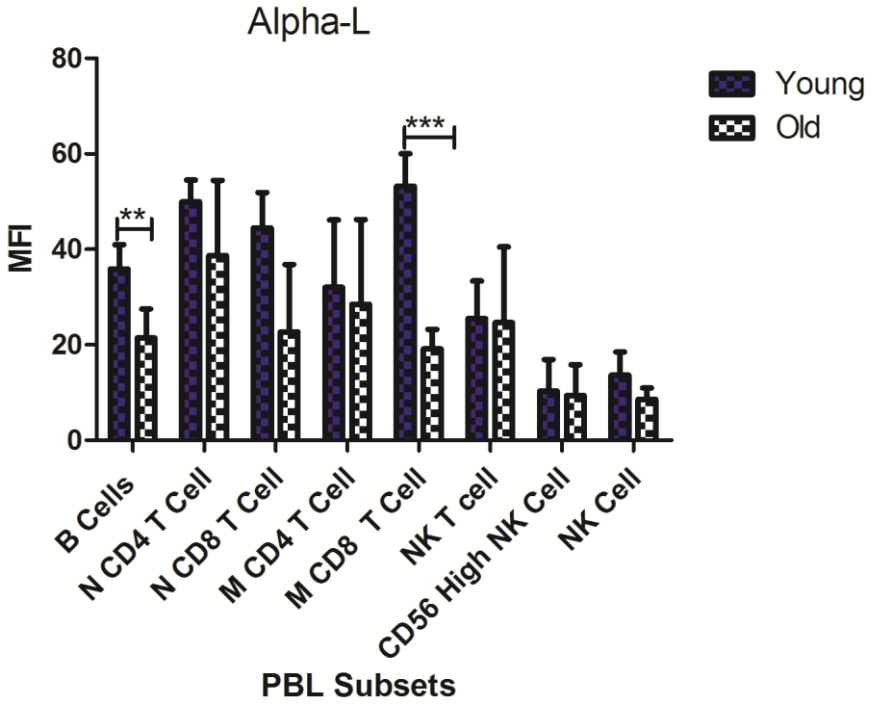


Figure 4-24: MFI of Alpha-L receptor on different subsets of migrated PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of Alpha L receptor on migrated PBL; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, Statin drug usage and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha -L expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

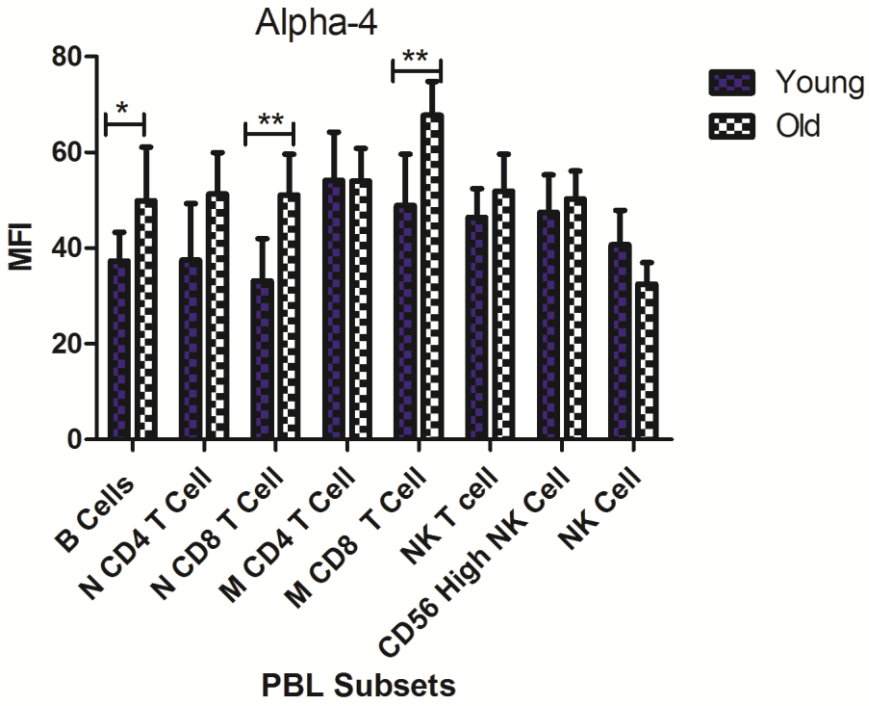


Figure 4-25: MFI of Alpha-4 receptor on different subsets of migrated PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of Alpha -4 receptor on migrated PBL; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, Statin drug usage and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha -4 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

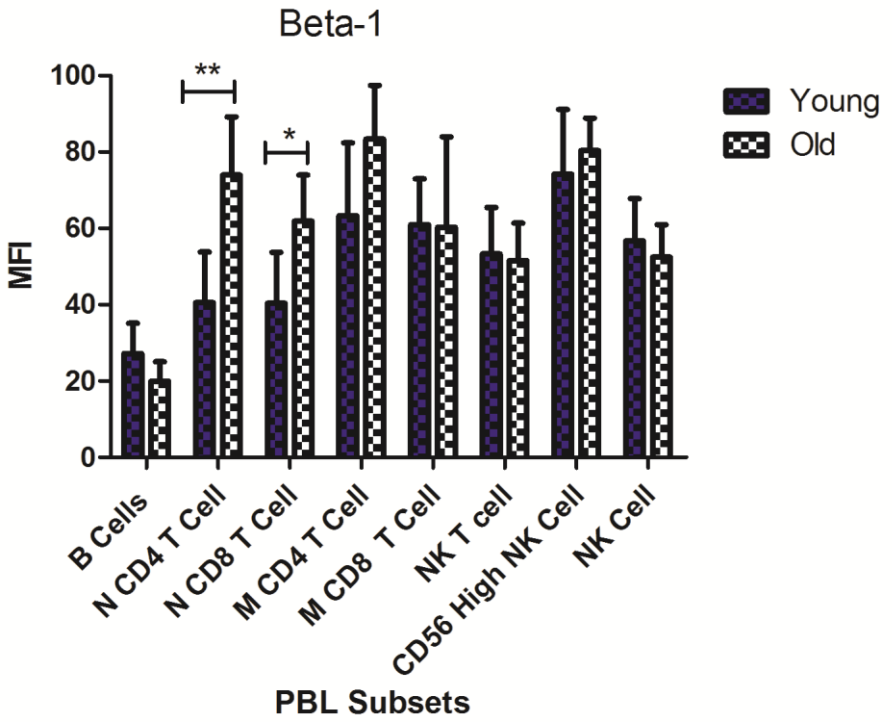


Figure 4-26: MFI of Beta-1 receptor on different subsets of migrated PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of Beta-1 receptor on migrated PBL; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, Statin drug usage and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

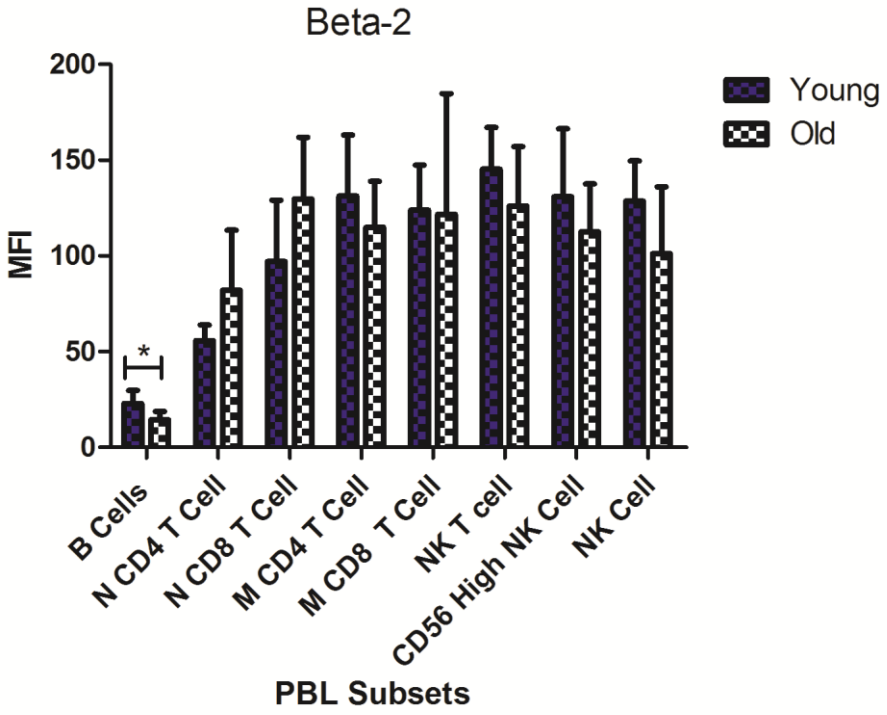


Figure 4-27: MFI of Beta-2 receptor on different subsets of migrated PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of Beta-2 receptor on migrated PBL; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, Statin drug usage and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-2 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

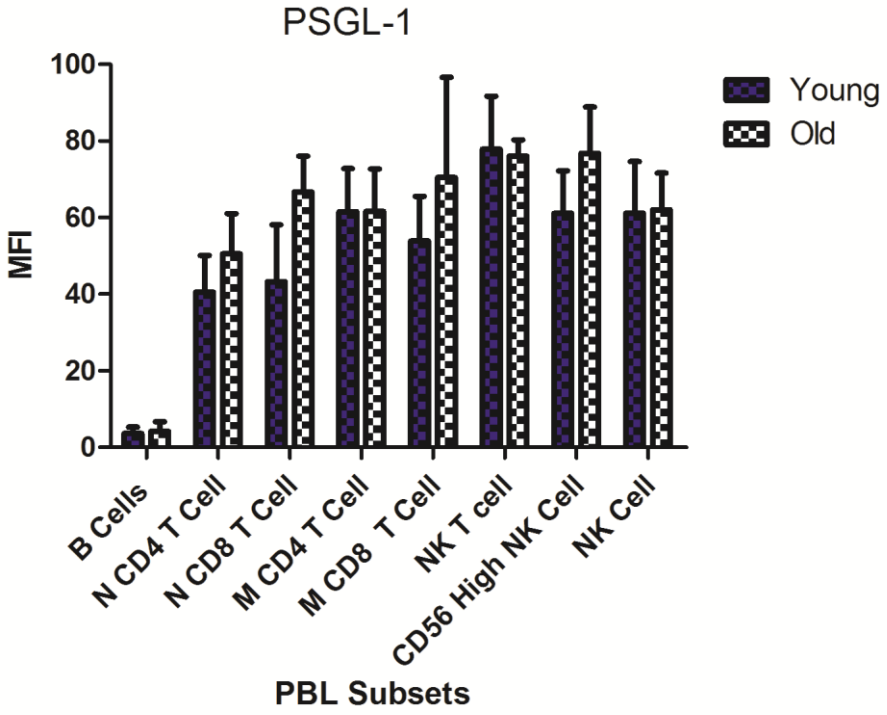


Figure 4-28: MFI of PSGL-1 receptor on different subsets of migrated PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of PSGL-1 receptor on migrated PBL; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, Statin drug usage and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. PSGL-1 expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

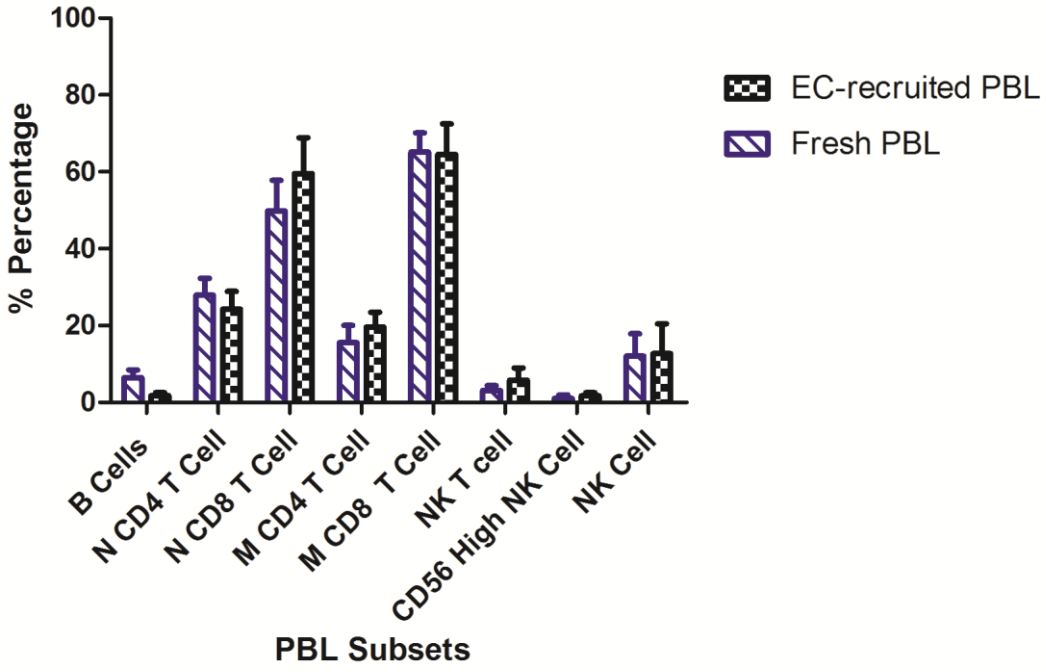


Figure 4-29: Circulating PBL vs Endothelium recruited (EC-recruited) PBL in young subjects.

Flow cytometry was used to measure the percentage of circulating PBL and EC-recruited PBL; Fresh blood was collected from young subjects. Young subjects aged from 20-35. Subjects were matched age, sex, ethnicity, and BMI. EC- recruited is the pool data for surface adherent PBL and migrated PBL. PBL was isolated, stained, and gated on their forward/side scatter profile. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

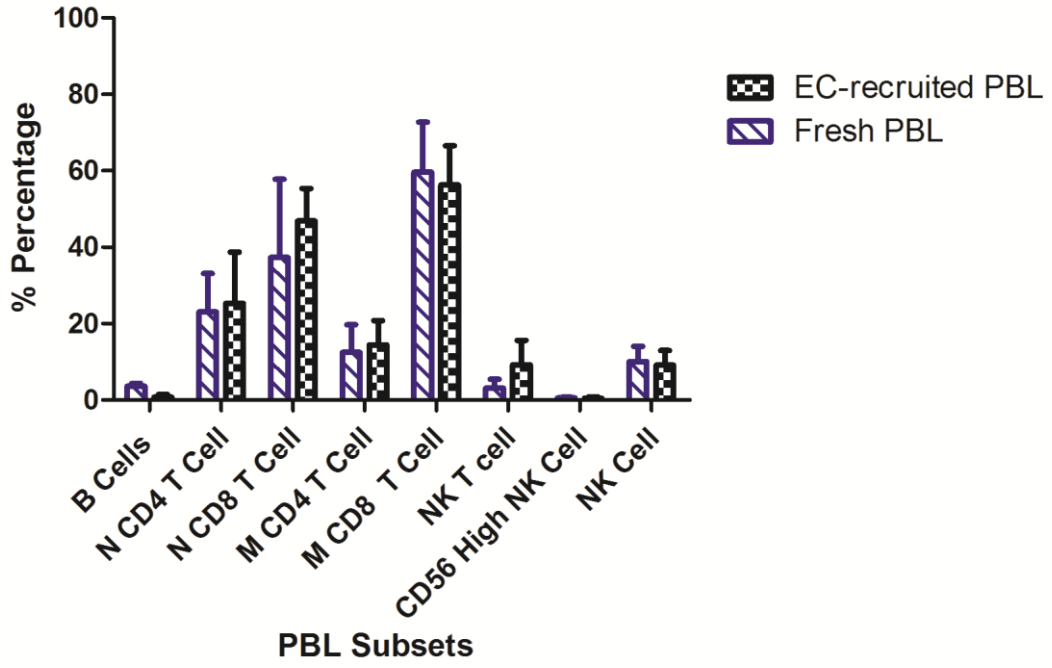


Figure 4-30: Circulating PBL vs Endothelium recruited (EC-recruited) PBL in old subjects.

Flow cytometry was used to measure the percentage of circulating PBL and EC-recruited PBL; Fresh blood was collected from old subjects. Old subjects aged from 60-75. Subjects were matched age, sex, ethnicity, and BMI. EC- recruited is the pool data for surface adherent PBL and migrated PBL. PBL was isolated, stained, and gated on their forward/side scatter profile. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.3.2. Surface expression of chemokine receptors expression on migrated PBL from young or old controls

In the first analysis of the chapter (2.1) we observed a marked and highly significant down regulation of all chemokine receptors assayed upon activation by EC. Here we could see no significant differences in the levels of chemokine receptors down regulation with age. Thus both cohorts of donors effectively lost surface expression of CCR5, CXCR3, and CX3CR1 receptors as seen in Figures 4-31, 4-32, 4-35.

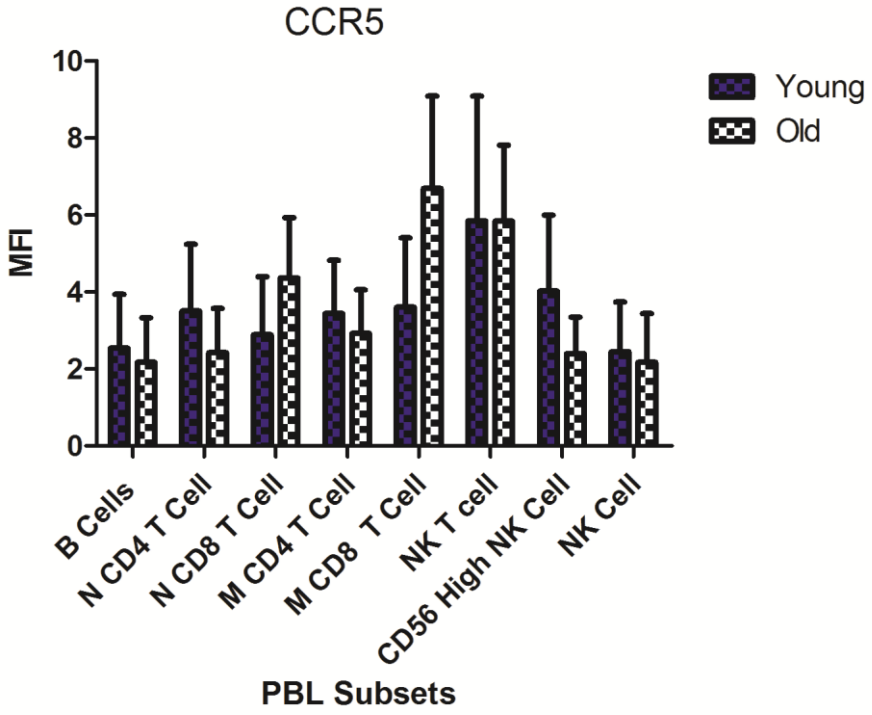


Figure 4-31: MFI of CCR5 receptor on different subsets of migrated PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of CCR5 receptor on migrated PBL; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, Statin drug usage and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

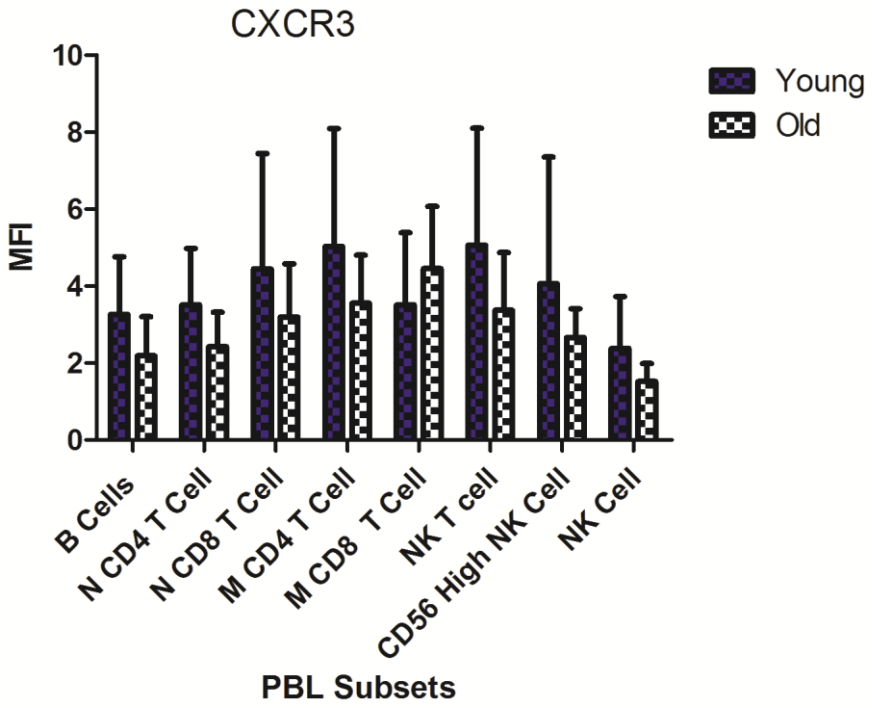


Figure 4-32: MFI of CXCR3 receptor on different subsets of migrated PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of CXCR3 receptor on migrated PBL; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, Statin drug usage and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

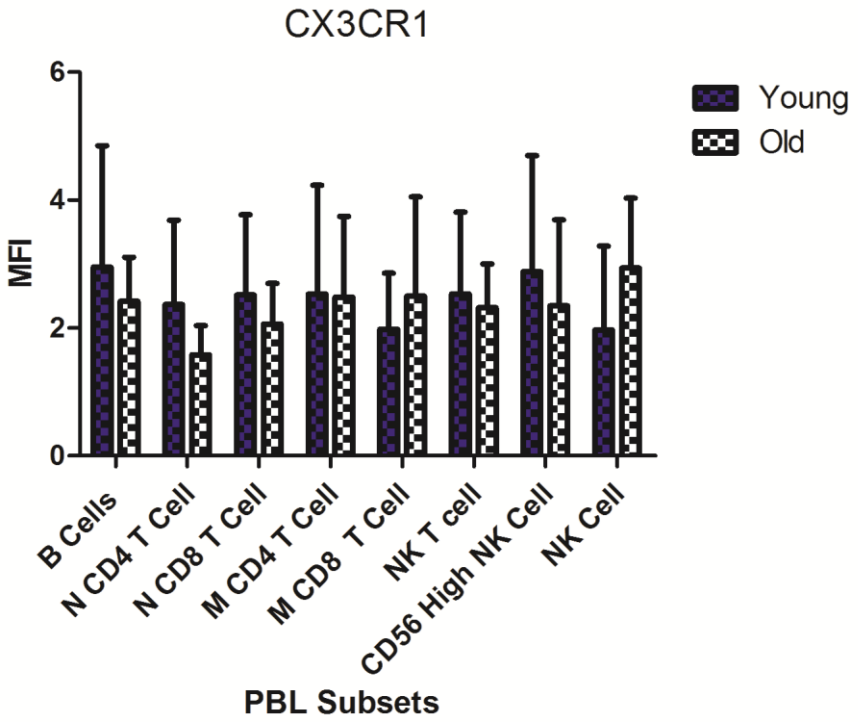


Figure 4-33: MFI of CX3CR1 receptor on different subsets of migrated PBL isolated from healthy donors.

Flow cytometry was used to measure the MFI of CX3CR1 receptor on migrated PBL; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, Statin drug usage and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.4. Enrichment ration of surface adherent and migrated PBL from young and old controls:

The effects of ageing and activation on the levels of adhesion receptors and chemokine receptors expression are complex and vary widely between lymphocyte subs sets. We wanted to see if they had any functional consequences. To do this we analysed the enrichment ratio of surface adherent and transmigrated PBL. Thus, we divided the number of surface adherent cells or transmigrated cells in each subset by the total number of cells of that subset added to the assay. Figure 4-34 and 35 show that there is no clear and obvious difference in the ratios of the subsets that are recruited in old and younger donors.

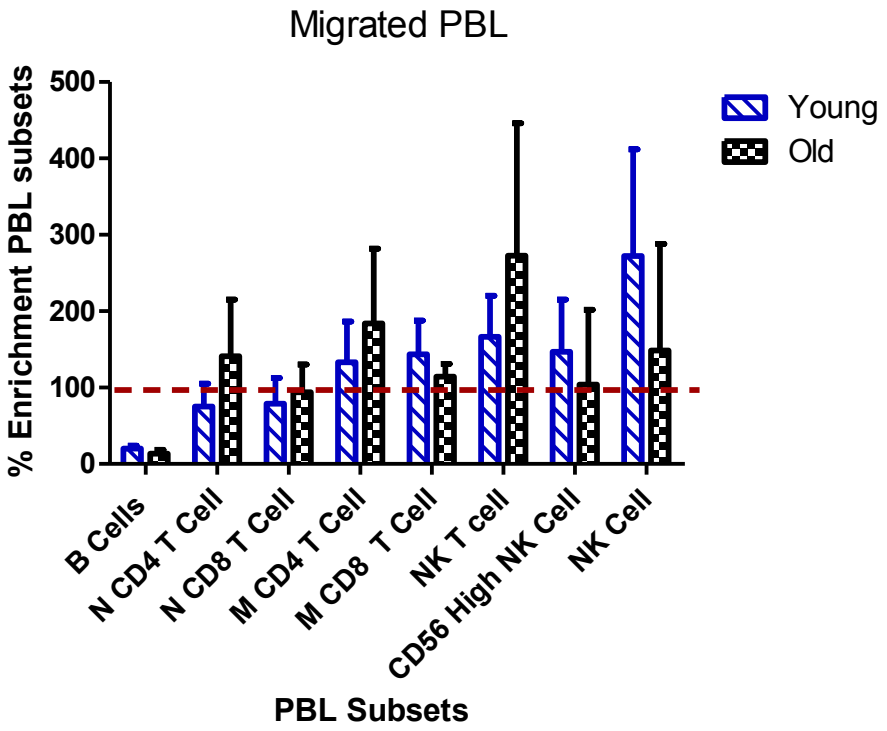


Figure 4-34: Enrichment ratio of migrated PBL in static condition:

The enrichment ratio for each subset from each group has been calculated. Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The number of transmigrated cells in each subset was divided by the total number of cells of that subset added to the assay. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

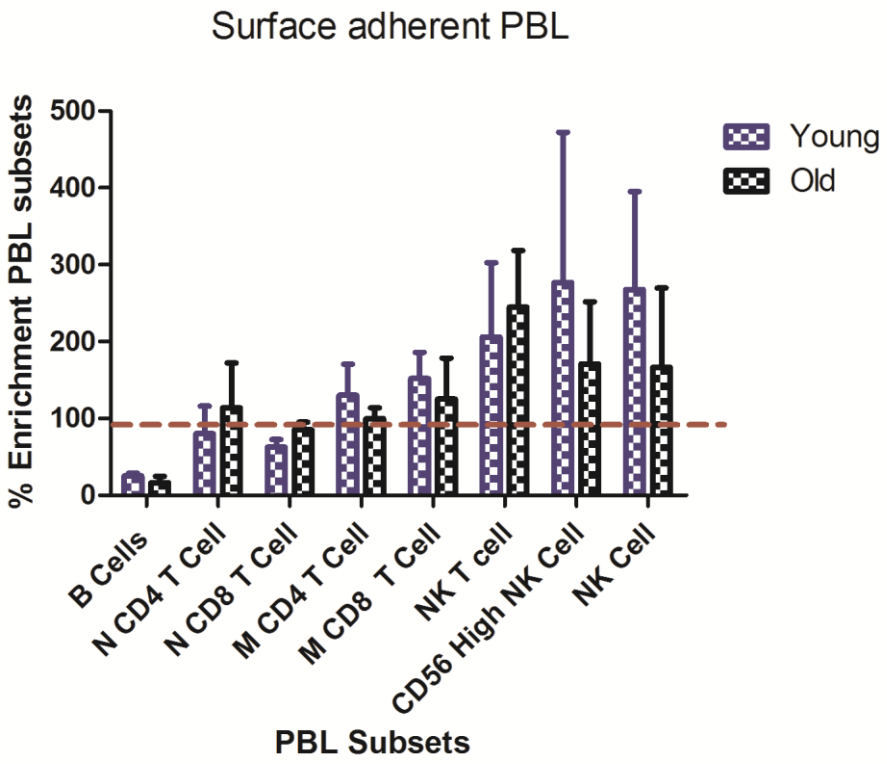


Figure 4-35: Enrichment ratio of surface adherent PBL in static condition:

The enrichment ratio for each subset from each group has been calculated. Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The number of surface adherent cells in each subset was divided by the total number of cells of that subset added to the assay. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

3. Discussion

We studied the effect of old age on the surface expression of eight adhesion molecules and three chemokine receptors. Firstly, we looked at the expression on freshly isolated PBL. Then, using static adhesion assay, we looked at the expression on PBL which had transmigrated through stimulated EC and on PBL that had firmly adhered to the EC surface. We did this experiment to investigate the effect of the transmigration process on the expression of integrins and chemokine receptors on PBL. As mentioned above, the mechanism which involves leukocytes capturing and rolling is well understood, but the mechanisms involved in leukocyte transmigration through the endothelium is still unclear. Our data showed a loss of the expression of chemokines CCR5, CXCR3, and CX3CR1 receptors upon activation by EC. This result is consistent with the data which had been established in our lab previously (Ahmed et al., 2011). Ahmed et al suggested that the chemokine signal is a short lived signal which is required during leukocyte trafficking to activate β1 and β2 intergrins which subsequently activate the release of Prostaglandin D2 (PGD2). At this point PGD2 takes over and induces the transmigration process (Ahmed et al., 2011). The current understanding of lymphocyte trafficking suggests that chemokine signalling is delivered to promote integrin mediated adhesion which is followed by actin cytoskeleton reorganization which enables the lymphocytes to spread and transmigrate. So it seems that chemokine signalling is essential for integrin activation and stabilization of lymphocytes, but that this signal needs to be stopped in order for PGD2 to facilitate the transmigration processes. Certainly, our data showed the loss of chemokine signalling very soon after activation on the EC surface and on both surface adherent and migrated cells.

One way in which chemokine receptors are down-regulated is internalization. It is well established that the control of internalization and recycling is a multistep step process and also that it is a complicated one. Usually it starts with receptor-ligand interactions followed by down-regulation of certain receptors. Thus, after chemokine- ligand binding, a transmembrane signal transduced via G protein-coupled receptors (GPCRs) promotes chemokine receptors uptake into endocytic vesicles, effectively deactivating the receptors. This explanation is supported by the findings by Ubogu et al (2006) who looked at the expression of CCR5 on monocytes and CD3+ T cells following CCL5 -driven transmigration through the blood brain barrier (BBB). The authors concluded that there was a noticeable down-regulation of CCR5 after transmigration (Ubogu et al., 2006). It should be noted that the migration through the BBB and through endothelium in other vascular beds is different due to the physiological and functional characteristics of these barriers. However, these author's findings are in agreement with ours; showing down regulation of CCR5 after activation at the EC surface. An interesting aspect of our observations is the down regulation of a number of chemokine receptors (CXCR3, CCR5 and CXC3R1) even though lymphocyte activation in this model is largely driven through signalling through CXCR3 (Personal communication with Dr. McGettrick). This activation of one receptor is sufficient to drive internalisation of multiple surface receptors.

After studying the chemokine receptor expression, we investigated the expression of adhesion molecules receptors after transmigration. In contrast to the down regulation of chemokine receptors we did not notice any dramatic regulation of adhesion molecules. This result was expected since those adhesion molecules receptors are needed in order to maintain and stabilize the adhesion; otherwise, lymphocytes would detach from EC. Our results concur with the data collected by Hourihan et al (1993) who reported that the expression of adhesion

molecules on lymphocytes is slightly altered apparently to increase the adhesiveness between lymphocyte and EC, (Hourihan et al., 1993). We also noticed an increase in the surface expression of Beta-1, Beta-2, Alpha-L, and Alpha-L receptors on a small number of subsets after transmigration which was an interesting observation. As we know from the literature that integrins modulate leukocyte crawling during adhesion cascade, but also that the integrin signal must then be attenuated in order to allow the transmigration process to start. Then, after the transmigration process is completed, the same signal is strengthened again to firmly adhere the leukocytes to EC (Barreiro and Sanchez-Madrid, 2009). So, we assume that the increase we noticed in integrin receptors expression reflects the strength of the integrin signal that is needed to keep PBL adhered to the EC.

Secondly, we looked at the effect of old age on the expression of adhesion molecules and chemokine receptors. The known slow deterioration of the immune system with age is called immunosenescence. Research has shown that every component of the immune system is negatively affected by age which in turn leads to functional impairment (Globerson and Effros, 2000). Many elucidations of the aging process support the hypothesis of oxidative stress which contributes to this increasing cellular dysfunction due to imbalances in the cellular redox mechanism (Zou et al., 2003). We noticed an increased expression only in Alpha-L and Alpha-4. This increase was significantly high on two subsets: CD56 high NK cell and NK cells. From the perspective of the existing literature, data regarding the effect of age on the expression of adhesion molecules and integrins receptors is conflicting. It has been suggested that there is an increase of adhesion molecules with age (Purschwitz et al., 2001) but data was challenged by another study by Nash et al which showed a decreased level of expression with age (Nash et al., 1996). Our data suggests a level of increase of expression with age only with Alpha-L and Alpha-4.

Regarding the Beta subunits of integrins molecules, we did not notice a significant change in the expression of β1 and β2 receptors, as mentioned above. In the case of the expression of PSGL-1, we found a decreased expression in the older age group. PSGL-1 is the most widespread P-selectin ligand on leukocytes and regulates leukocytes rolling. Current literature has shown that an over-expression of PSGL-1 is usually associated with diseases (Zarbock et al., 2009). For example, over expression of PSGL-1 has been noticed in patients with chronic obstructive pulmonary disease (COPD) (Schumacher et al., 2005). Moreover, it has also been reported in patients with MS (Engelhardt, 2009). These diseases are examples of the inflammatory response when it is over exuberant. The loss of PSGL-1 with age may indicate a deficiency in leukocyte recruitment which would be consistent with the increased incidence of opportunistic infections that are associated with age.

Regarding chemokine receptors expression on fresh blood PBL, we documented that there is a noticeably higher expression of CCR5 and CX3CR1 receptors on some PBL subsets of older subjects. This increase in chemokine receptors expression in circulation could be an indication of uncontrolled inflammation which leads to higher susceptibility to inflammatory disease. In fact many reports have suggested an increase in the level of chemokine receptors and cytokines in circulation with age (Hu et al., 2004). Moreover mice studies have also shown that aged mice have higher expressions of chemokine receptors (Yung and Mo, 2003).

In the case of the expression on migrated PBL and surface adherent PBL, we found that there is no old age associated observable difference in the expression of adhesion molecules?, but at the same time we revealed a dramatic down regulation of chemokines CCR5, CXCR3, and CX3CR1 in both groups.

Finally we wanted to look at the ability of PBL subsets to bind to endothelial, measured by an enrichment study. We did not notice clear and obvious differences in the ratios of the subsets that are recruited in old and younger donors which could be a result of technical problems because previous work in our? lab showed a significant enrichment ratio (personal communication with Dr. Myriam Chimen and Professor Ed Rainger).

4. Conclusion

From our data, we conclude that the chemokine receptors of CCR5, CXCR3, and CX3CR1 are down regulated during the transmigration process on the majority of PBL subsets. So it seems that chemokine signalling is essential for integrin activation and stabilization of lymphocytes, but that this signal needs to be stopped in order for PGD2 to facilitate transmigration processes. Further clarifying the regulation process which facilitates this may help in the development of antagonists for treating conditions involving chronic inflammation such as atherosclerosis or neuro-inflammation such as MS. Finally, the age related differences in the expression of adhesion molecules and integrins could be an indication of an early loss of homeostatic mechanisms.

CHAPTER 5: STUDYING THE BEHAVIOUR OF PEPITEM AND ADIPONECTIN ON PBL MIGRATION IN ATHEROSCLEROSIS PATIENTS.

1. Introduction

A novel peptide has been discovered in our lab. It is the Peptide inhibitor of trans-endothelial migration called PEPITEM and inhibits T cell trafficking by a multistep mechanism (Chimen et al., 2015). The work in our lab shows that PEPITEM is released exclusively from B cells after they are stimulated by Adiponectin (AQ). After that PEPITEM binds to EC by Cadherin-15; this binding stimulates the release of a bioactive lipid mediator called sphingosine-1-phosphate. Since T cells express receptors for sphingosine-1-phosphate (S1PR1 and S1PR4), the release of S1P from EC inhibits T cell trafficking as it is well-established that S1P is a key player in T cell trafficking. The finding is suggestive of high levels of S1P in circulation, where it has very high binding affinity for albumin and high density lipoprotein (HDL), and is considered to be the stem of S1P role in pathogenesis of atherosclerosis (Maceyka et al., 2012). There is a large variety of cells in which S1P has multiple effects which are pivotal to atherosclerosis development such as: activation of NF-κB pathway resulting in pro inflammatory cytokines production, vascular tone, smooth muscle cell proliferation, and migration and attachment of monocytes (Daum et al., 2009).

Sphingosine-1-phosphate (S1P) has an inhibitory and stimulatory effect on atherosclerosis because of the pleiotropic effect of sphingosine-1-phosphate (S1P) on macrophages, SMCs and ECs which are the principle cells in atherosclerosis development (Wang et al., 2010). Macrophages/monocytes express the two major S1P receptor types, S1PR1 and S1PR2 (Hughes et al., 2008). It has been illustrated by current studies that S1PR1 receptor stimulation by S1P containing HDL and S1P induces anti-inflammatory phenotypes, including the inhibition of pro inflammatory cytokine production in ECs and macrophages, and leucocyte adhesion (Hughes et al., 2008, Whetzel et al., 2006). Development of

atherosclerosis is inhibited in apolipoprotein E-deficient mice and by a phosphorylation product of FTY-720 which is considered as an agonist with high affinity for S1PR3, S1PR4, S1PR5, S1PR1 but not S1PR2 (Nofer et al., 2006) (Keul et al., 2007). Marked inhibition of atherosclerosis has been demonstrated in compound mutant mice deprived of S1PR2 and apoE (S1pr2-/-Apoe-/-mice) (Wang et al., 2010).

After a long debate regarding the physiological significance of S1P binding to plasma albumin versus its transport in HDL, it has been suggested that S1P may be pro-atherogenic when localized in blood bound to albumin and anti-atherogenic when transported in HDL (SamarM. Hammad, 2012) (Alewijnse and Peters, 2008) (Okajima, 2002). It is most significant to note that in patients having coronary artery disease, S1P distribution between protein fraction of blood and plasma lipoprotein tends to be altered significantly (Sattler et al., 2010).

SIP and S1PR1, the main SIP receptors that are expressed on lymphocytes, are significant regulators of lymphocyte trafficking (Im et al., 2000). The migration of mature thymocytes from the thymus into blood and of T and B cells from the lymph nodes (LN) into the efferent lymph depends on signalling of the SIP1 receptor by SIP (Ikeda et al., 2004). A key role is performed by the circulation of T cells between blood and secondary lymphoid organs (SLO) regarding the development of the adaptive immune response. Naïve T cells develop in the thymus, and they migrate into the systematic circulation and re-circulate between blood and SLO looking for antigens (Means and Brown, 2009). After antigen encounters in SLO, T cells obtain effector functions and then home to areas of inflammation to organize an immune-regulated reaction (Graeler et al., 2003). According to the existing evidence, with respect to antigen-specific activation within the LN, S1P1 receptors on T cells are rapidly down-regulated. Consequently, such cells show a lack of response to the egress signal created

by the prevailing SIP positive concentration gradient between lymphoid tissues and circulatory fluids and therefore stay in the LN (Wendler and Rivkees, 2006). Following their expansion and differentiation, effector T cells carry out up-regulation of SIPR1 expression and, as a reaction to the SIP gradient, progress through the sinus-lining endothelium into lymphatic sinuses, and then within the efferent lymph into the blood via the thoracic duct (Yatomi et al., 1997). SIP may also offer a further regulatory method for lymphocyte egress from the LN by signalling at the S1P1 receptors on sinus-lining endothelial cells (Okamoto et al., 1999). Selective SIP1 agonists, however, can restrict the transendothelial migration of T cells over the lymphatic endothelial barrier in the LN, without modifying cell motility (Kohama et al., 1998).

2. Results

In this chapter we wanted to test the action of PEPITEM on PBL isolated from atherosclerosis patients. It is well known that atherosclerosis is characterised by prolonged PBL trafficking, so we designed an in vitro static adhesion assay, where isolated the PBL was put on stimulated EC and after a few minutes, PBL migration behaviour was studied. Based on previous studies our hypothesis was that if we treated PBL with PEPITEM, we would see a lower number of transmigrated PBL. Also, we wanted to measure the surface expression of AR1 and AR2 on B cells which are responsible for releasing PEPITEM in to the extracellular environment. In addition to the surface expression, we measured the gene expression of AR1 and AR2 on PBMCs. Finally, we measured the serum level of AQ. Changes in any of these parameters could affect the function of the PEPITEM pathway and lead to exaggerated lymphocyte recruitment.

To conduct this experiment, we recruited Atherosclerosis patients (Athero patients) and healthy subjects and put them into two different groups (n=6). All subjects were matched for age, sex, ethnicity, BMI, illness, and Statin usage. See clinical parameters of the study on Table 4. These Subjects are healthy and not taking any medications which might affect our results.

	Control subjects	Atherosclerosis patients
N	6	6
AGE	68	70
BMI	31	29

Table 5-1: Clinical parameters of study subjects
Data is represented as median (n=6); subjects were divided into two groups: control and atherosclerosis patients and subjects in both groups were matched for age, sex and ethnicity, BMI, Statin use.

2.1. In vitro static transmigration assay

First, we looked at the effect of PEPITEM on the total levels of PBL adhesion. Figure (5-1) shows that PEPITEM did not affect the levels of adhesion of PBL form either diseased or healthy donors. This is in agreement with our previous observations. Thereafter the effect of PEPITEM and AQ treatment on the migration of PBL was observed. Figure (5-2) shows that there was no effect of treatment on PBL isolated from atherosclerosis patients. However, the treatment of PBL from healthy donors with AQ and PEPITEM inhibited the transmigration of PBL significantly, in line with our previously published responses to these agents. This is an extremely surprising and interesting observation because in other patient cohorts (e.g. Type-1

diabetics and rheumatoid arthritis patients) loss of the response to adiponectin can be rescued by addition of exogenous PEPITEM. However, in atherosclerosis patients this is not the case.

2.2. Surface expression of AR1, AR2 on B cell isolated from atherosclerosis patients

Briefly, a flow cytometry assay was used to investigate the surface expression of AR1 and AR2 on B cells. PBL was freshly isolated from subjects and processed to isolate PBL from whole blood. Subsequently, PBL was stained with specific antibodies to identify each cell subset. Our analysis shows that the number of AR1 positive B cells was unchanged in patients (Figure 5-3). In addition there was no significant change in the MFI of positive cells comparing donor cohorts (Figure 5-4). There was a significant increase in the frequency of B cells positive for AR2 (Figure 5-5), although there was not an increase in the MFI of these positive cells (Figure 5-6).

2.3. Levels of gene expression of AR1and AR2 in patients with atherosclerosis in healthy controls.

PBMCs were isolated from blood and RNA was extracted to study the gene expression of AR1 and AR2 in PBMCs. Figure (5-7) shows the gene expression of AR1 and AR2 on both groups. The graph shows that gene expression for both receptors was unchanged in PBMCs isolated from atherosclerosis patients when compared to the control group.

2.4. Levels of adiponectin in patients with atherosclerosis and in healthy controls.

Serum isolated from atherosclerosis patients or healthy controls was assayed for levels of adiponectin using a commercial ELISA. Figure (5-8) shows that adiponectin levels in patients are significantly lower than in healthy controls. Indeed, patients are considered hypoadiponectinaemic when levels fall below 4g/ml.

2.5. Surface expression of S1PR1 and SIPR4 on whole PBL subset:

To establish protocol, blood was taken from random donors and PBL's were isolated and stained by direct conjugated antibodies to measure SIPR1 and SIPR4 on PBL subsets. Since SIPRs are expressed both on the surface and internally, we measured the expression accordingly both on the surface and internally. Our analysis shows that the receptors were widely expressed inside lymphocyte subsets. However, only circulating B cells and some NK subsets showed any marked expression of S1PR1on their surface, while S1PR4 was largely absent from the surface of circulating lymphocytes. (Figures 5-9, 5-10, 5-11, and 5-12).

2.6. PBL binding on ICAM-SIPR1/4 expression:

As the PBL of patients with atherosclerosis were refractory to the effects of PEPITEM, but had normal levels of AR1 and AR2 on their B cells, we hypothesised that an alternative defect was inhibiting responses to the pathway. We wondered if patient T cells were unable to respond to S1P. One way this might occur is by an inability of their T cells to express S1P receptors on the surface in response to inflammatory stimulation. We have previously assayed for this expression by inducing S1P receptor expression on T cells bound to ICAM-1 and stimulated by chemokines. Here we used a 16 well plate coated with ICAM-1 to bind lymphocytes, these were then activated by CXCL10 to induce the up-regulation of SIPR1 and SIPR4. Incubated, the PBL was collected by rough pipetting with cold PBS in FACS tube and then stained for flow cytometry. Unfortunately we were unable to demonstrate S1P receptor mobilisation using this assay, even in the T cell of healthy control donors. Furthermore, the data do not indicate a deficiency in response. (Figures 5-13, 5-14, 5-15, 5-16).

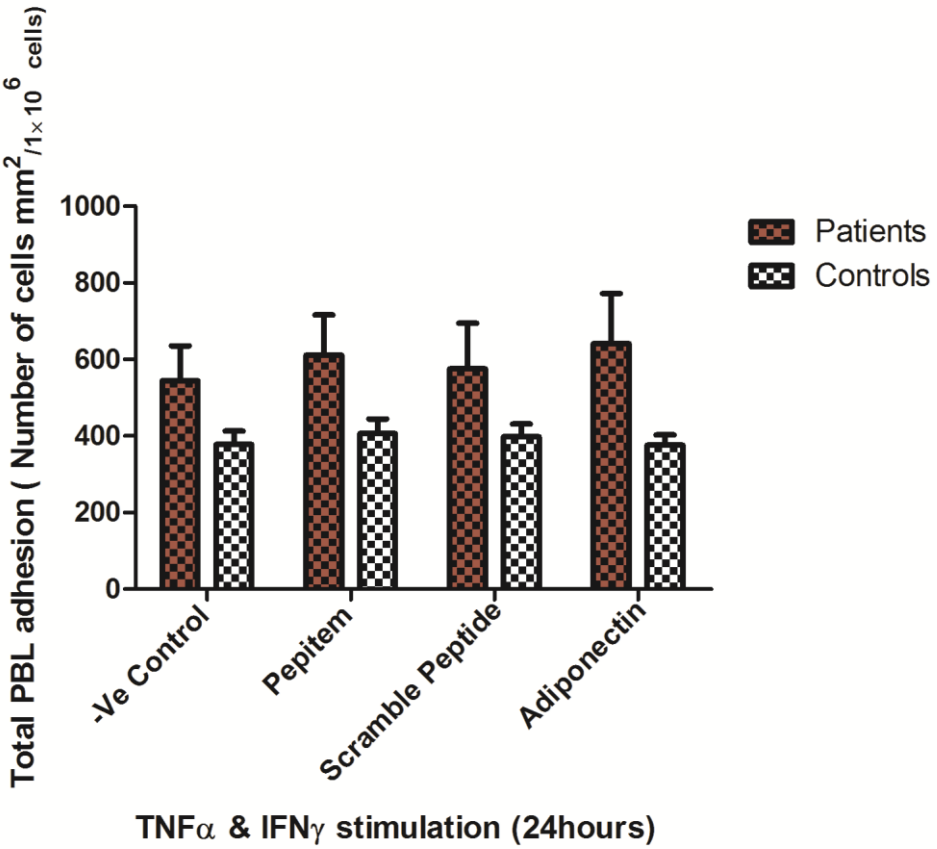


Figure 5-1: Total adhesion of PBL from atherosclerosis patients or age match control.

Static transmigration assay was used to calculate the total adhesion of PBL isolated from atherosclerosis patients and control subjects**.** Subjects were aged from 60-75 years old and they were matched for sex, ethnicity, BMI, and Statin usage. DMEC were cultured in culture medium, and then stimulated with TNF-α/IFN-γ for 24 hours. First, PBL was either treated by Pepitem, scramble peptide, or AQ. Second, PBL was added to EC and the total adhesion was calculated. Data is mean ± SEM (N=6) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

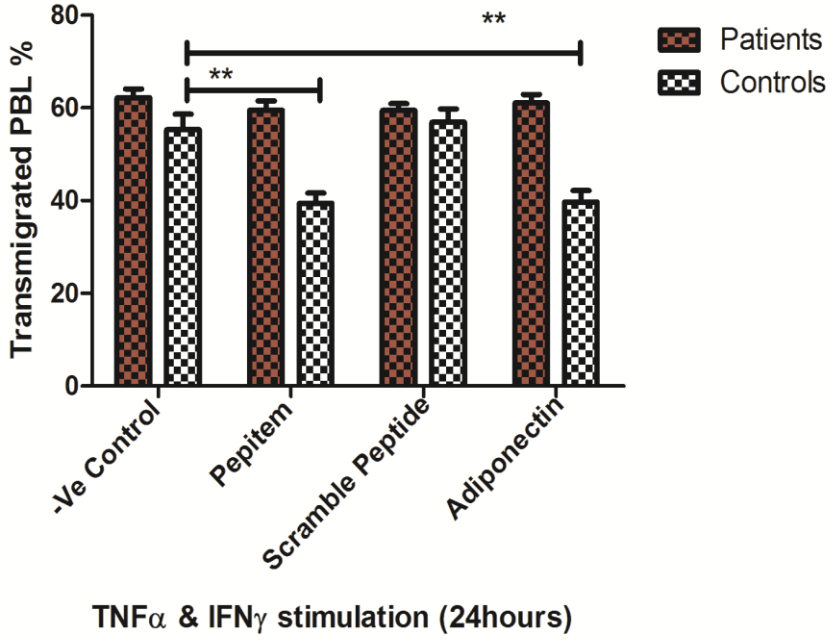


Figure 5-2: Transmigration percentage of PBL from atherosclerosis patients or age match control.

Static transmigration assay was used to calculate the percentage of transmigration of PBL isolated from atherosclerosis patients and control subjects**.** Subjects were aged from 60-75 years old and they were matched for sex, ethnicity, BMI, and Statin usage. DMEC were cultured in culture medium, and then stimulated with TNF-α/IFN-γ for 24 hours. First, PBL was either treated by Pepitem, scramble peptide, or AQ. Second, PBL was added to EC and the percentage of transmigration was calculated. Data is mean ± SEM (N=6) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$). The effect of PEPITEM and AQ treatment on the migration of PBL was then considered. This figure shows that there was no effect of treatment on PBL isolated from atherosclerosis patients. However, the treatment of PBL from healthy donors with AQ and PEPITEM inhibited the transmigration of PBL significantly, in line with our previously published responses to these agents. This is an extremely surprising and interesting observation because in other patient cohorts (e.g. Type-1 diabetics and rheumatoid arthritis patients) loss of the response to adiponectin can be rescued by addition of exogenous PEPITEM. However, in atherosclerosis patients this is not the case.

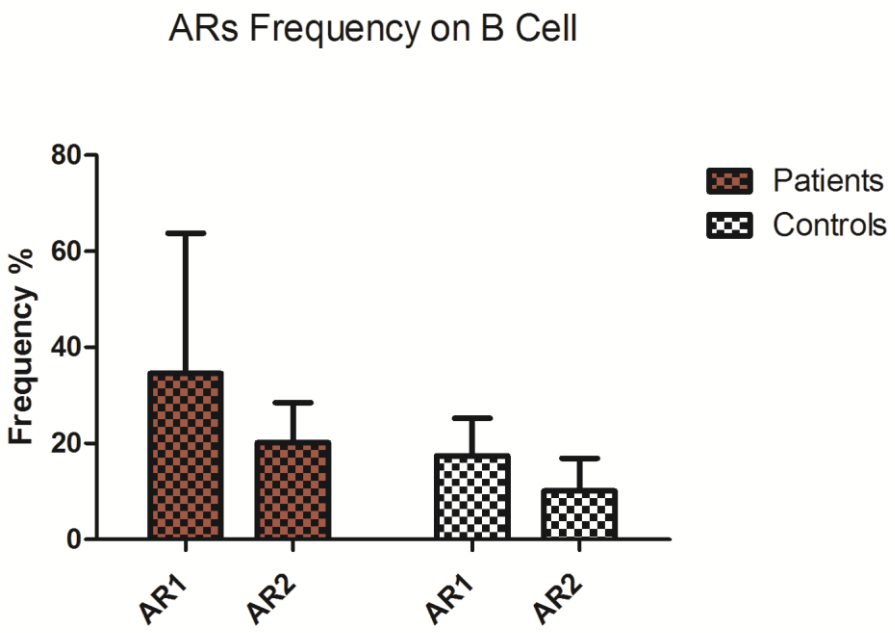


Figure 5-3: ARs frequency on B cells from atherosclerosis patients or age match control.

Flow cytometry was used to measure the frequency of AR1 and AR2; PBL was isolated from atherosclerosis patients or age match control. **.** Subjects were aged from 60-75 years old and they were matched for sex, ethnicity, BMI, and Statin usage. Fresh blood samples, stained, then gated on their forward/side scatter profile. CD19 antibody was used to identify B cells. ARs receptors expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an? independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

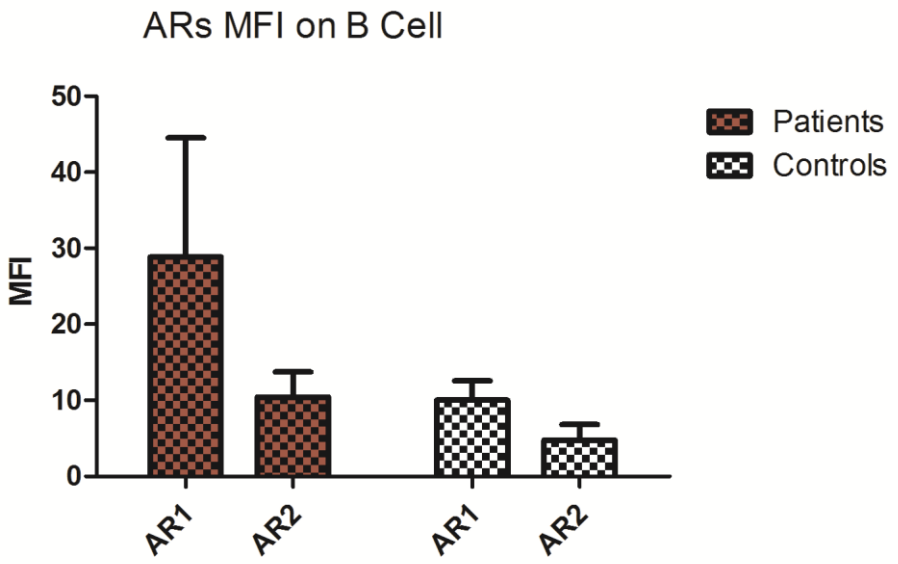


Figure 5-4: ARs frequency on B cells from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of AR1 and AR2; PBL was isolated from atherosclerosis patients or age match control**. .** Subjects were aged from 60-75 years old and they were matched for sex, ethnicity, BMI, and Statin usage. Fresh blood samples were stained, then gated on their forward/side scatter profile. CD19 antibody was used to identify B cells. ARs receptors expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

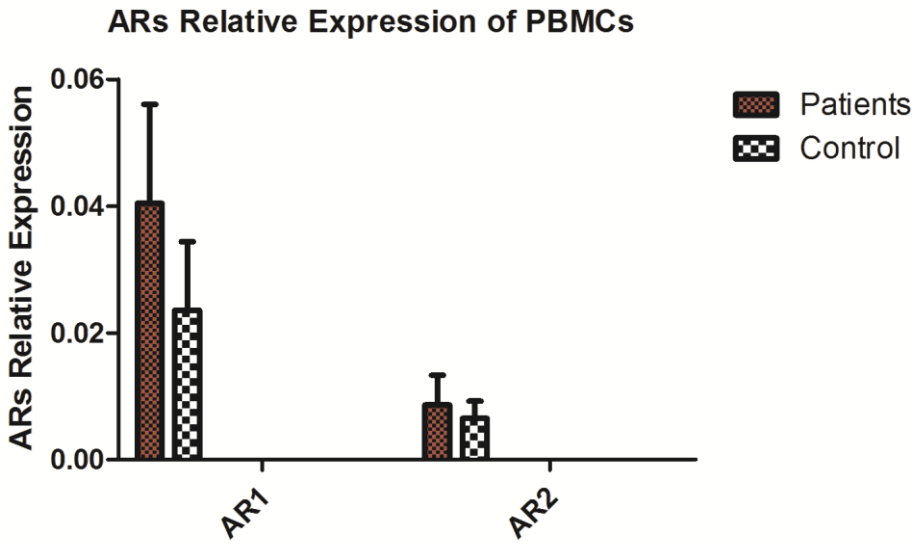


Figure 5-5: AR1/2 gene expression on PBMCs from atherosclerosis patients or age match control.

qPCR was used to measure the gene expression of AR1/2 on PBMCs from atherosclerosis patients or age match control**.** Subjects were aged from 60-75 years old and they were matched for sex, ethnicity, BMI, and Statin usage. PBMCs were isolated from fresh blood samples and gene expression was measured and quantified using qPCR. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

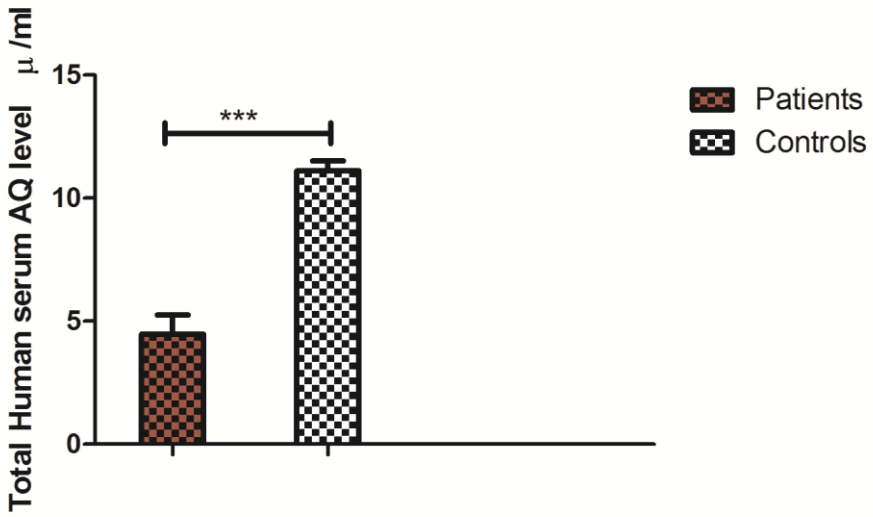


Figure 5-6: Circulating AQ levels in atherosclerosis patients or age match control.

The ELISA technique was used to measure the Circulating levels of Adiponectin in atherosclerosis patients or age match control. Subjects were aged from 60-75 years old and they were matched for sex, ethnicity, BMI, and Statin usage. ELISA kit and the numeric values were calculated using a standard curve as described by the manufacture protocol. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

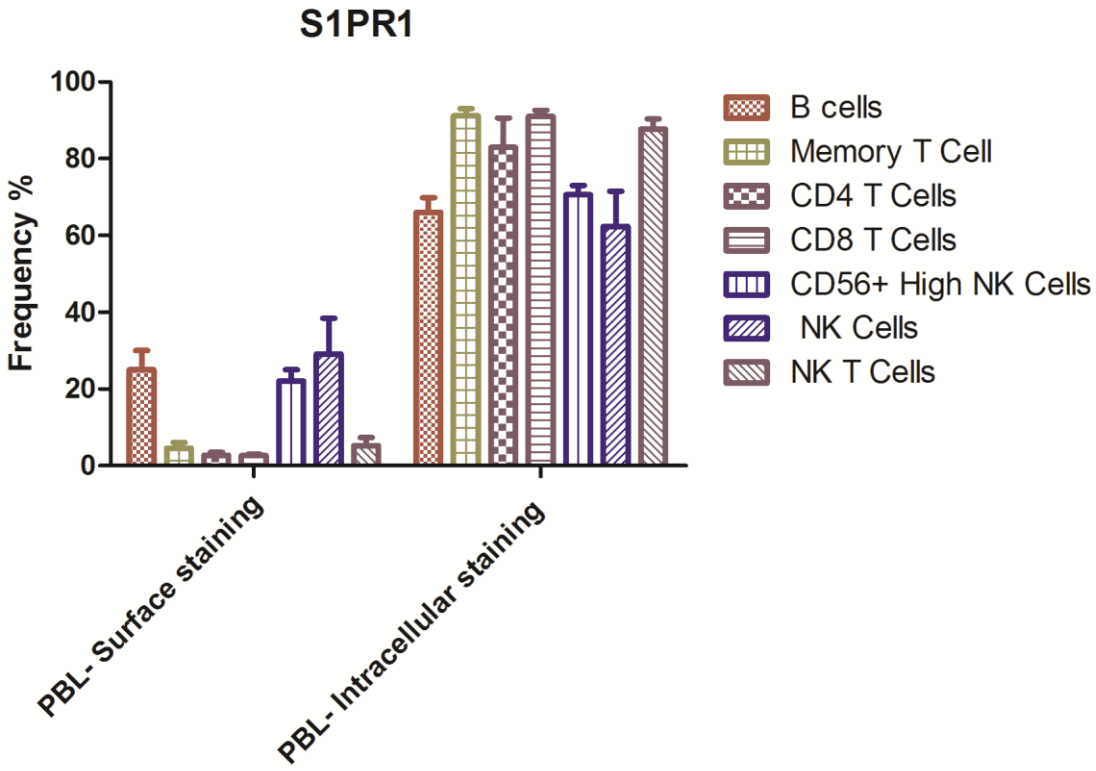


Figure 5-7: Surface expression of SIPR1 on fresh PBL subsets from random healthy donors.

Flow cytometry was used to measure the frequency of SIPR1 receptor on fresh blood PBL; blood was collected from random donors and PBL surface staining figures represent the surface expression of the receptors whereas PBL- intracellular staining represents the intracellular expression of receptors. Data is mean ± SEM (N=6). Since the experiment did not work as planned, it was not carried out on patients' blood.

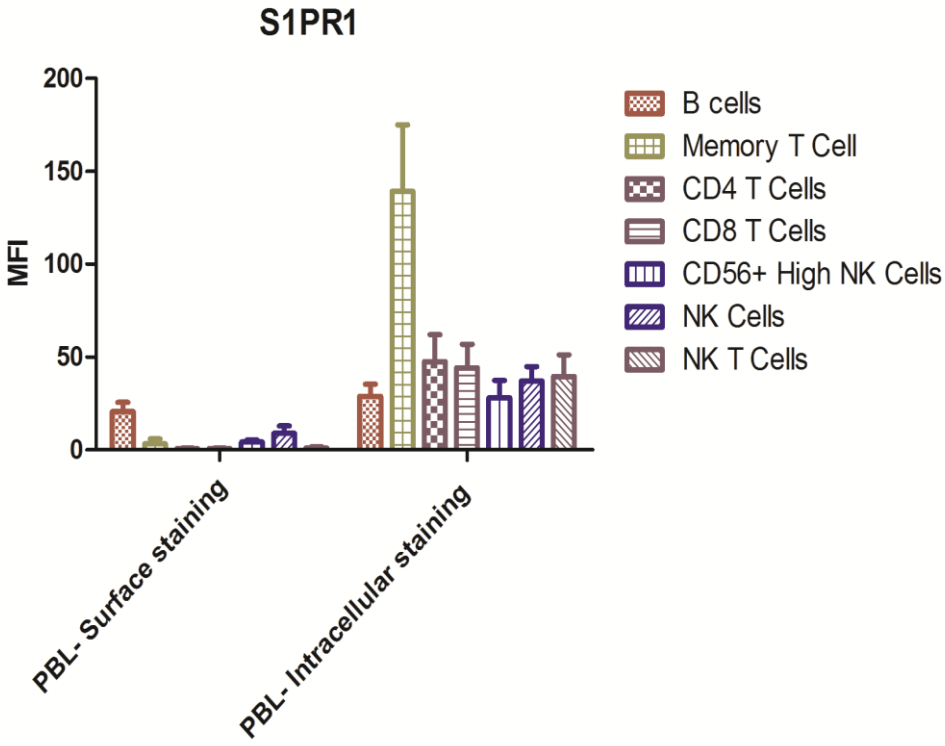


Figure 5-8: Surface expression of SIPR1 on fresh PBL subsets from random healthy donors.

Flow cytometry was used to measure the MFI of SIPR1 receptor on fresh blood PBL; blood was collected from random donors and PBL surface staining figures represent the surface expression of the receptors whereas PBL- intracellular staining represents the intracellular expression of receptors. Data is mean ± SEM (N=6). Since the experiment did not work as planned, it was not carried out on patients' blood.

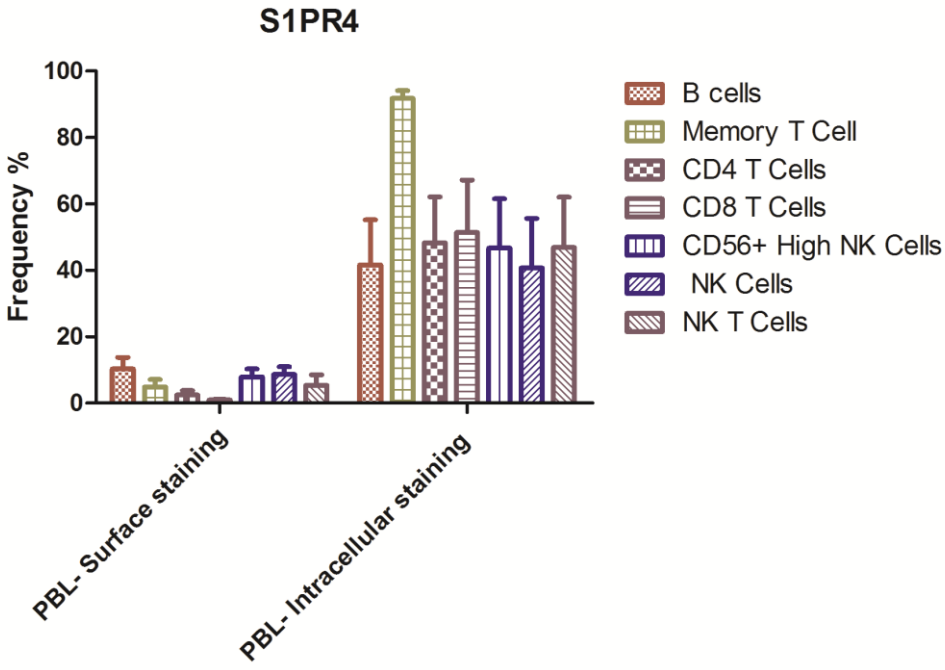


Figure 5-9: Surface expression of SIPR4 on PBL subsets from random healthy donors.

Flow cytometry was used to measure the frequency of SIPR4 receptor on fresh blood PBL; blood was collected from random donors and PBL surface staining figures represent the surface expression of the receptors whereas PBL- intracellular staining represents the intracellular expression of receptors. Data is mean ± SEM (N=6). Since the experiment did not work as planned, it was not carried out on patients' blood.

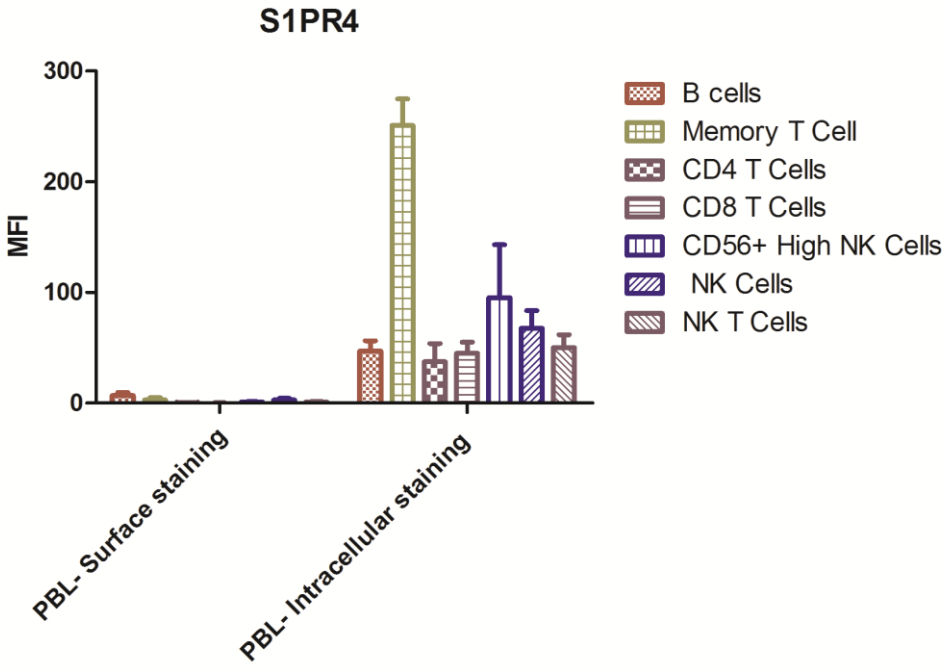


Figure 5-10: Surface expression of SIPR4 on PBL subsets from random healthy donors.

Flow cytometry was used to measure the MFI of SIP4 receptor on fresh blood PBL; blood was collected from random donors and PBL surface staining figures represent the surface expression of the receptors whereas PBL- intracellular staining represents the intracellular expression of receptors. Data is mean ± SEM (N=6). Since the experiment did not work as planned, it was not carried out on patients' blood.

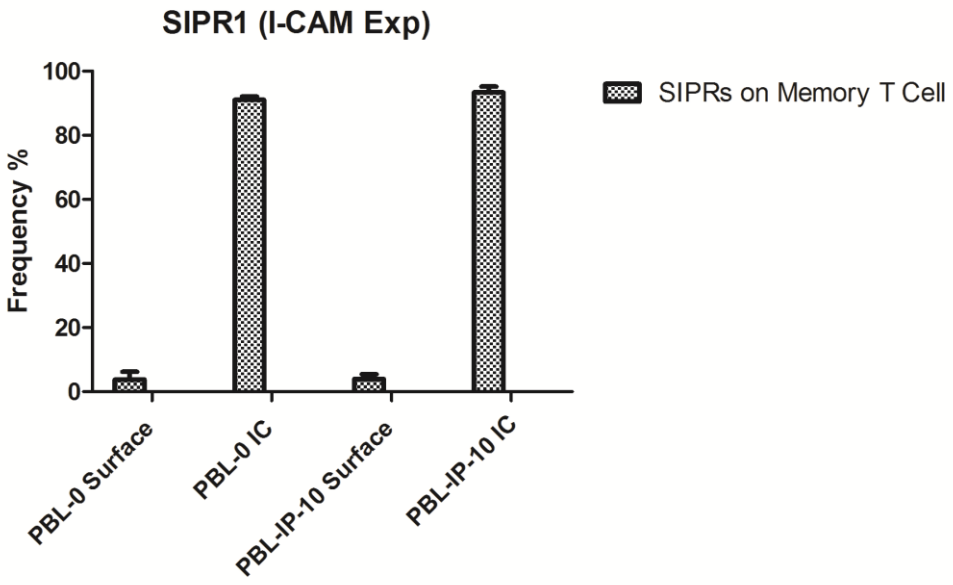


Figure 5-11: The frequency of SIPR1 receptor on migrated PBL of random healthy donors using ICAM surface.

Static adhesion assay was used to measure the frequency of the up-regulation SIPR1 on the surface of PBL after binding to I-CAM. PBL-0 represents PBL without any treatment. PBL-IP10 represents PBL treatment with IP10. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$). Since the experiment did not work as planned, it was not carried out on patient's blood.

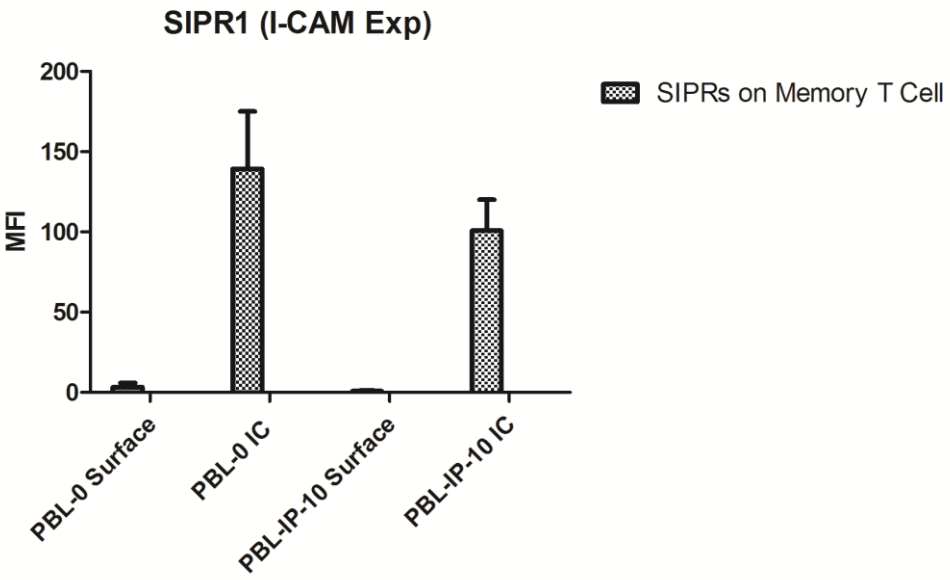


Figure 5-12: The MFI of SIPR1 receptor on migrated PBL of random healthy donors using ICAM surface.

Static adhesion assay was used to measure MFI of the up-regulation SIPR1 on the surface of PBL after binding to I-CAM. PBL-0 represents PBL without any treatment; PBL-IP10 represents PBL treatment with IP10. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$). Since the experiment did not work as planned, it was not carried out on patients' blood.

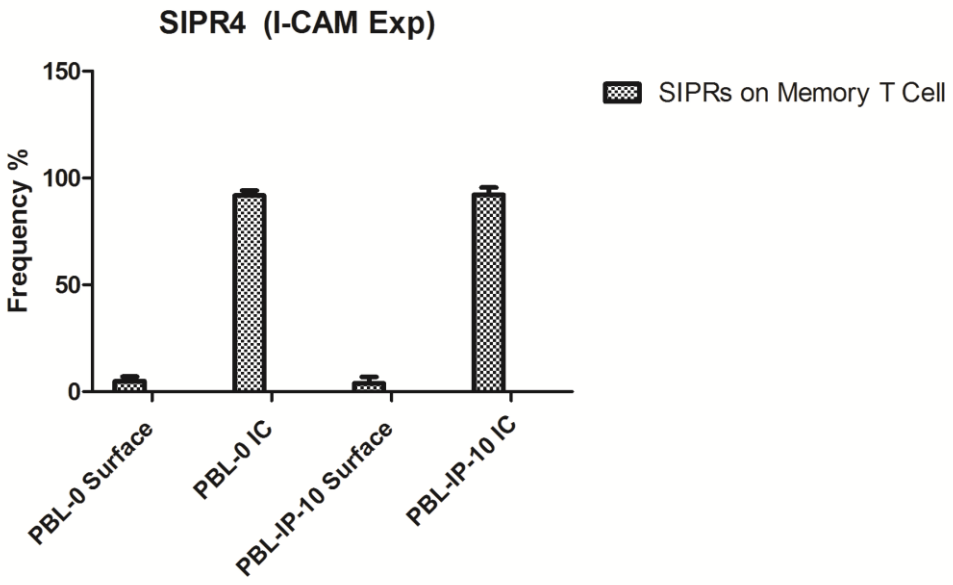


Figure 5-13: The frequency of SIPR4 receptor on migrated PBL of random healthy donors using ICAM surface.

Static adhesion assay was used to measure the frequency of the up-regulation SIPR4 on the surface of PBL after binding to I-CAM. PBL-0 represents PBL without any treatment; PBL-IP10 represents PBL treatment with IP10. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$). Since the experiment did not work as planned it was not carried out on patient's blood.

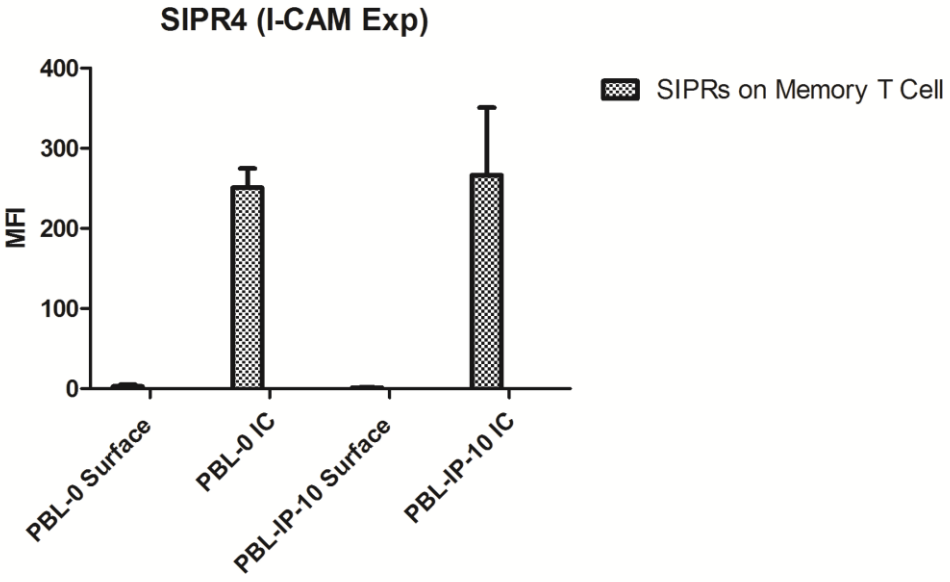


Figure 5-14: The MFI of SIPR4 receptor on migrated PBL of random healthy donors using ICAM surface.

Static adhesion assay was used to measure the frequency of the up-regulation SIPR4 on the surface of PBL after binding to I-CAM. PBL-0 represents PBL without any treatment, PBL-IP10 represents PBL treatment with IP10. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$). Since the experiment did not work as planned, it was not carried out on patients' blood.

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3. Discussion

In this chapter we discuss the effect on migration of treating PBL which have been isolated from atherosclerosis patients (Athero patients) with PEPITEM or AQ. Firstly, when looking at the total adhesion of PBL; we noticed no significant change in the number of PBL adhering to the endothelium when these were isolated from patients with atherosclerosis.. Studies on mouse atherosclerotic aortas have shown that lymphocyte adhesion is affected by lesion topology and depends of VCAM-1 expression on the atherosclerotic lesions (Moller et al., 2005). Moreover, studies using targeted mutations to E or P selectins or adhesion molecules such as ICAM-1, chemokines such as MCP-1 and their receptors, suggested that leukocyte recruitment in the case of atherosclerosis is mediated by their actions (Rosenfeld, 2002).

Furthermore, we planned to investigate the effect of the treatment of PBL isolated from atherosclerosis patients with PEPITEM and AQ. Interestingly, PBL isolated from atherosclosis patients did not show any sign of inhibition of transmigration after treatment, whereas PBL isolated from healthy controls showed a significant inhibition of transmigration after treatment with either agent. Previous work in our lab studied the effect of PEPITEM and AQ treatment on PBL isolated from patients with type 1diabetes (T1D). The behaviour of PBL in response to AQ was lost in these patients. The molecular basis of this deficiency was loss of ARs on B cells, leading to loss of PEPITEM release in response to AQ stimulation. Importantly, regulation of T cell trafficking could be restored with exogenous PEPITEM. (Chimen et al., 2015). Interesting, exactly the same defect was evident in patients with rheumatoid arthritis. Thus in both T1D and RA, T cell migration was defective on the same basis, and it is therefore extremely interesting that in atherosclerosis, although there is a loss

of response to AQ, this cannot be rescued with PEPITEM and does not appear to be due to the same changes observed in T1D or RA.

Thus, in our study, PEPITEM and AQ did not have any obvious effect on the migration of PBL which may suggest that the signal downstream of PEPITEM release from B cells was likely to have been lost or impaired. As mentioned above, PEPITEM works by binding to cadherin-15 on EC which activates the synthesis and the release of SIP. Following this, SIP binds to its receptors SIPR1 and SIPR4 on T cells? to inhibit T cell transmigration. We subsequently hypothesized that SIP production or the signalling from S1PR might be defective.

We therefore focused our work on SIP and SIP receptors. As a result, we aimed to develop an assay enabling us to measure the up-regulation of SIPR1 and SIPR4 on PBL surfaces? which would enable us to measure their expression on atherosclerosis patients' PBL. The first step was characterising SIPR1 and SIPR4 expression on PBL, since these receptors are expressed internally and externally, expressions were measured in both pools. SIP receptors are widely expressed internally but show only very limited expression externally; meaning we wanted to develop an assay to stimulate the surface expression of the receptors which would allow us to measure this expression on atherosclerosis patients and then compare that to the healthy control. For that reason, we wanted PBL to bind to ICAM-1 and anticipated that this binding in association with stimulation with chemokine CXCL10, would increase SIPR1 and SIPR4 on the surface. Indeed, we have reported positive results from this assay previously. Unfortunately only very low surface expression was achieved. It is possible that SIPRs were up-regulated, but because of the high SIP concentration in the medium (in the serum), the surface expression was down-regulated very quickly (Ikeda et al.,

2004). So, another assay needed to be developed to enable us to measure SIPRs' expression on the surface.

For flow cytometry analysis, we measured the expression of AR1 and AR2 on B cells because PEPITEM is produced from this source. We actually saw no change of these receptors on the B cells from atherosclerosis patients when compared to healthy donors. A higher expression of AR1 and AR2 is usually associated with healthy individuals and loss of the receptors has been reported in diseases such as RA and T1D (Kadowaki et al., 2006). Here however, the expression of AR1 and AR2 on B cells did not change in either groups. Clearly the pathogenesis of these diseases varies markedly, but it is interesting that the PEPITEM system, although deregulated in all 3 diseases, does not demonstrate the same molecular basis of deficiency.

We also looked at the gene expression of AR1 and AR2 on PBMCs as this has been shown to be a sensitive marker for changes in disease. The gene expression in the atherosclerosis group compared to the healthy group was unchanged. Moreover, previous studies from unpublished work by Dr. Helen McGettrick in our lab showed the same pattern of the gene expression of AR1 and AR2 in arthritis patients.

Most interestingly AQ serum levels in atherosclerosis patients are significantly lower compared to the healthy control. As mentioned above existing literature has concluded that any AQ serum level less than 4 μg/ ml can indicate hypoadiponectinemia. Our data shows AQ serum levels as 4.4 μg/ ml which is very close to the cut-off value for hypoadiponectinemia whereas AQ serum levels in the healthy group is 11.1 μg/ ml. This value is consistent with other data which shows hypoadiponectinemia is associated with T1D and atherosclerosis (Weyer et al., 2001).

It's worth mentioning however, that there are some studies which show a decrease in the surface expression of AR1 and AR2 in T1D. Kollias et al measured the expression of AR1 and AR2 on monocytes from a patient with coronary artery disease and reported a decrease of the surface expression (Kollias et al., 2011). So, there is no general consensus about the gene or surface expression of AR1 and AR2 in case of diseases. Additionally this appears to vary in a disease specific manner.

4. Conclusion

Since PEPITEM is a novel peptide discovered in our lab; this is the first time to our knowledge, that the effect of PEPITEM and AQ on PBL isolated from Atherosclerosis patients has been reported. We demonstrated for the first time that the AQ-pepitem pathway is impaired in these patients. In addition, we have reported that the surface expression and the gene expression of AR1 and AR2 is unchanged in atherosclerosis patients Furthermore, in contrast to genetic analysis and flow cytometry analysis, the AQ serum level is very low in those patients.

CHAPTER 6: STUDYING THE EFFECT OF ATHEROSCLEROSIS ON CHEMOKINE AND INTEGRIN RECEPTORS EXPRESSION ON PBL SUBSETS

1. Introduction

The recruitment of inflammatory cells present in blood and their subsequent migration in a transendothelial manner is one of the initial steps in the progression of atherosclerosis. The cellular adhesion molecules which are involved in regulating this function are upregulated by inflammatory substances acting on the vascular endothelium, and on circulating leukocytes the importance of adhesion molecules in the development of atherosclerosis and plaque stability, however, has been demonstrated through a number of evidential studies. On atherosclerotic plaques, VCAM-1, ICAM-1 and E-selectin have been consistently observed (Blankenbrg et al., 2003). Studies provide proof soluble versions of these molecules, e.g. sICAM-1 sVCAM-1 in the blood is associated with high risk of developing cardiovascular disease or can be found in CAD patients (Blankenbrg et al., 2003).

1.1. Adhesion molecules and Integrins

Adhesion molecules are expressed with distinct patterns. For example L-selectin is constitutively expressed most leukocytes, P-selectin is found in granule stores in endothelium and in platelets and can be rapidly (minutes) mobilised on the surface of the cell after activation (Smith, 2008). E-selectin and VCAM-1 are transcriptionally unregulated upon endothelial cell activation by cytokines and other inflammatory agonists (Olofsson et al., 1994). The processes supporting down-regulation of these molecules are essential to terminate inflammatory processes; it is vital for them to be removed from the surface of the cell after the activation otherwise inflammation can become a chronic process (Blankenbrg et al., 2003). Selectin molecules and VCAM-1 are eliminated from the cell surface after activation of the cell though the process of internalisation and lysosomal targeting (P- and E-selectin) or by shedding by proteolytic cleavage (Blankenbrg et al., 2003). It is this processes

that produces soluble forms of the adhesion molecules, which can be measured in the blood (Hafezi-Moghadam et al., 2001). P-selectin glycoprotein ligand (PSGL-1) is the most important ligand for P-selectin because it has more affinity to P selectins on myeloid cells (Borges et al., 1997). It is displayed on the leukocyte surface, specifically on microvillous tips (Sako et al., 1994).

Integrin molecules are hetrodimeric proteins made of α chain and β chain. There is complexity in their patterns of association with many α and β chains demonstrating promiscuity in their associating partners. For example, β1 integrin, may be linked to any of 12 α chains (Blankenbrg et al., 2003). The function of integrins is regulated by their confirmation state which can be regulated by cellular activation. Their nature in resting cells is mostly non-adhesive, due to the low affinity interactions that occur with ligands. As a result of variations in their confirmation which is directly associated with the activation of these cells, this affinity increases.. This process of regulated integrin activity is based on inside-out signalling to these receptors (Blankenbrg et al., 2003).

Intergins are also capable of providing outside-in signals following ligand binding (Faull and Ginsberg, 1996). The important aspect here, are the leukocyte integrins that support leukocyte trafficking. Beta-2 integrins are exclusively displayed only in white blood cells and they are particularly important for the trafficking of these cells (Rodriguez et al., 1996). During this process they can interact with endothelial counter receptors such as ICAM-1 as well as matrix proteins in the vessel wall and the tissue, which allow leukocytes to migrate in these environments (Lynam et al., 1998). Soluble adhesion molecules may have utility as biomarkers for CAD (Gearing and Newman, 1993). The soluble forms increase in the circulation in association with a number of cardiovascular risk factors, such as smoking

(Blann et al., 1997), hypercholesterolemia and/or hypertriglyceridemia. (Hackman et al., 1996) (Lupattelli et al., 2000).

Soluble ICAM-1 and soluble VCAM-1 and proatherogenic lipid profiles are considered markers for CVD (Ridker et al., 2000). Statin therapy was discovered to decrease the soluble adhesion molecules soluble ICAM-1 and soluble P-selectin (Blann et al., 2001). Soluble adhesion molecules have also been proposed to be biohumoral markers for disease severity in atherosclerosis (Blann and McCollum, 1994), and carotid intima-media thickness correlates to the levels of sVCAM-1 and sICAM-1(Rohde et al., 1998).

1.2. Chemokines and Atherosclerosis

In atherosclerosis chemokines are involved in the recruitment of leukocytes into atherosclerotic plaques (Burke-Gaffney et al., 2002). These are a super family of proteins which are structurally associated with a molecular weight of 8-10 kDa. (Baggiolini et al., 1997). Based on the distance between the first two of four conserved cysteine residues, the 40 or more chemokines so far identified can be categorized into four groups (Burke-Gaffney et al., 2002). The CC, CXC, CX3C, XC chemokines. It has been observed that chemokines stimulate leukocyte activation through seven transmembrane domain G protein-coupled cell-surface receptors present on target cells (Braunersreuther et al., 2007). The chemokine receptor is responsible for regulating the chemokine's biological functions. There is promiscuity in the ligand receptor interactions of chemokines with for example several chemokines forming bonds with one receptor, or one receptor binding more than one chemokine ligand (Burke-Gaffney et al., 2002). In atherosclerosis, the major roles of chemokines is to regulate leukocyte extravasation (Reape et al., 1999) and studies in mice

with genetic deletion of chemokines or their receptors show reduced burden of atherosclerotic disease (Wan et al., 2013) .

There are no antichemokine therapeutic interventions currently accessible to treat atherosclerosis. Despite this, in clinical use a number of agents, such as the lipid-lowering statins and insulin-sensitizer glitazones, regulate the appearance of chemokines and these present potential in development of drugs (Ray et al., 2006).

2. Results

2.1. Surface expression of adhesion molecule receptors on PBL from patients with atherosclerosis or age matched controls

The aim of this chapter was to compare the surface expression of adhesion molecules and chemokine receptors on the PBL of atherosclerosis patients and compare this to age-matched healthy control subjects. We used the same two cohorts we had recruited in the previous chapter (Chapter 5). Here we have shown MFI as measure of expression. The percentage of positive cells for each receptor measured can also be found in the appendix section. We did not include the data of the surface expression of both adhesion molecules and chemokine receptors on surface adherent PBL, as it was not deemed necessary to mention the difference from the results for transmigrated PBL as these were essentially the same as the expression patterns on migrated cells. This data is not included in the appendix section to avoid distracting the reader and to follow the guidelines of the thesis word limit.

The surface expression of Alpha-L receptors was generally the same between cohorts, however there was a significantly lower expression on naïve CD8 T cells in patients. There was also a marked reduction in expression on some NK populations in patients (Figure 6-1). Figure (6-2) shows Alpha-4 receptor surface expression; the expression was significantly

higher on NK cells in healthy subjects, whereas the expression on other subsets was similar. The surface expression of Beta-receptor family (Beta-1 and Beta-2) was significantly higher in some PBL subsets in atherosclerosis patients (Figure 6-3, 6-4). The same trend of increase expression in atherosclerosis patients was seen in the case of PSGL-1 receptors on some subsets (figure 6-5). In general these significant increases in some PBL subsets indicates an increase in the expression of capture receptors such as PSGL-1 and a shift in integrin from alpha4/B1 to a beta-2 dominated expression, although this was most consistently marked in NK populations.

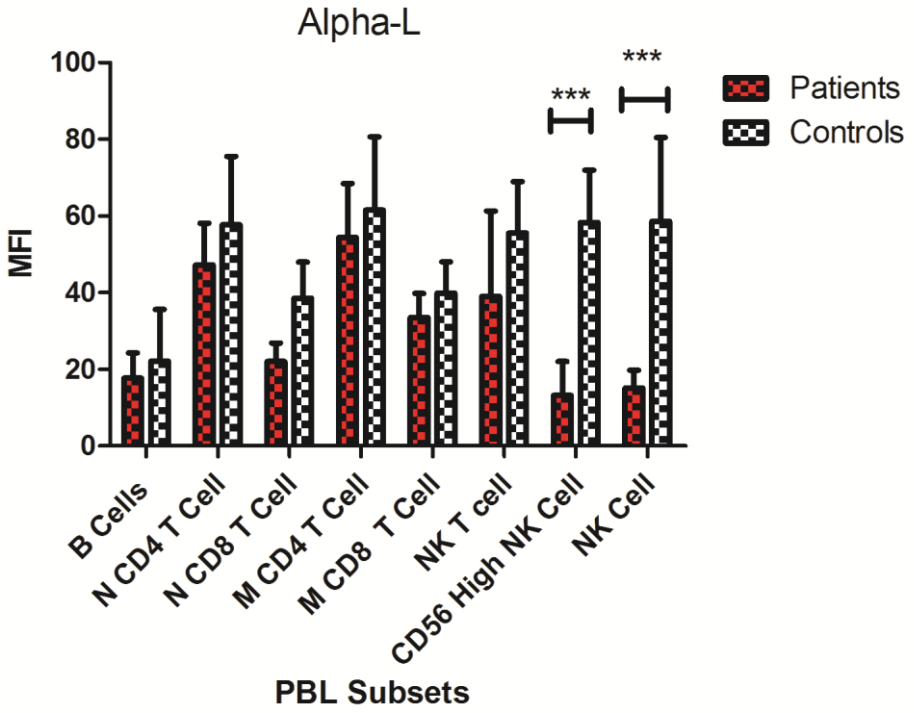

Figure 6-1: MFI of Alpha-L receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of Alpha-L receptors? on fresh blood PBL subsets. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha L expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

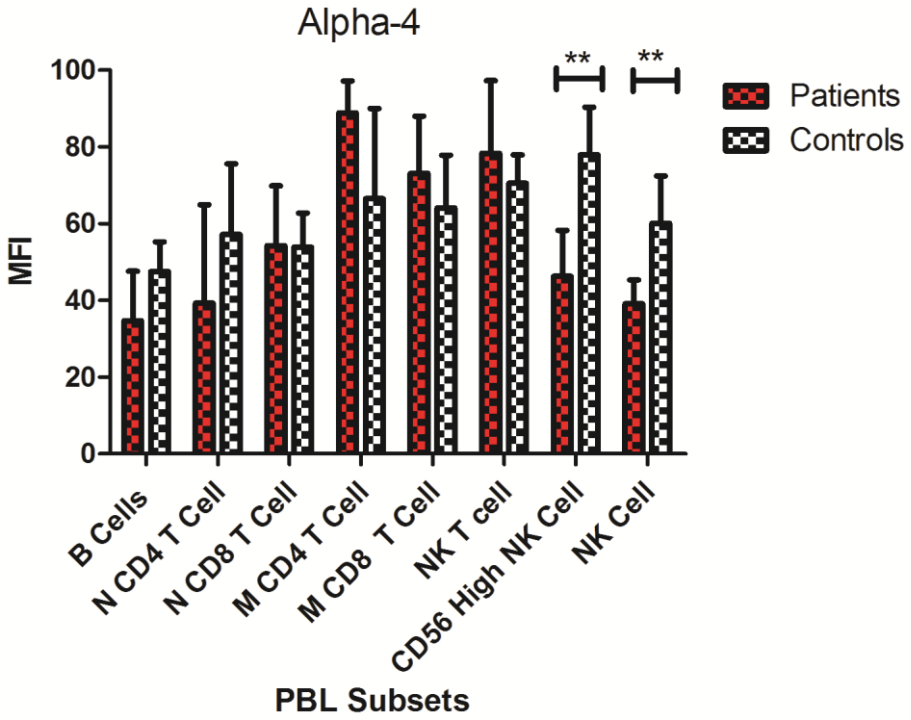

Figure 6-2: MFI of Alpha-4 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of Alpha-4 receptor on fresh blood PBL subsets. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-4 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

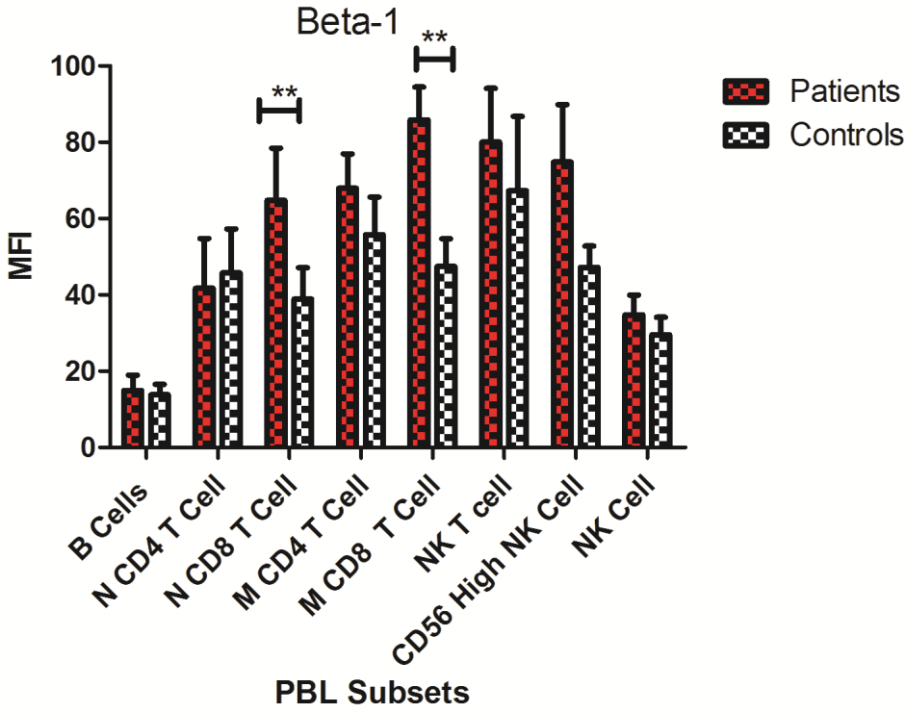

Figure 6-3: MFI of Beta-1 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of Beta-1 receptor on fresh blood PBL subsets. Fresh blood was collected fromatherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

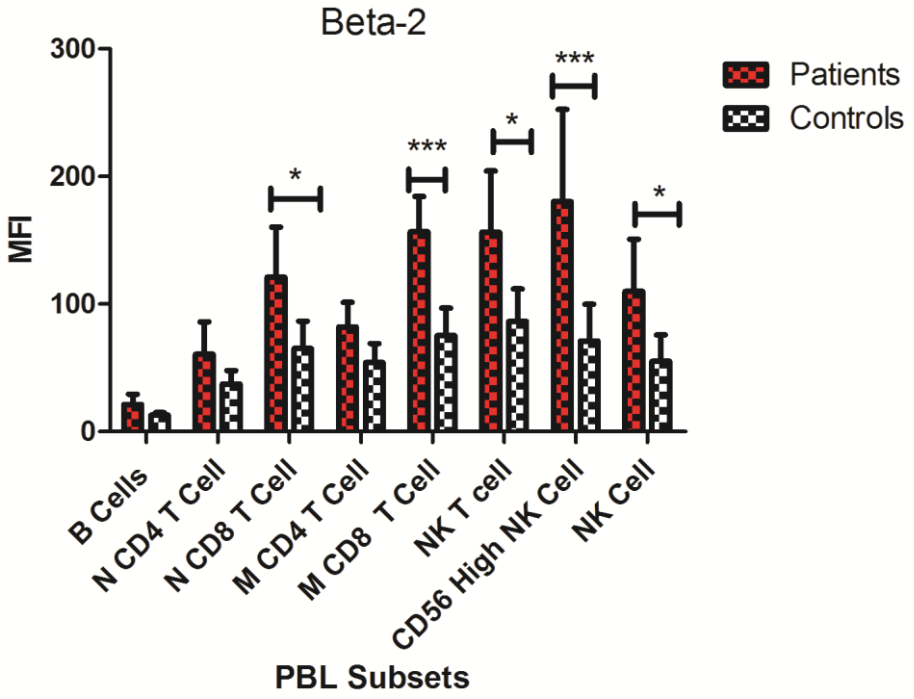

Figure 6-4: MFI of Beta-2 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of Beta-2 receptor on fresh blood PBL subsets; Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-2 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent an sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

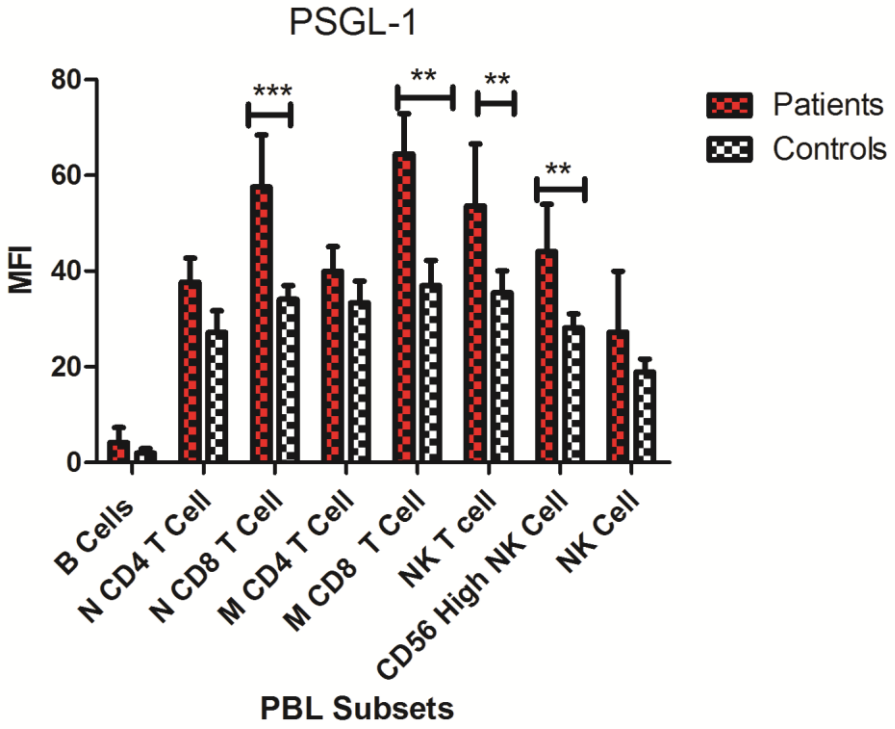

Figure 6-5: MFI of PSGL-1 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of PSGL-1 receptor on fresh blood PBL subsets. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. PSGL-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.2. Surface expression of chemokine receptors on fresh PBL from atherosclerosis patients or age matched controls.

In this section we looked at chemokine receptors expression on PBL subsets. We put them into a separate section because in the literature they are classified as a distinct family, and their activation, down-regulation and role in leukocyte trafficking is different from that of adhesion molecules. We used the same PBL subsets markers we had used for adhesion molecules; we observed that there was a large degree of variation within populations of donors for both CCR5 and CXCR3 receptors. However, there was a strong trend for lower expression of these receptors in patients and this was significant for some subsets. Again changes in NK cell populations seemed most consistent (Figure 6-6, 6-7). Surprisingly, the expression of fractalkine receptor (CX3CR1) showed a reciprocal response and was in general markedly higher on patient cells. (Figure 6-8).

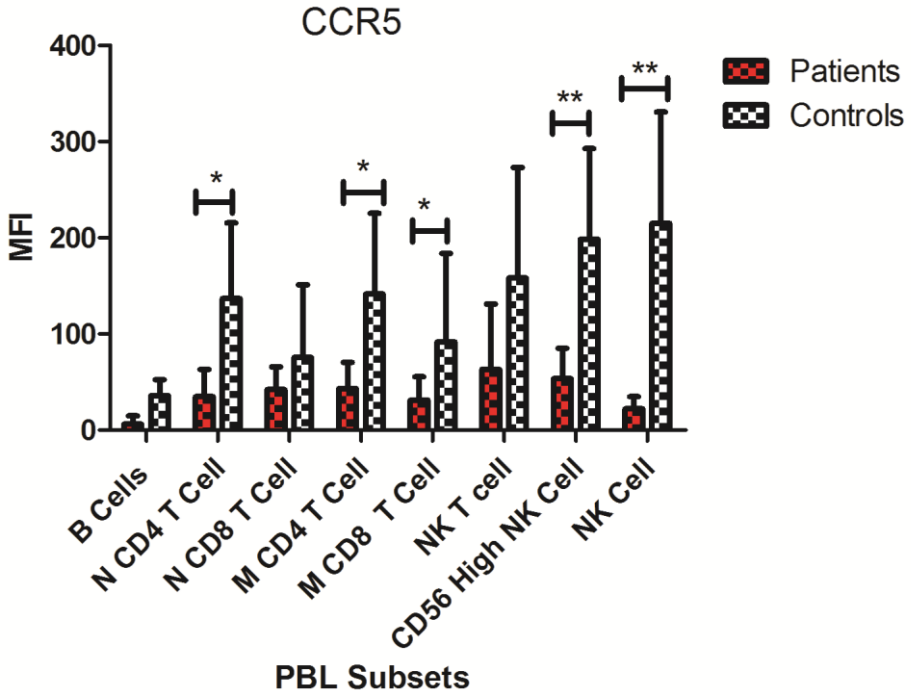

Figure 6-6: MFI of CCR5 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of CCR5 receptor on fresh blood PBL subsets. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

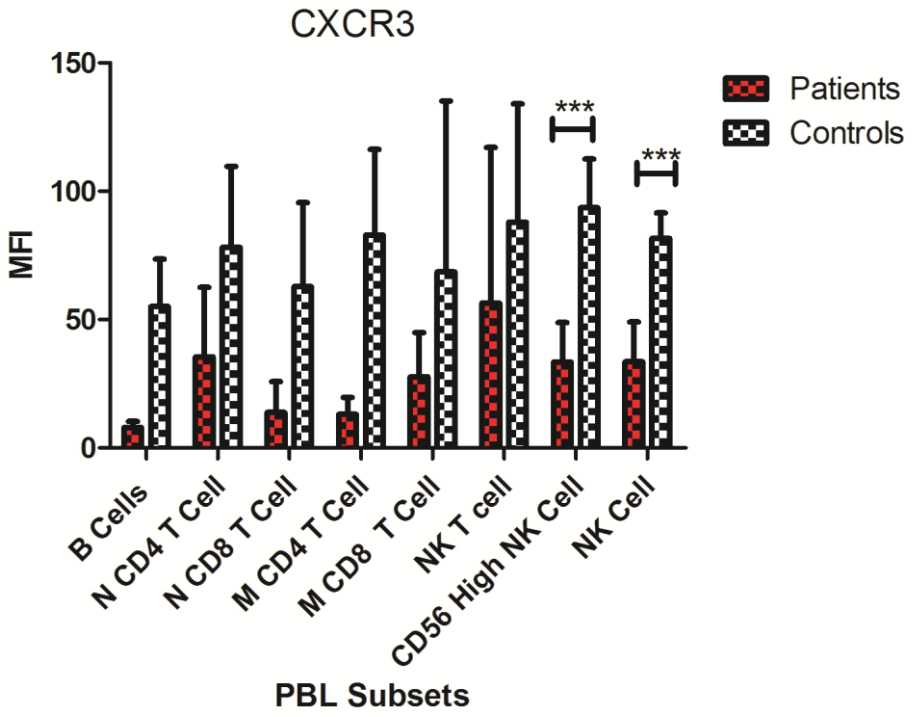

Figure 6-7: MFI of CXCR3 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of CXCR3 receptor on fresh blood PBL subsets. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent an sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

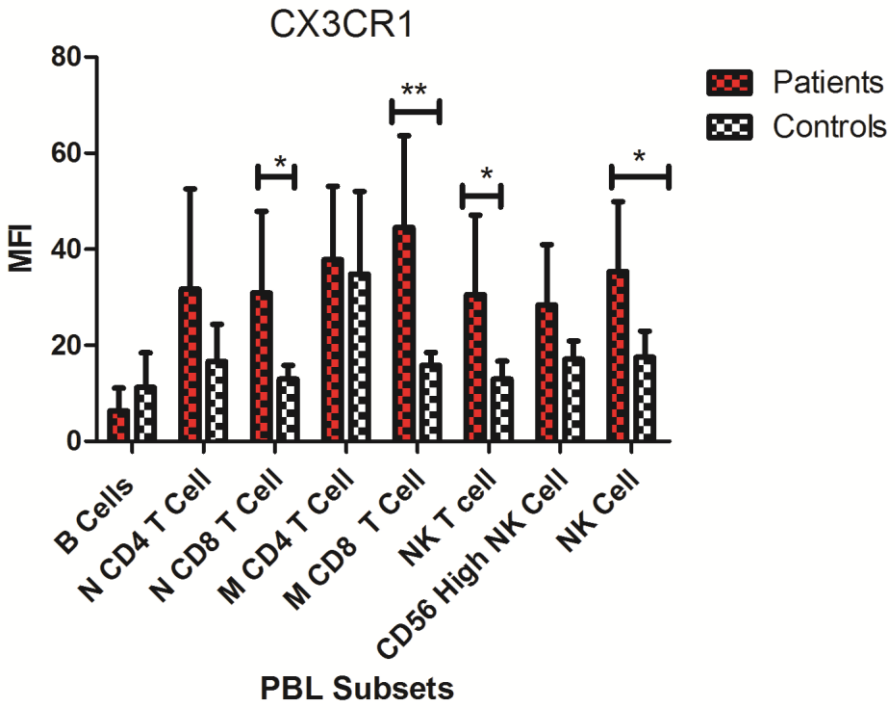

Figure 6-8: MFI of CX3CR1 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of CX3CR1 receptor on fresh blood PBL subsets. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent an sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.3. Surface expression of adhesion molecules receptors on migrated PBL from patients atherosclerosis or age matched controls

The following graphs show the expression of the same set of adhesion molecules on PBL that were collected after in vitro transmigration assay (see Chapter 2 for a detailed protocol). The data analysis shows that the expression of Alpha-L receptors on PBL subsets after transmigration is higher in atherosclerosis patients compared to the healthy controls (figure 6-9). However, the same pattern was not seen in the case of Alpha-4 receptors; in fact the surface expression was almost equal and there was only a small variation on some subsets (figures 6-10). Both the Beta receptors family (Beta-1 and Beta-2) and PSGL-1 surface expression showed no clear difference after transmigration (figures 6-11, 6-12, and 6-13).

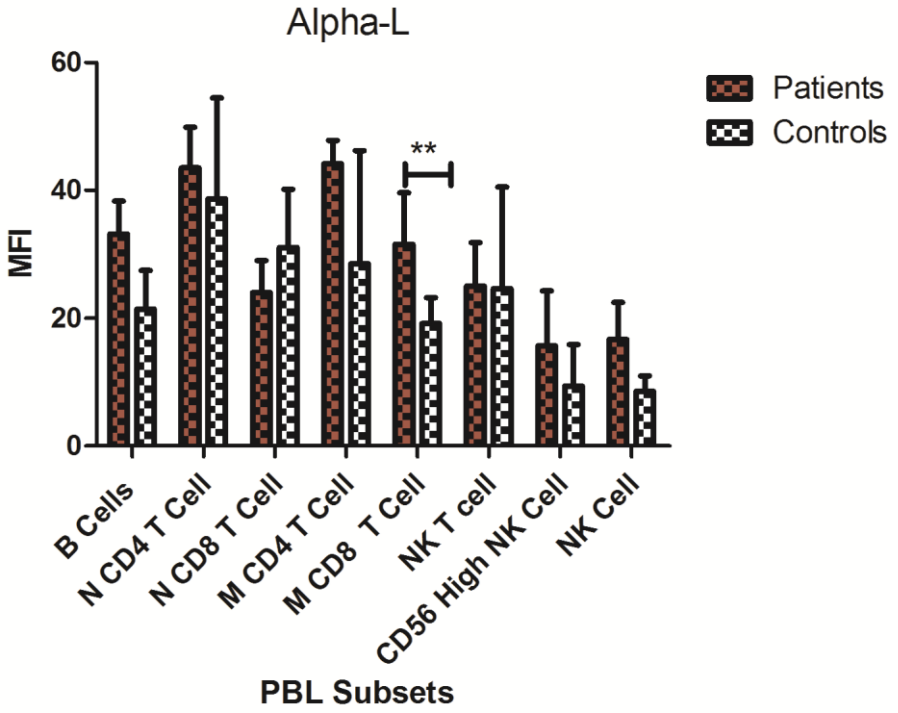

Figure 6-9: MFI of Alpha-L receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of Alpha L receptors. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. The Alpha L receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

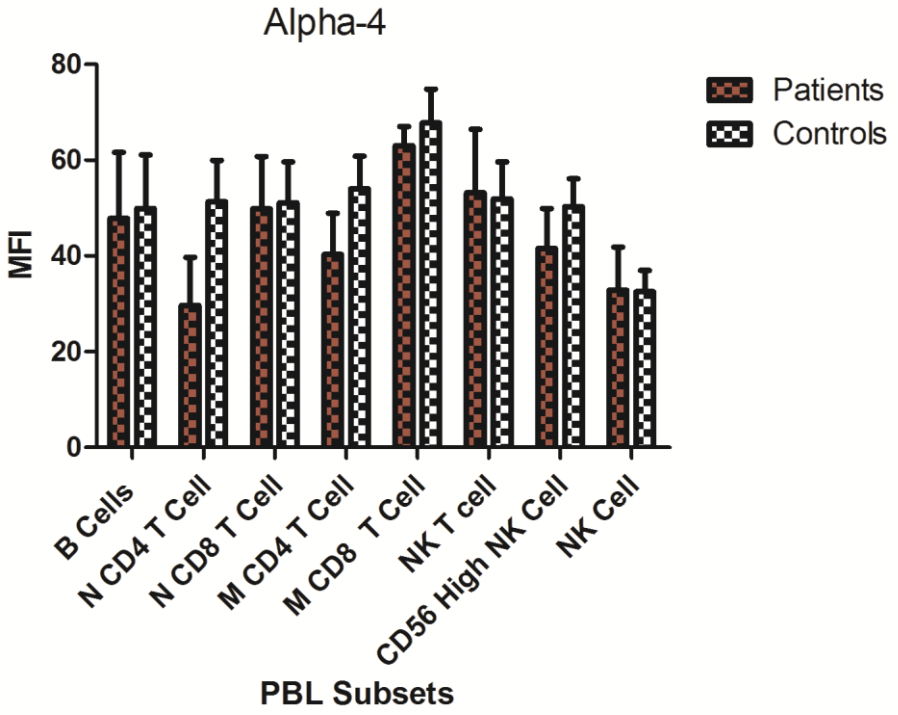

Figure 6-10: MFI of Alpha-4 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of Alpha-4 receptors. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. The Alpha-4 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

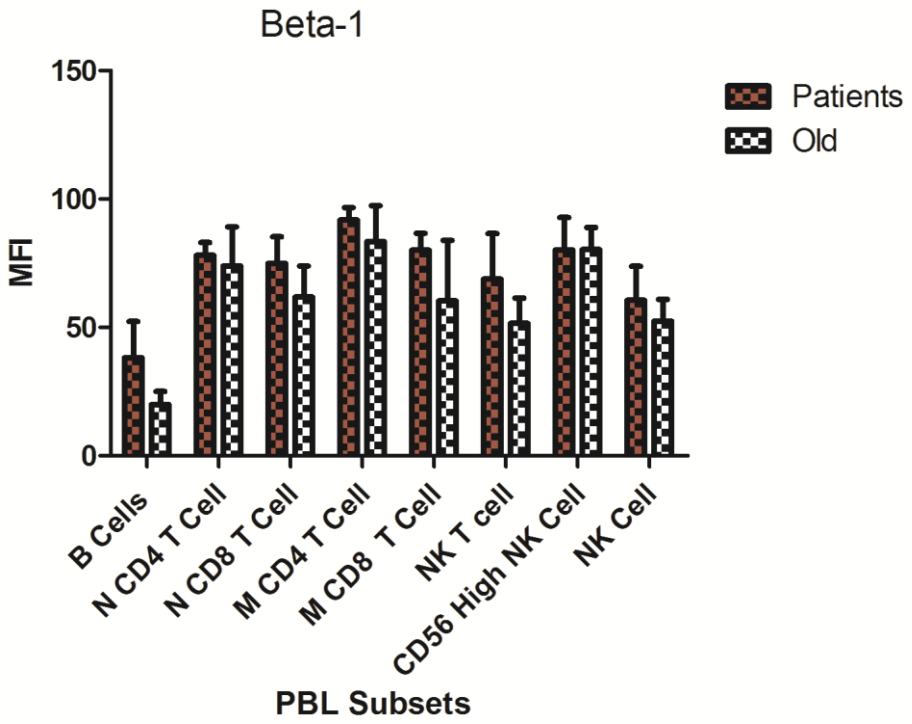

Figure 6-11: MFI of Beta-1 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of Beta-1 receptors. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. The Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

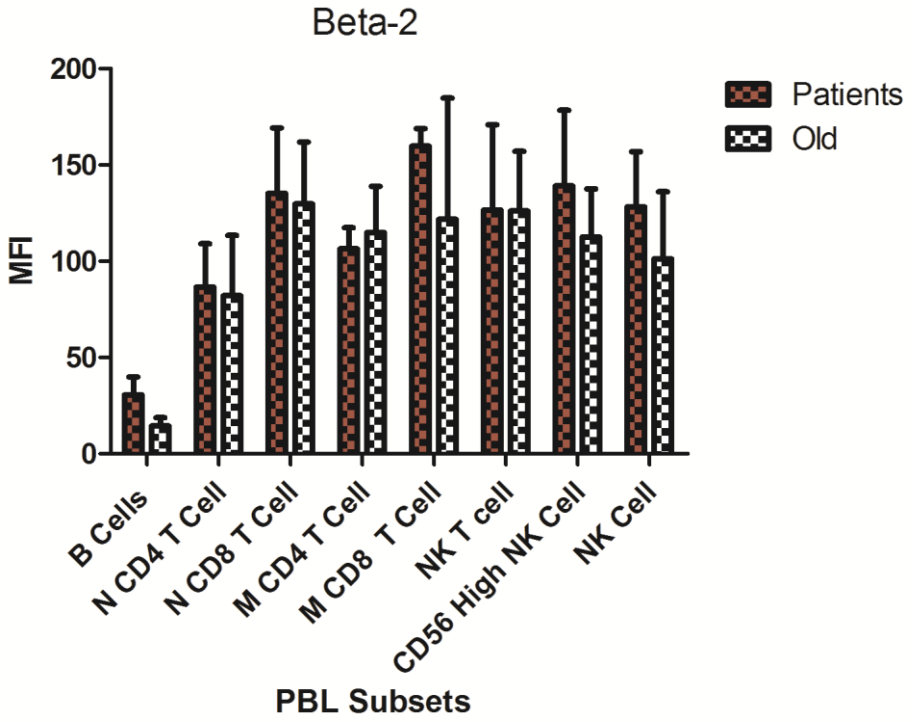

Figure 6-12: MFI of Beta-2 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of Beta-2 receptors. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. The Beta-2 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

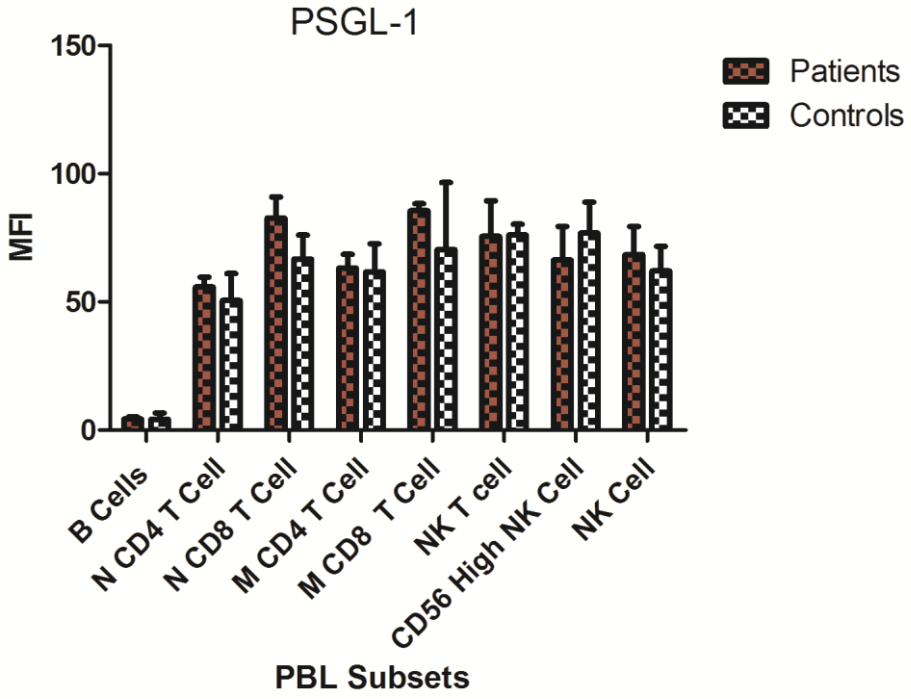

Figure 6-13: MFI of PSGL-1 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of PSGL-1 receptors. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. The PSGL-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.4. Surface expression of chemokine receptors on migrated PBL from atherosclerosis patients or age matched controls

As mentioned above, this section provided a discussion regarding chemokine receptors expression on PBL subsets. We hypothesized that Atherosclerosis patients have higher expressions of chemokin receptors than healthy controls. They were put into separate sections because the literature classifies them as a distinct family and their activation, down-regulation, and role in leukocyte trafficking is different from that of adhesion molecules. Our analysis shows a dramatic down-regulation of the surface expression of CCR5, CXCR3, CX3CR1 receptors after transmigration when compared to that in fresh blood PBL. This did not vary between cohorts (Figure 6-14, 6-15, and 6-16).

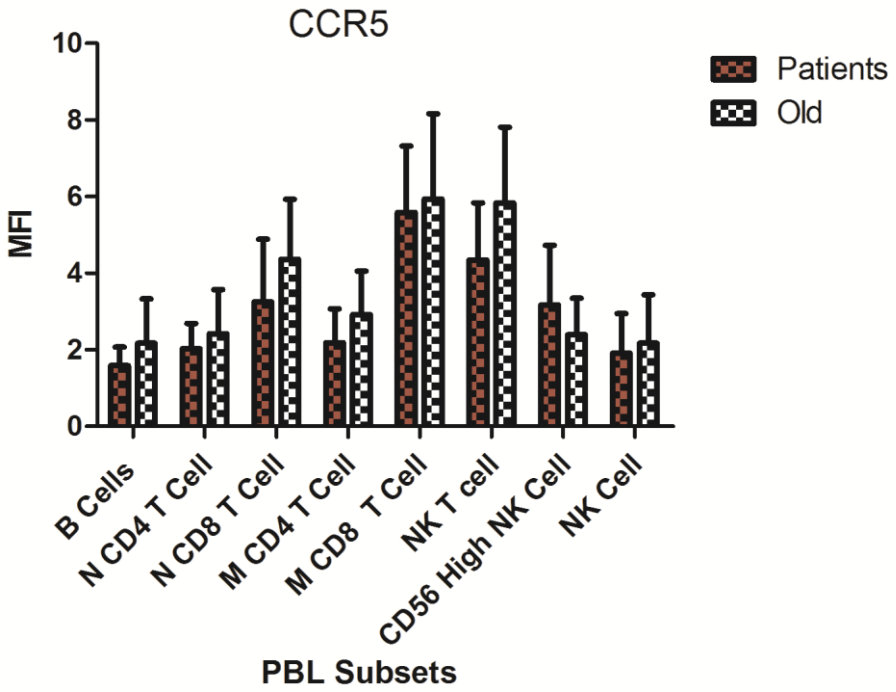

Figure 6-14: MFI of CCR5 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of CCR5 receptors. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. Finally The PBL was collected and stained with the same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. The CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

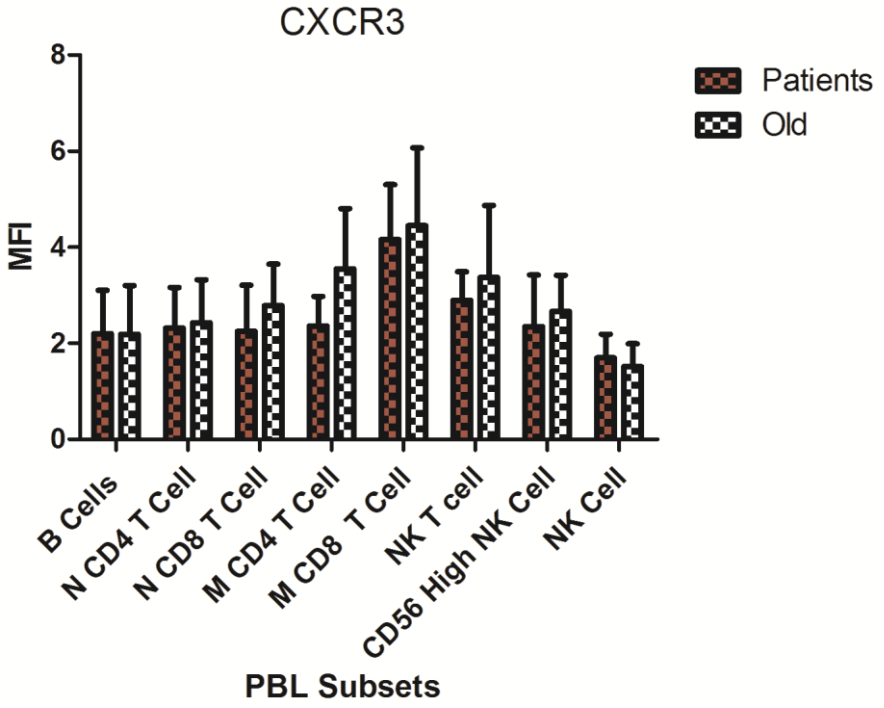

Figure 6-15: MFI of CXCR3 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of CXCR3 receptors. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. Finally The PBL was collected and stained with the same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. The CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

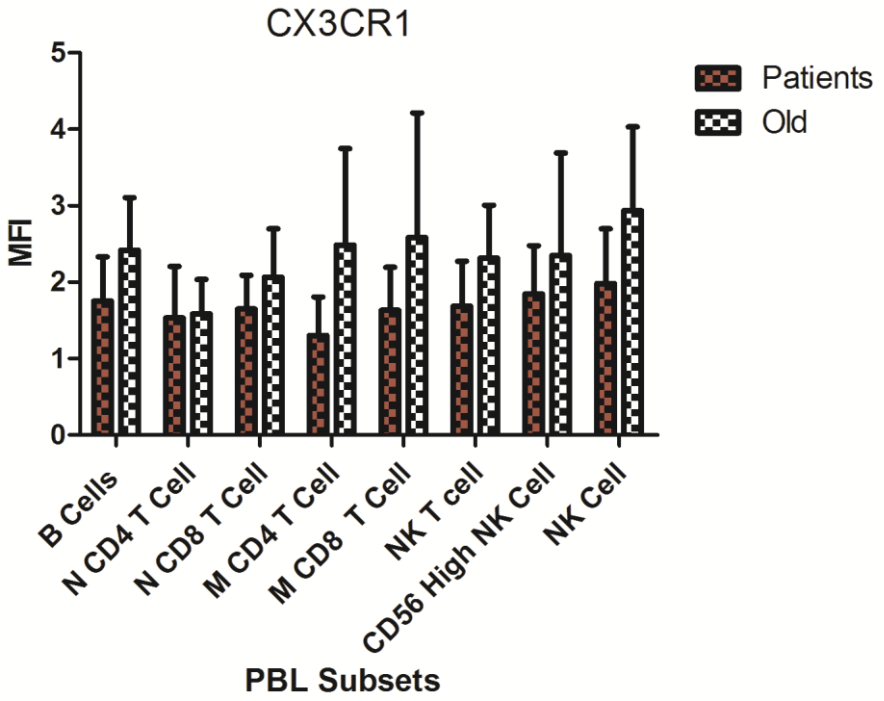

Figure 6-16: MFI of CX3CR1 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.

Flow cytometry was used to measure the MFI of CX3CR1 receptors. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. Finally The PBL was collected and stained with the same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

2.5. Enrichment ratio of surface adherent and transmigrated PBL from atherosclerosis patients or age matched controls.

Here, we aimed to measure the response of PBL subsets to the cytokines. In our static adhesion assay, we stimulated EC with TNF-α and INF-ɤ, and then PBL was added to adhere. We wanted to investigate the response of these PBL to cytokines and also their ability to bind to EC. We made this measurement by dividing either the number of surface adherent cells or transmigrated cells by the total number of added cells. Figures 6-17 and 6-18 show the enrichment ration of PBL subsets between atherosclerosis patients and healthy control subjects. The figures reveal no clear and obvious difference in the ratios of the subsets that are recruited in atherosclerosis patients and age matched controls.

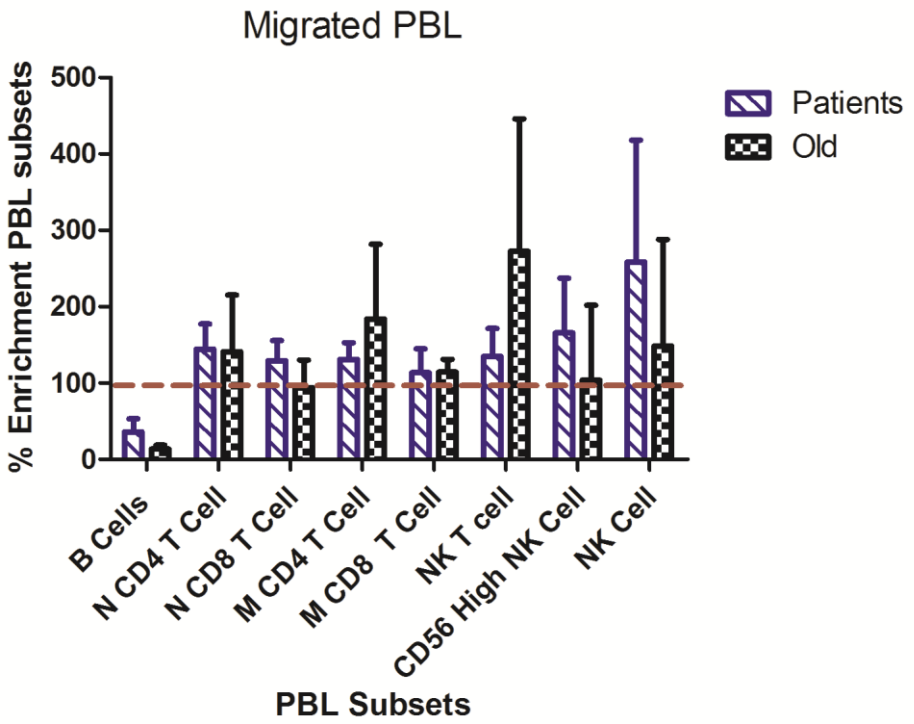

Figure 6-17: Enrichment ratio of migrated PBL from atherosclerosis patients or age match control in static condition:

The enrichment ratio for each subset from each group has been calculated. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. The number of transmigrated cells in each subset was divided by the total number of cells of that subset added to the assay. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).The transmigrated cells in each subset was divided by the total number of cells of that subset added to the assay. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

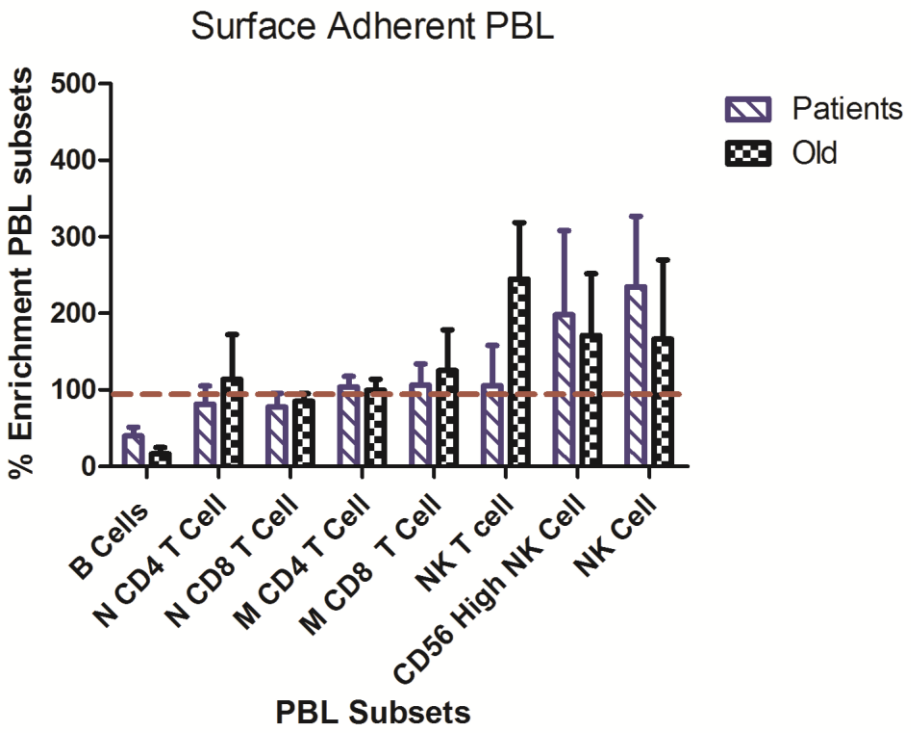

Figure 6-18: Enrichment ratio of Surface adherent PBL from atherosclerosis patients or age match control in static condition:

The enrichment ratio for each subset from each group has been calculated. Fresh blood was collected from atherosclerosis patients or age match control subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. TrypLE was subsequently used as cell-dissociation enzymes that make transmigrated PBL floating. The number of transmigrated cells in each subset was divided by the total number of cells of that subset added to the assay. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).The number of surface adherent PBL in each subset was divided by the total number of cells of that subset added to the assay. Data is mean ± SEM (N=6) and the differences between groups were determined by an using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

3. Discussion

In this section, we discuss the results of our investigations of the effect of atherosclerosis on the surface expression of adhesion molecules and chemokines receptors. Our panel of receptors was chosen because it represented the classical receptors that are responsible for T cell trafficking and therefore might lead to the progression of atherosclerosis. Our hypothesis was that after patients develop atherosclerosis, the process of T cell trafficking becomes prolonged, and that this might be caused by an increased expression of adhesion molecules. Our data shows that Alpha-L receptor surface expression was highly expressed on some PBL subsets in healthy subjects when compared to atherosclerosis patients. In the case of alpha-4 receptor surface expression, it was found to be higher on healthy subjects, but only on NK cells. As mentioned above, the majority of the integrins involved in leukocytes trafficking are β2 and α4, and usually α and β members bind together to form one main receptor, such as LFA-1, which consists of αLβ2. It is also worth mentioning that α4β1 is called VLA-4 (Galkina and Ley, 2007). The fact that alpha-L receptor expression was low in the atherosclerosis patients is also interesting. We speculate that the reason for this anomaly is because once the manifestation of atherosclerosis is recognized by the immune system, it adapts by down regulating the expression of adhesion molecules. Another reason could relate to the medications used by the patients; studies have shown for instance, that Statins reduce the expression of some adhesion molecules (Rezaie-Majd et al., 2003), and all of these patients will be well served with such drugs.

The increased expression of Beta-1, Beta-2, and PSGL-1 receptors surface expression on some PBL subsets in atherosclerosis patients was not very surprising; the involvement of the Beta integrins family is well established. In fact, Beta-1/Alpha-5 is known to mediate the

inflammation in the early stages of atherosclerosis (Yurdagul et al., 2014). One of the main characteristics of PSGL-1 is its interaction with the selectins (Schumacher et al., 2005). PSGL-1 plays a key role in monocyte recruitment in atherosclerosis; in fact, it is considered to be the favoured ligand of leukocytes P and E section (An et al., 2008). Additionally, it has been shown that PSGL-1 regulated selective T cell recruitment to an atherosclerosis site (An et al., 2008). Its increase here, therefore, was interesting in the atherosclerosis patients.

Chemokines receptor expression was significantly higher on some PBL subsets in healthy subjects than in atherosclerosis patients. The expression of chemokines receptors is regulated by internalization and shedding mechanisms, which is controlled by levels of ligand in the environment. This data contradicts another study, however, which showed that inflammatory Ly-6Chi monocytes highly express certain chemokines such as CCR2 and CX3CR1 when compared to healthy ones (An et al., 2008). Furthermore another study by Burke-Gaffney et al concluded that in inflammatory conditions, the interaction between leukocytes and EC leads to amplification of chemokines release (Burke-Gaffney et al., 2002). On the other hand, a study on menopausal women revealed that menopausal women have higher expressions of certain pro-inflammatory serum markers (Sivro et al., 2013). Moreover, animal studies have shown an increase in chemokine expression on T cell subsets (Chen et al., 2003).

Migrated PBL data on the expression of adhesion molecules was not conclusive; their levels of expression on most PBL subsets in both groups were almost equal, except for Alpha-L receptors which were highly expressed in atherosclerosis patients when compared to healthy controls. In terms of chemokines receptors surface expression, as we have seen above, their expression declined sharply, which indicates they had been down regulated.

Similarly to the enrichment data in chapter 4, we did not notice any difference in the ratios of the subsets that are recruited in at atherosclerosis patients and age matched controls which could be a result of technical problem.

4. Conclusion

Some PBL subsets which were freshly isolated from atherosclerosis patients highly express Beta-1, Beta-2, and PSGL-1 receptors, whereas their surface expression of Alpha-L and Alpha-4 receptors is low when compared to healthy subjects. Moreover, chemokines surface expression on freshly isolated PBL subsets is low compared to healthy controls. In the case of migrated and surface adherent PBL subsets, no conclusive difference in surface expression of adhesion molecules between atherosclerosis patients and healthy control was noticed. Finally, both groups recorded down-regulated chemokine receptors after the transmigration process.

CHAPTER 7: GENERAL DISCUSSION

1. General discussion

The main goal for this thesis was to study the PEPITEM pathway and its dysregulation in atherosclerosis patients. We showed for the first time that the PEPITEM pathway is impaired in atherosclerosis patients, evidenced by the lack of the inhibitory effect of PEPITEM on PBL isolated from patients. In addition, we showed the lack of AQ inhibitory effect on PBL from the same patients. These data suggest a missing link along the PEPITEM pathway which needs more investigation. Previous published work from our lab shows that the treatment of PBL isolated from T1D patients by PEPITEM and AQ, rescues the PEPITEM pathway and restores its effect which is seen by the inhibition of T cell migration in static adhesion assay (Chimen et al., 2015). So in this instance we tried to focus on the pathological changes between both diseases atherosclerosis and T1D. The literature shows that atherosclerosis starts in very early ages and some studies have also reported signs of sub-endothelium changes in the right coronary artery (Tabas et al., 2007). This fact suggests that the endothelium injury which initiates atherosclerosis has been progressing for a long period of time without prevention in contrast to T1D which can be predicated by a blood test. We now suggest that this chronic and long-term progression of endothelium lesion formation might make it advisable to increase the stock concentration of PEPITEM and AQ when treating PBL in the adhesion assay. In this experiment we used the same concentration of PEPITEM and AQ which has been used for the T1D study, so we also suspect that increasing the dose might restore the inhibitory effect of the PEPITEM pathway.

Adiponectin is very important in respect of the PEPITEM pathway because of its binding to its receptors (AR1 and AR2) on B cells which is essential for PEPITEM production. However, we demonstrated that both AR1 and AR2 surface and gene expression were

unchanged in atherosclerosis patients, and that neither PEPITEM nor AQ inhibit T cell transmigration. At the same time we found a significant decrease in AQ serum levels in atherosclerosis patients. It appeared that the unchanged surface expression and the gene expression of AR1 and AR2 could be a compensation mechanism from the immune system to resist any down regulation of AR1 and AR2 surface expression. The AQ serum level supports this speculation since the serum level is decreased in atherosclerosis patients; meaning that the vasculature protective mechanism of AQ is impaired. When we looked at AQ serum levels on older subjects we found out a significant increase in AQ serum levels which we believe is the compensation mechanism which intervenes again to maintain AQ vasculature protective function.

Another suggestion is to explore the other components of the pathway. S1P receptors expression is a potential target for further investigation but the lack of SIP serum level measurement kit makes it difficult to study the whole pathway. Worse still, the rapid internalization of S1P receptors prevents us from measuring the expression of S1PR1 and S1PR4 on the PBL surface. On account of our assay, we know that AQ, PEPITEM, and CHD-15 are present in the system but due to these technical issues we were unable to investigate SIP and SIPRs behaviour. It is worth mentioning that CHD-15 is expressed on the endothelium we used (DMEC), this information is confirmed by the manufacturer's certificate and previous work in our lab.

In addition to the percentage of transmigration, we showed that the adhesive properties of PBL in atherosclerosis patients are similar to that in healthy subjects. These findings are in contrast with another study which showed the high expression of adhesion molecules VCAM-1 using an in vitro assay, with the authors reporting a higher expression of VCAM-1 on EC when stimulated by TNF-α, interleukin-1 and lipopolysaccharide (Carlos and Harlan, 1994).

Even though it is well known that β-2 is an important adhesion molecule on PBL surface helping to orchestrate its adhesion to EC, we did not notice a significant increase in atherosclerosis patients PBL adhesive behaviour. We showed an increase in β-2 surface expression on some PBL subsets isolated from atherosclerosis patients. Since β-2 interaction is mediated by ICAM-1 on EC, this increase in β-2 on the PBL surface is highly likely to be accompanied by an increase on EC expression of ICAM-1. A result of this should be an increase in the percentage of PBL total adhesion on EC which is something we were unable to demonstrate.

Furthermore, we looked at the impact of old age on the adhesive properties of PBL. There is a well-established relationship between old age and the development of atherosclerosis. We hypothesized that older subjects may more highly express adhesion molecules on their peripheral PBL than younger subjects, and we expected this high expression to be wide and consistent on all PBL subsets. Our data, however, did not support this hypothesis. We therefore considered the affinity and activation state of the adhesion molecule receptors. Research has shown an increase in the number of infiltrating leukocytes to inflamed tissue when there is prolonged expression of chemokine receptors (Scholten et al., 2012). Also, the authors of this study showed that there is a redundancy in the chemokine system because of the fact that a single receptor has multiple binding sites which might lead to differential signalling. This means that the outcome of signalling pathway probably depends not only on ligand-receptor interaction but also on the activation state of the receptors and the presence of signalling proteins.

Transmigrated PBL showed down-regulation of chemokine receptors CCR5, CXCR3, and CX3CR1. To the best of our knowledge in this thesis we revealed for the first time that surface adherent PBL down-regulate chemokine receptors as well, just like transmigrated

PBL. This temporarily down-regulation is necessarily for integrin activation which as a result completes the leukocyte adhesion cascade.

In the case of adhesion molecules receptors after transmigration, we did not see the same down-regulation which was expected, as these molecules keep PBL adhered to the EC. Moreover, we showed that the process of down-regulation of chemokine receptors during transmigration does not change with age or with atherosclerosis.

In respect of the impact of old age on the surface expression of chemokine receptors on PBL subsets, we showed a significant increase in the expression on peripheral PBL subsets of older subjects compared with that of atherosclerosis patients. This was not anticipated because atherosclerosis is associated with an increase of surface expression of chemokine receptors (Blankenberg et al., 2003). Chemokine receptors have been implicated in many chronic diseases including atherosclerosis, allergy, and malaria (Murdoch and Finn, 2000).

This increase could be a sign of the beginning of EC stimulation because the expression of chemokine receptors or chemokines by EC recruits inflammatory cells (Braunersreuther et al., 2007). In addition to leukocyte recruitment, chemokines play a role in platelets activation, something which is considered pro-inflammatory because it secretes inflammatory mediators, one of which is chemokine platelet factor-4. Furthermore, platelets release another chemokine called RANTES which is known to recruit monocytes (Li et al., 1996) (Davenpeck et al., 2000). In our studies we have shown an increase in the expression of both CCR5 and CXCR3 receptors, and the literature has also previously revealed an association between atherosclerosis and the expression of both of these receptors (Warnock et al., 1998). Polymorphism studies have also shown reductions of early MI macrophages when the CCR5 gene is depleted (Hughes and Pfaff, 1998). Our data suggests possible therapeutic

uses of chemokine receptors to cure atherosclerosis. Interestingly TAK-799 (which is considered both a CCR5 and CXCR3 antagonist) has revealed a reduction in disease burden in a carotid artery atherosclerosis model (Chen et al., 1997).

2. Further experiments

Further investigation in some areas is clearly required.

- As mentioned above atherosclerosis is more complicated than other chronic diseases, using higher PEPITEM concentration with atherosclerosis patients in static adhesion assay could restore PEPITEM function.
- One of the limitations of our experiments is that we measured AR1 and AR2 gene expression on PBMCs not B cells. The fact that monocyte subsets highly express ARs (see chapter 1), make our data very general, so measuring ARs gene expression on B-cells using magnetic beads would provide us with accurate and specific results regarding the role of B-cellse in PEPITEM releases.
- Although we used static adhesion assay in this thesis, it would be very interesting to see what difference could be seen when using flow adhesion assay instead of static. Flow adhesion assay enables us to reconstruct leucocyte recruitment cascade in the presence of shear stress which is important in EC adhesion molecules expression.
- Another future experiment which would help us understand the dysfunction in PEPITEM-Adiponectin pathway in atherosclerosis patients is to measure the surface expression of SIPR1 and SIPR4 on transmigrated PBL. We think that SIPRs are not up-regulated in transmigrated PBL which prevents the inhibitory effect of PEPITEM upon T-cells.

APPENDIX

Appendix

✓ List of publications arising from this work:

CHIMEN, M., MCGETTRICK, H. M., APTA, B., KURAVI, S. J., YATES, C. M., KENNEDY, A., ODEDRA, A., **ALASSIRI, M.,** HARRISON, M., MARTIN, A., BARONE, F., NAYAR, S., HITCHCOCK, J. R., CUNNINGHAM, A. F., RAZA, K., FILER, A., COPLAND, D. A., DICK, A. D., ROBINSON, J., KALIA, N., WALKER, L. S. K., BUCKLEY, C. D., NASH, G. B., NARENDRAN, P. & RAINGER, G. E. 2015. **Homeostatic regulation of T cell trafficking by a B cell-derived peptide is impaired in autoimmune and chronic inflammatory disease**. *Nature Medicine,* 21**,** 467-U244.

✓ Conference Abstracts:

1. 26th UK Adhesion Society meeting (21/11/ 2014):

STUDYING THE BEHAVIOUR OF PEPITEM ADIPONECTIN MECHANISM ON LYMPHOCYTE MIGRATION IN ATHEROSCLEROSIS PATIENTS

Mohammed Alassiri, Ed Rainger, Parth Narendran

2. 17th imperial college London symposium: Vascular endothelium; Role in disease pathogenesis & as a therapeutic target (19/11/2015)

STUDYING THE EFFECT OF ATHEROSCLEROSIS ON THE SURFACE EXPRESSION OF INTEGRI AND CHEMOKINE RECEPTORS ON PBL.

Mohammed Alassiri, Ed Rainger, Parth Narendran

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Chapter 4: Studying the effect of age on Chemokine and Integrin recptors expression on PBL subsets.

- **Characterisation of surface expression of adhesion molecules and chemokine receptors on fresh blood PBL, migrated PBL, and surface adherent cells.**

First we started by comparing the difference in the expression of adhesion molecules and integrins on fresh blood PBL, migrated PBL, and surface adherent PBL. Migrated PBL and surface adherent PBL are the PBL after migration assay where PBL placed on stimulated EC. After that, Number of migrated PBL and surface adherent PBL was calculated and finally the expression of integrins and adhesion molecules have been analysed to see which molecules have been utilised during migration process.

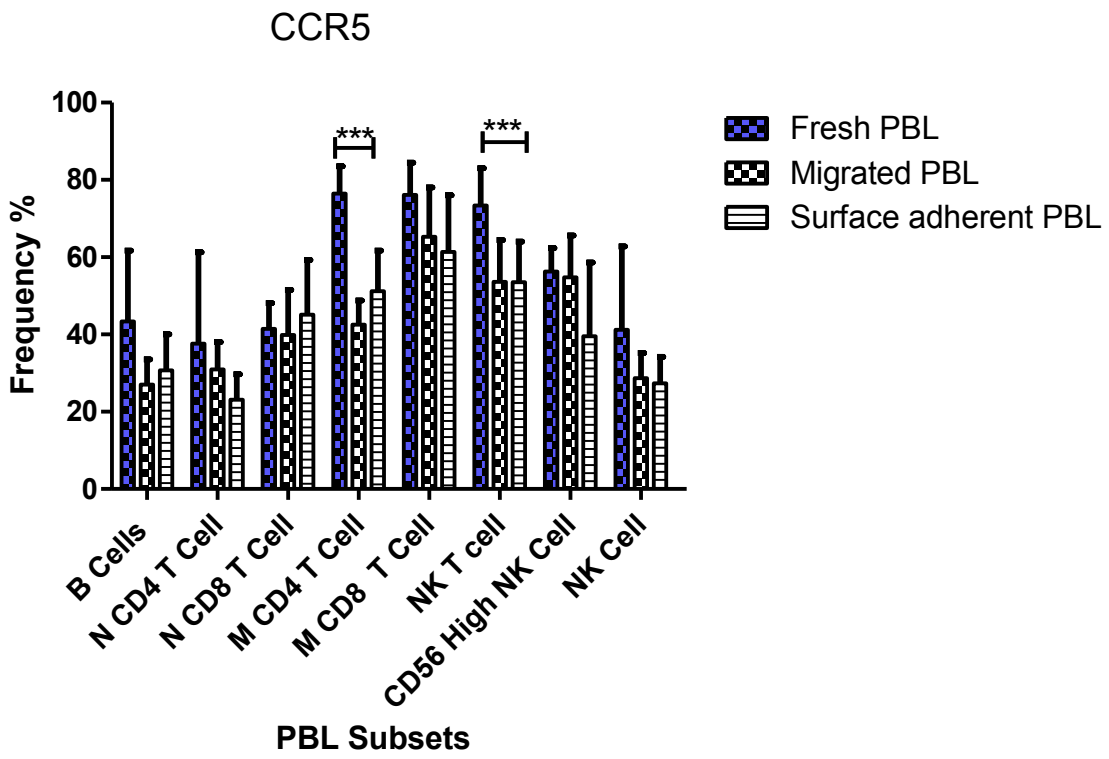

Figure 0-1: **Frequency of CCR5 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).**

Flow cytometry was used to measure the frequency of CCR5 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

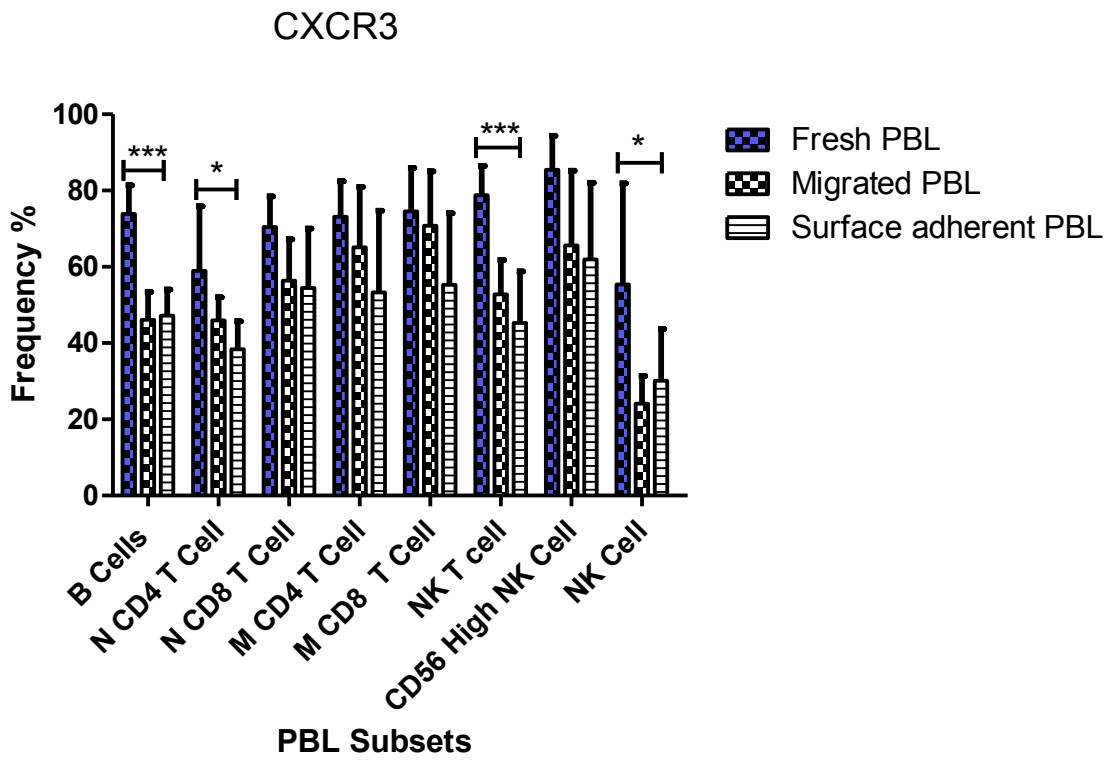

Figure 0-2: **Frequency of CXCR3 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).**

Flow cytometry was used to measure the frequency of CXCR3receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

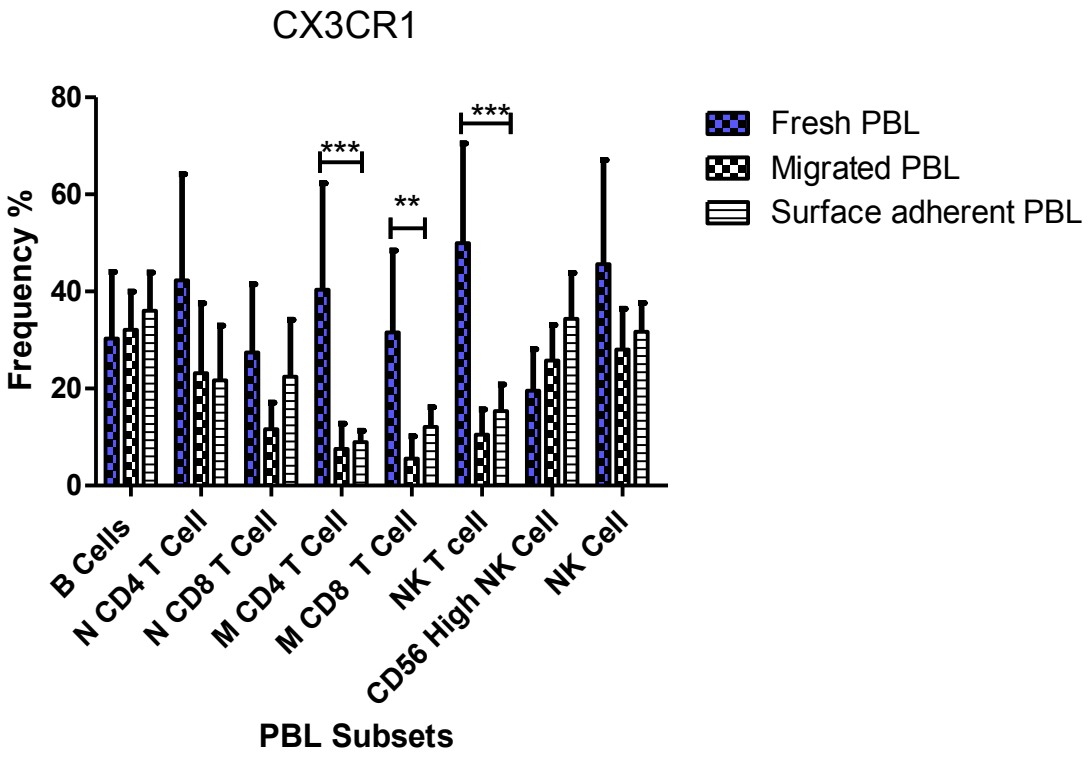

Figure 0-3: **Frequency of CX3CR1 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).**

Flow cytometry was used to measure the frequency of CX3CR1 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

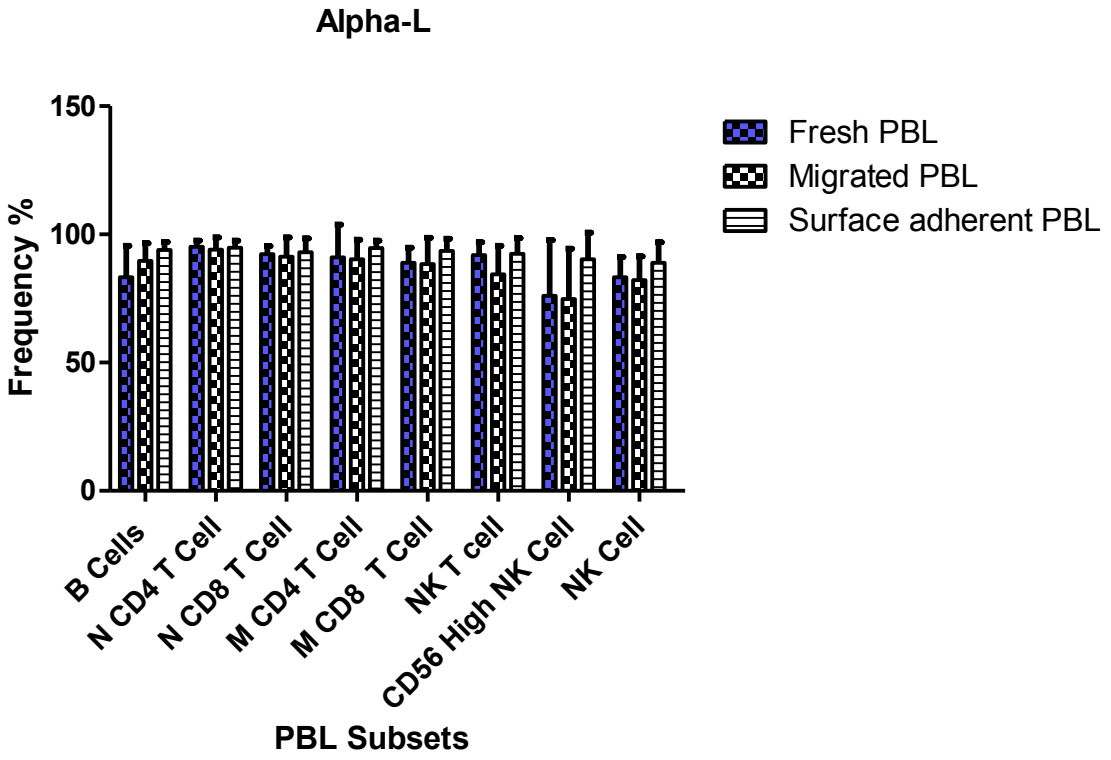

Figure 0-4: **Frequency of Alpha-L receptor on different subsets of PBL isolated from healthy donors (20-75 years old).**

Flow cytometry was used to measure the frequency of Alpha-L receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. Alpha-L receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

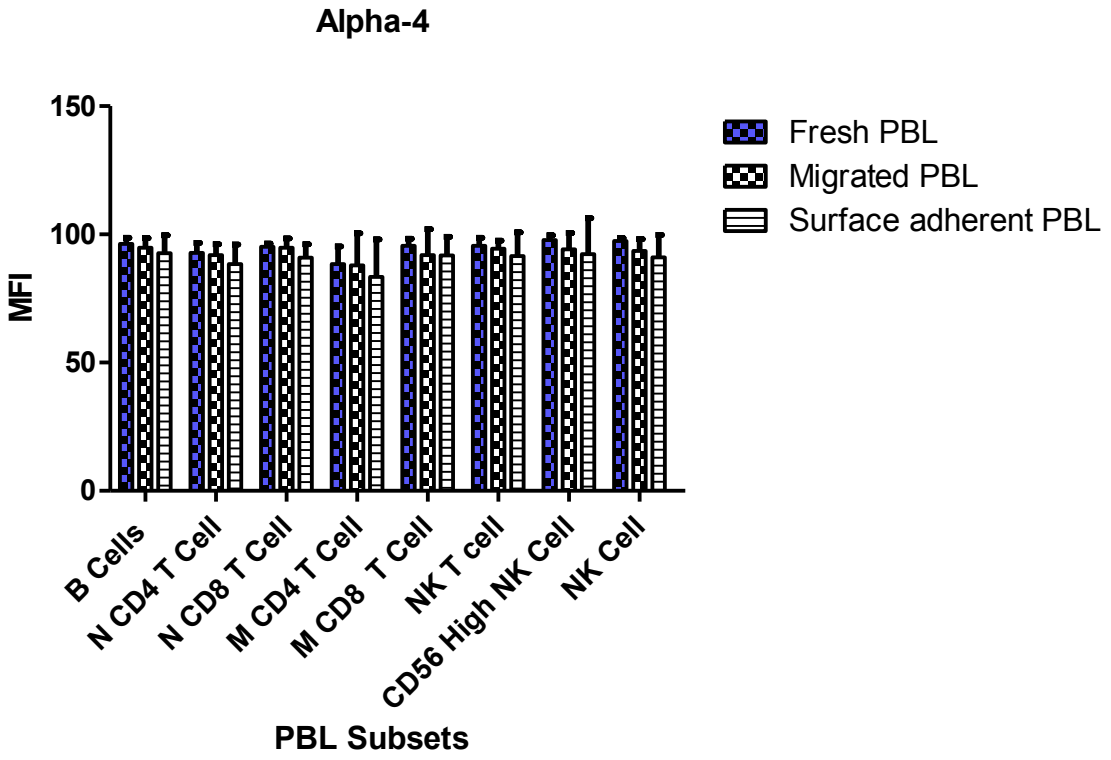

Figure 0-5: **Frequency of Alpha-4 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).**

Flow cytometry was used to measure the frequency of Alpha-4 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. Alpha-4 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$

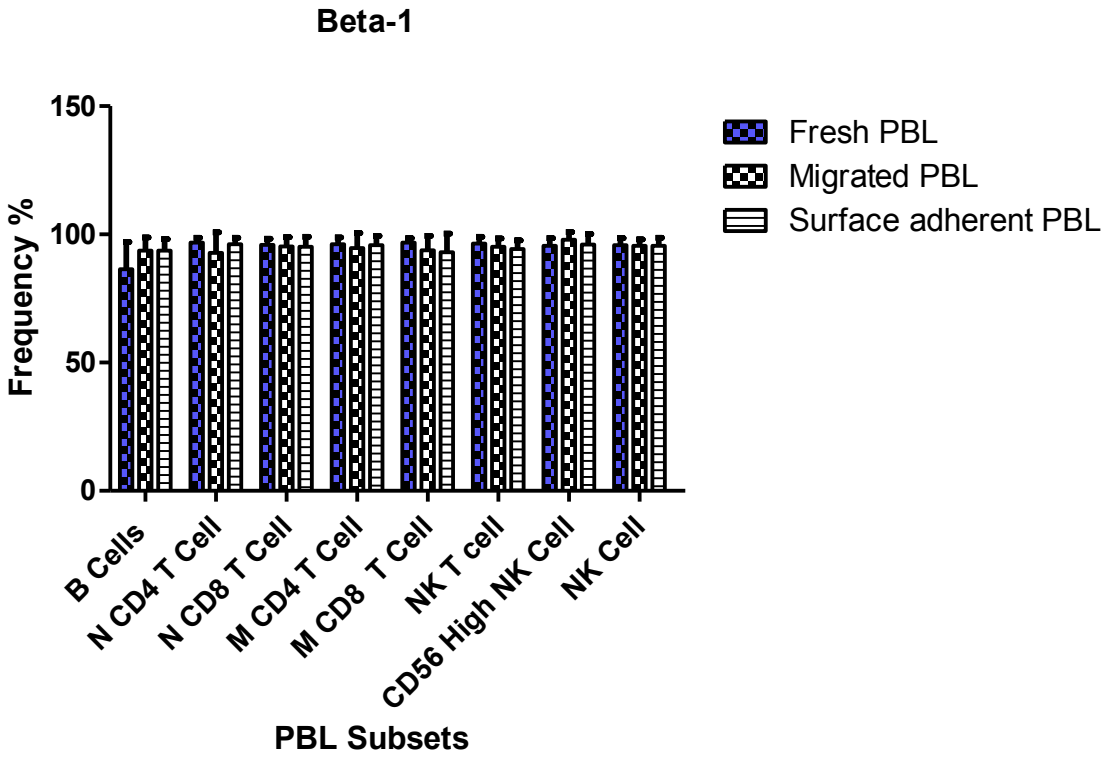

Figure 0-6: **Frequency of Beta-1 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).**

Flow cytometry was used to measure the frequency of Beta-1 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

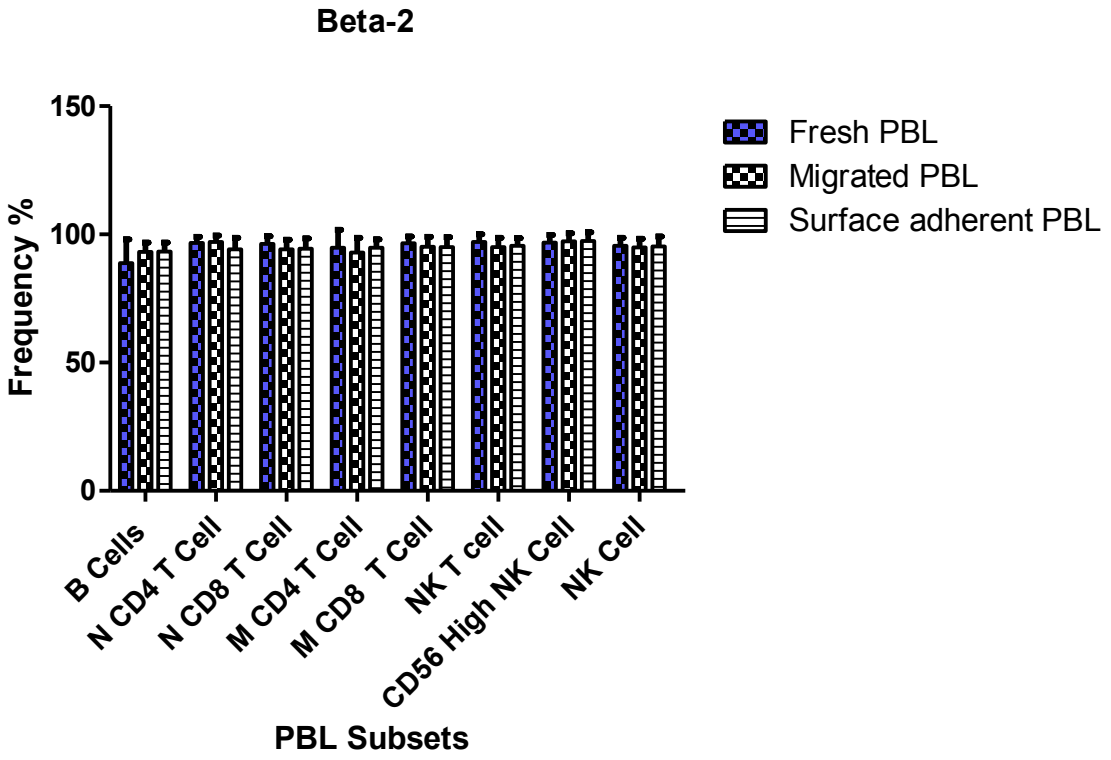

Figure 0-7: **Frequency of Beta-2 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).**

Flow cytometry was used to measure the frequency of Beta-2 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. Beta-2 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

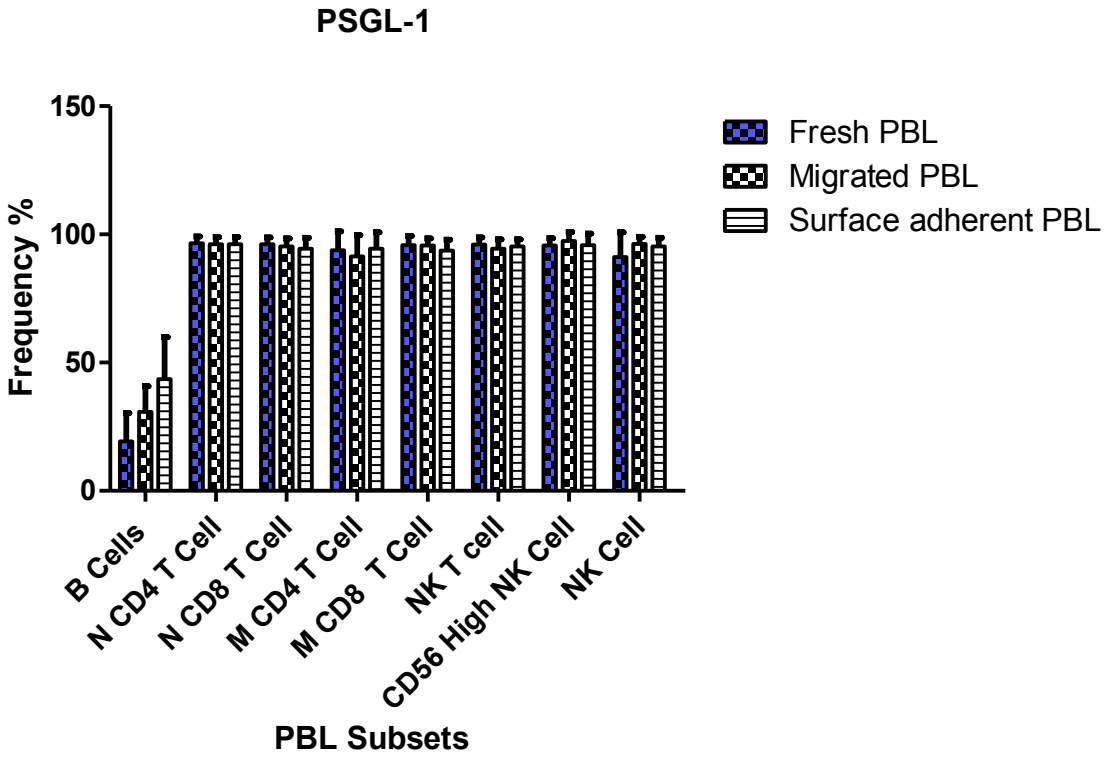

Figure 0-8: **Frequency of PSGL-1 receptor on different subsets of PBL isolated from healthy donors (20-75 years old).**

Flow cytometry was used to measure the frequency of PSGL-1 receptor; PBL was isolated from fresh blood samples, stained with same panel of antibodies, and gated on their forward/side scatter profile. Surface adherent PBL is collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. Finally, The PBL was stained with same panel. Regarding migrated PBL, they are PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same panel of antibodies. Direct conjugated antibodies were used for staining. PSGL-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=12) and were analysed using one way Anova, with a Bonferroni post hoc test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression of adhesion molecule receptors on fresh blood PBL from young or old controls:**

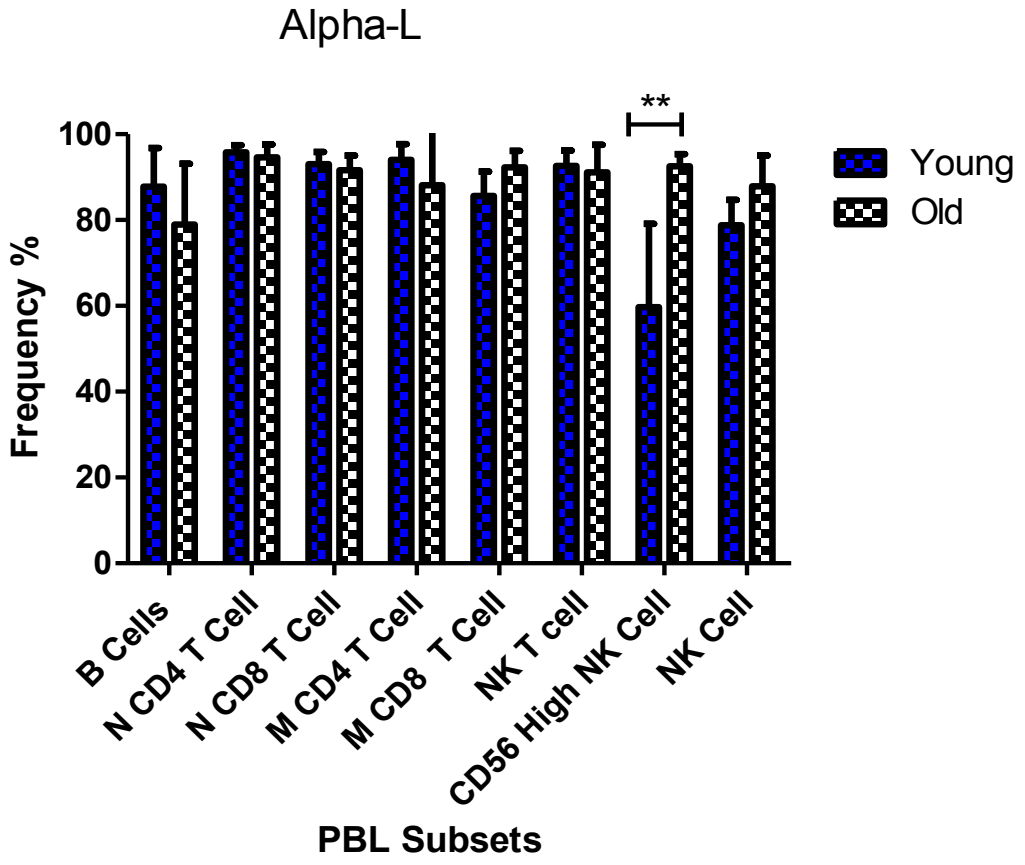

Figure 0-9: **Frequency of Alpha-L receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Alpha-L receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha L receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

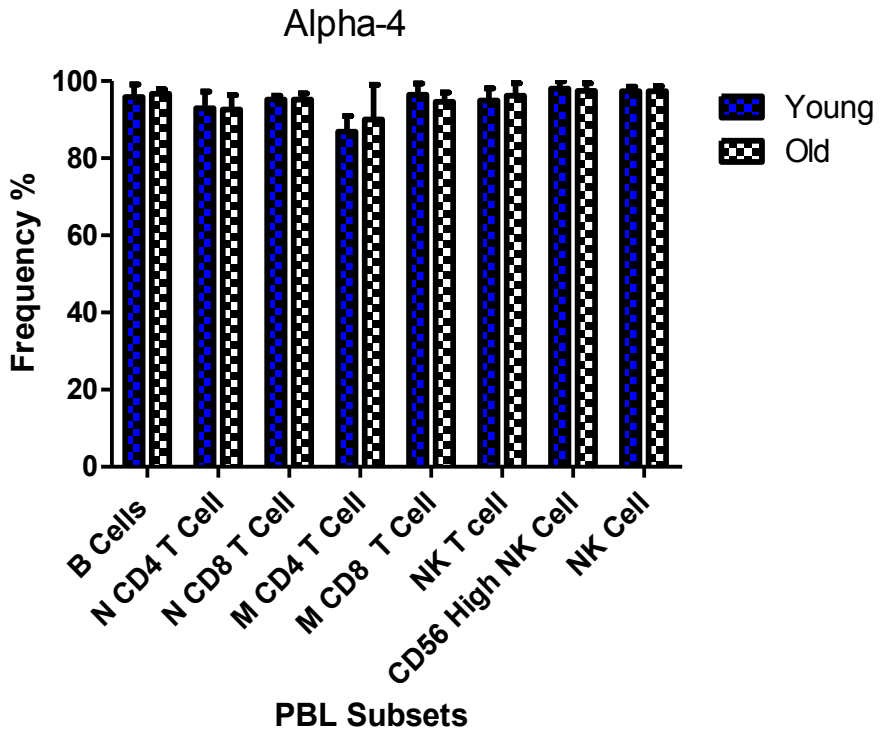

Figure 0-10: **Frequency of Alpha-4 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Alpha-4 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha 4 receptor expressions was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

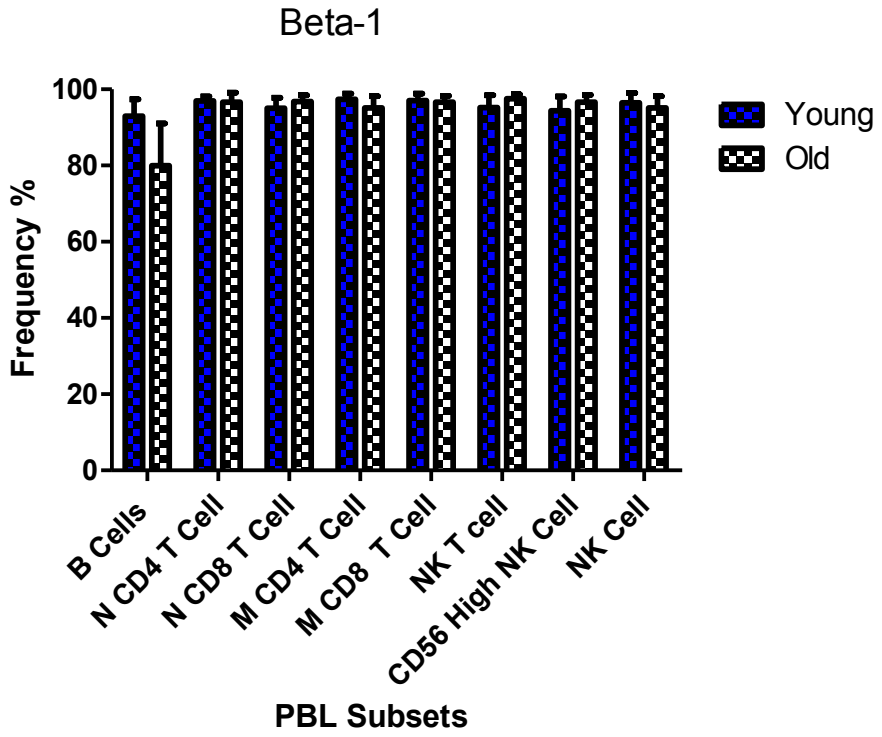

Figure 0-11: **Frequency of Beta-1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Beta-1 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-1 receptor expressions were measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

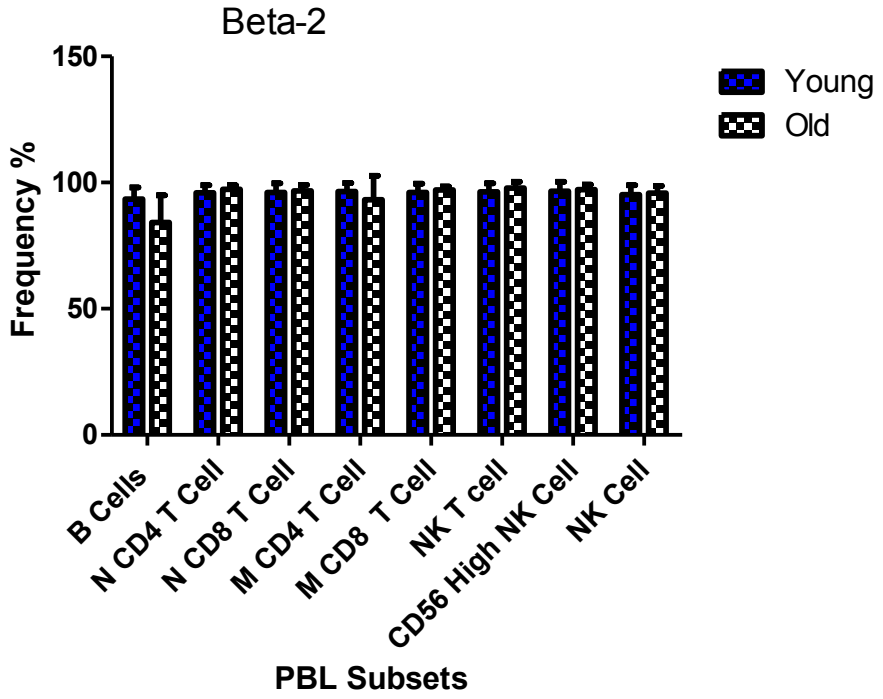

Figure 0-12: **Frequency of Beta-2 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Beta-2 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-2 receptor expressions were measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

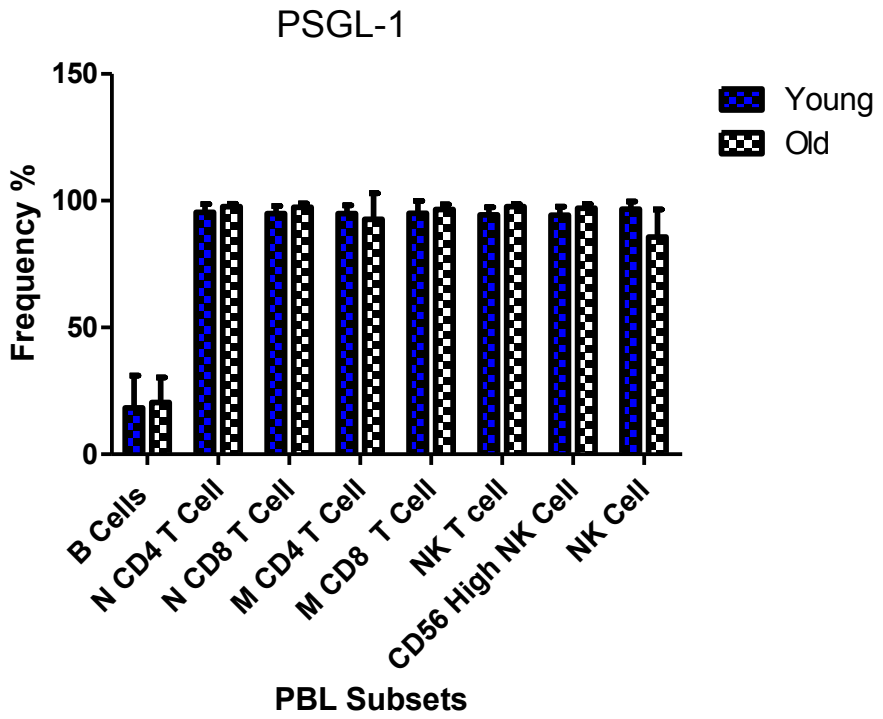

Figure 0-13: **Frequency of PSGL-1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of PSGL-1 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. PSGL-1 receptor expressions were measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression of chemokine receptors on fresh blood PBL from young or old controls:**

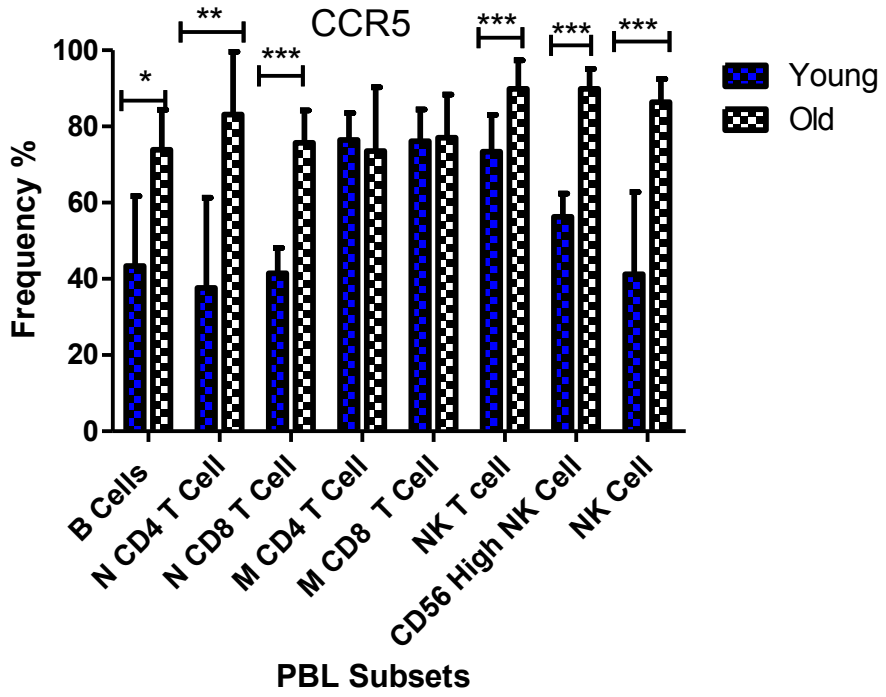

Figure 0-14: **Frequency of CCR5 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of CCR5 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CCR5 receptor expressions were measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

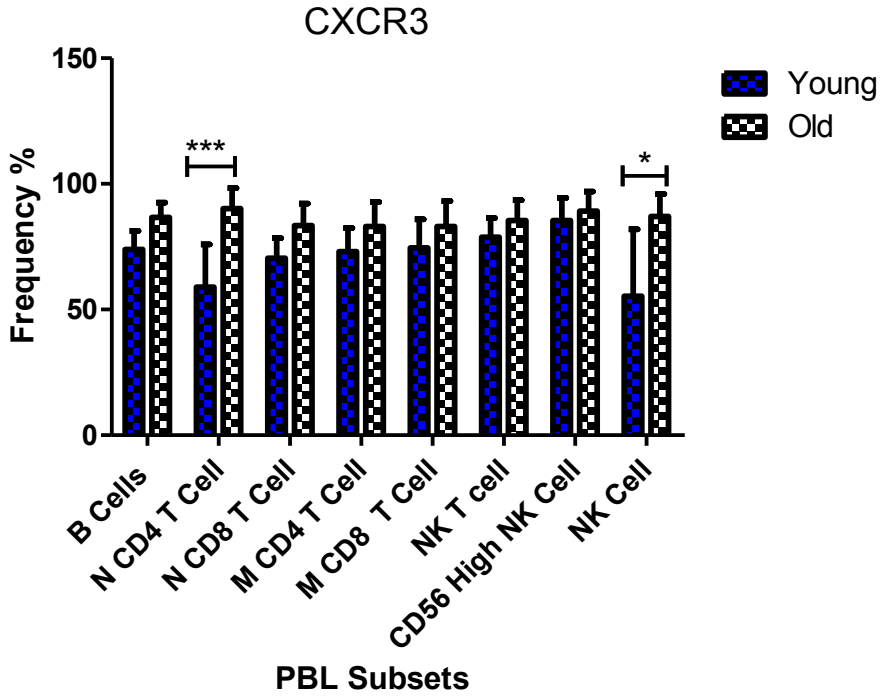

Figure 0-15: **Frequency of CXCR3 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of CXCR3 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expressions were measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

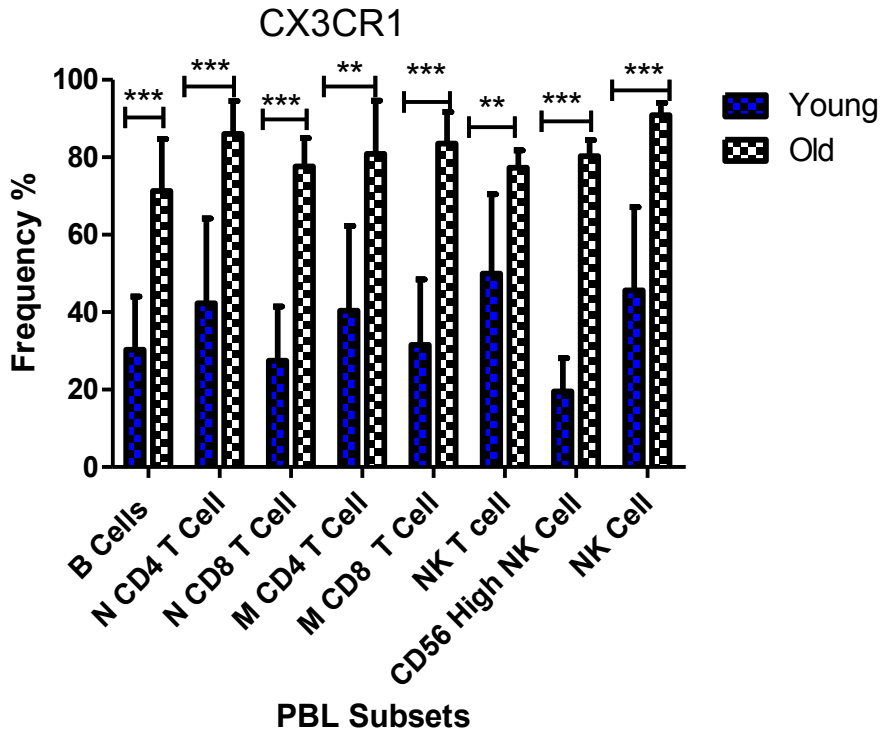

Figure 0-16: **Frequency of CX3CR1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of CX3CR1 receptor on fresh blood PBL subsets; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched for sex, ethnicity, and BMI, Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expressions were measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression of adhesion molecule receptors on migrated PBL from young or old controls:**

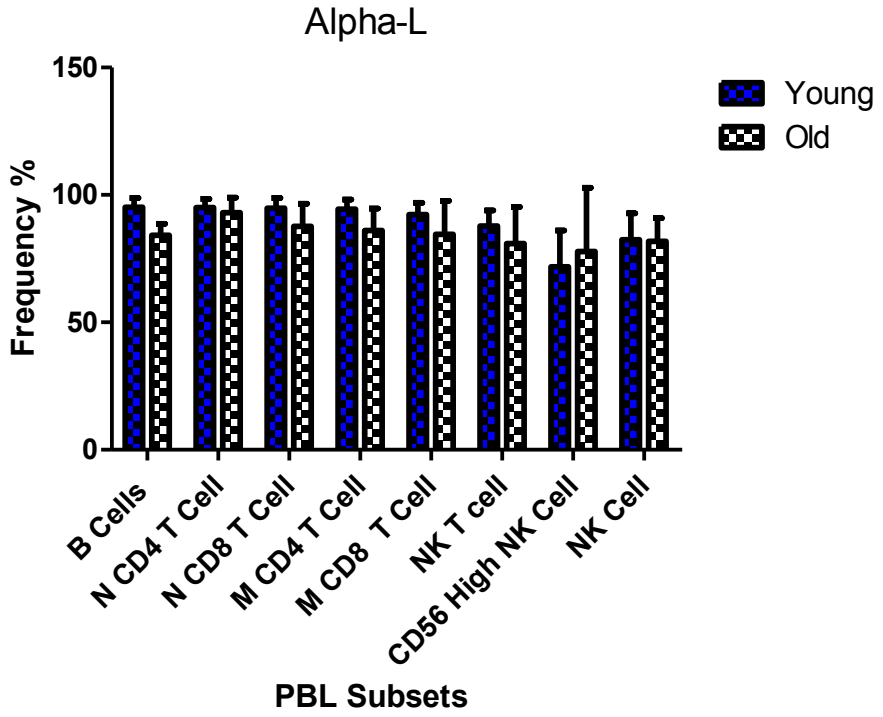

Figure 0-17: **Frequency of Alpha-L receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Alpha-L; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-L receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

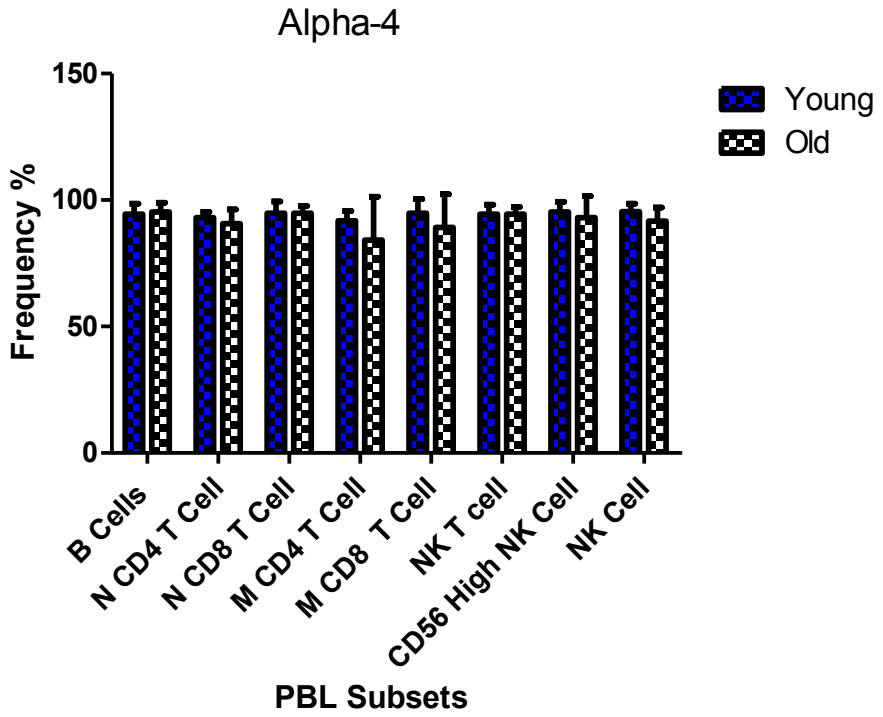

Figure 0-18: **Frequency of Alpha-4 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Alpha-4; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-4 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

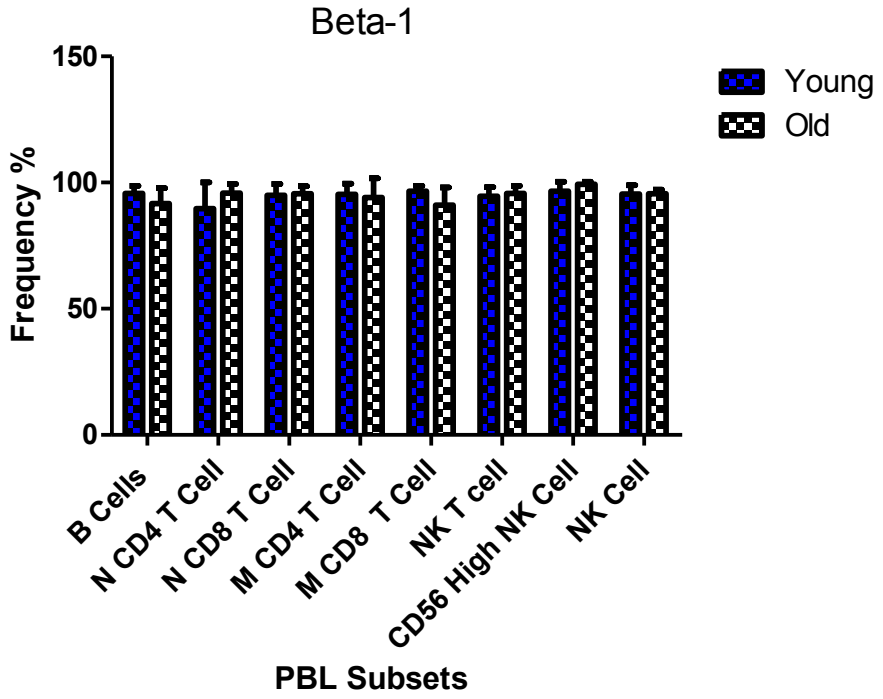

Figure 19: **Frequency of Beta-1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Beta-1; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

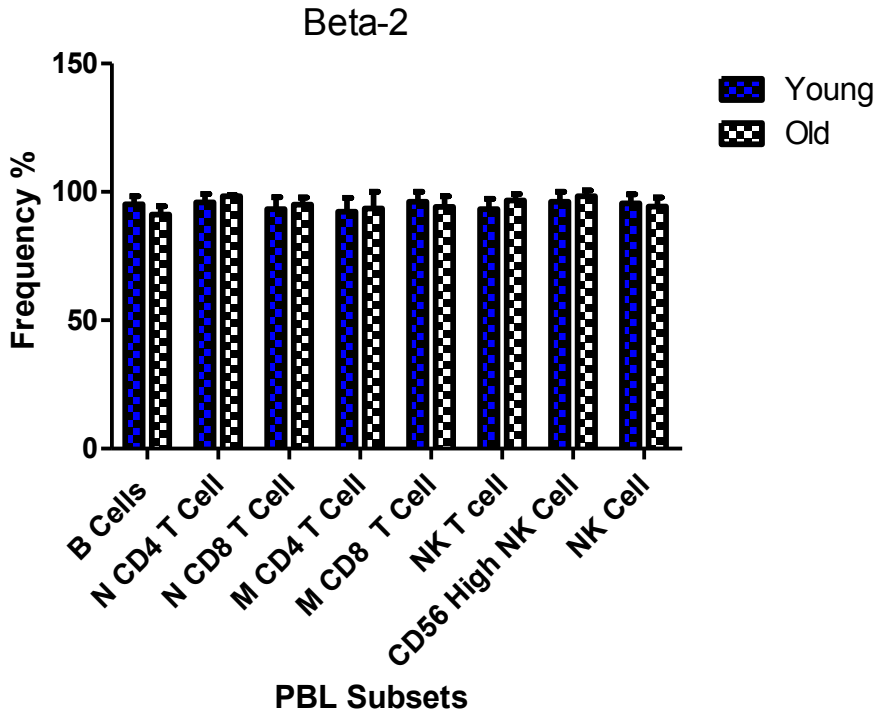

Figure 0-19: **Frequency of Beta-2 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Beta-2; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining.Beta-2 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

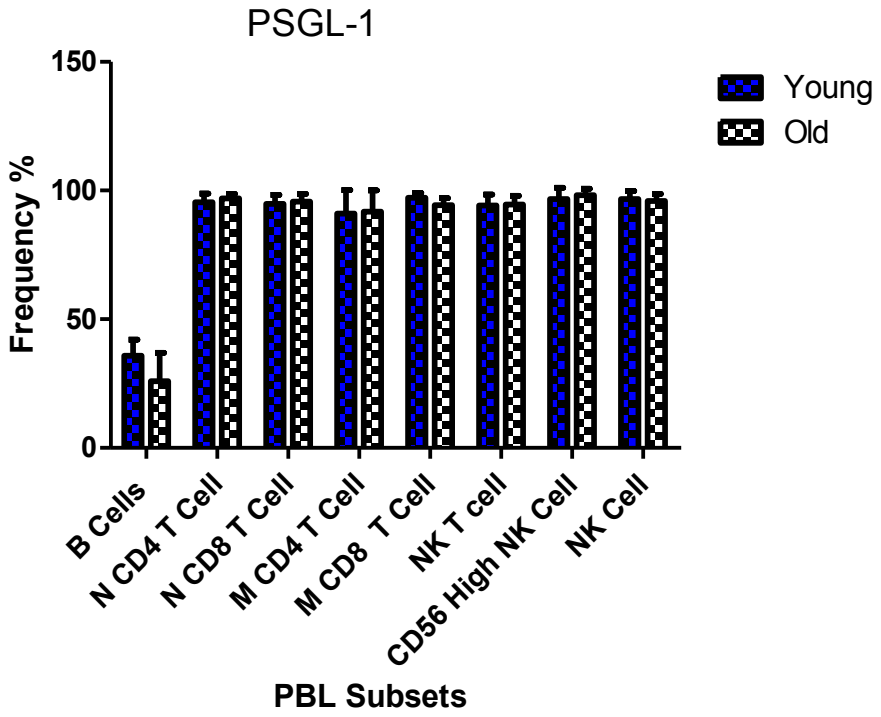

Figure 0-20: **Frequency of PSGL-1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of PSGL-1; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. PSGL-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression of chemokine receptors on migrated PBL from young or old controls:**

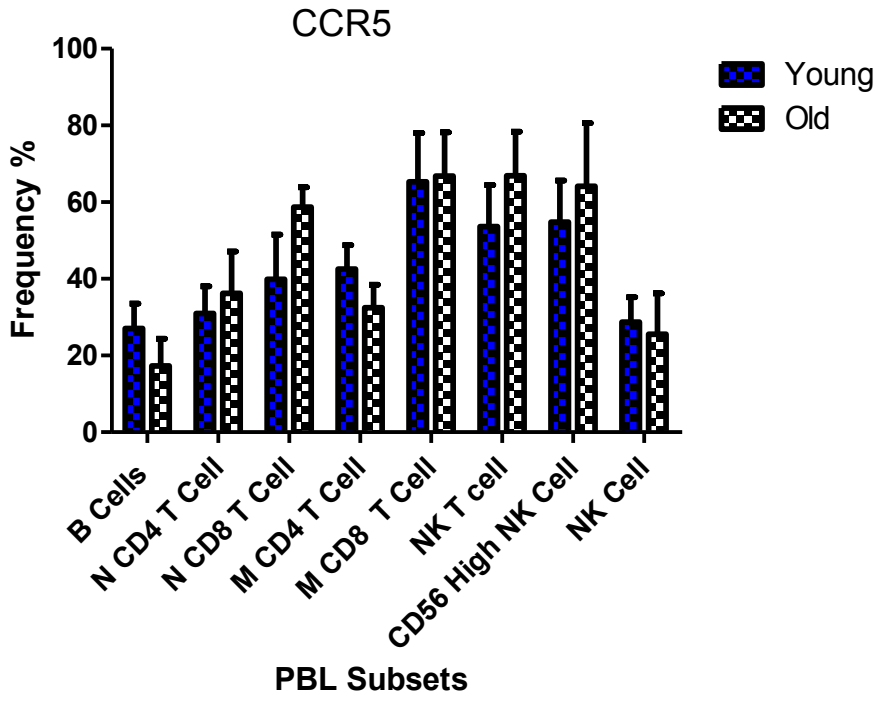

Figure 0-21: **Frequency of CCR5 receptor on different subsets of migrated PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of CCR5; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

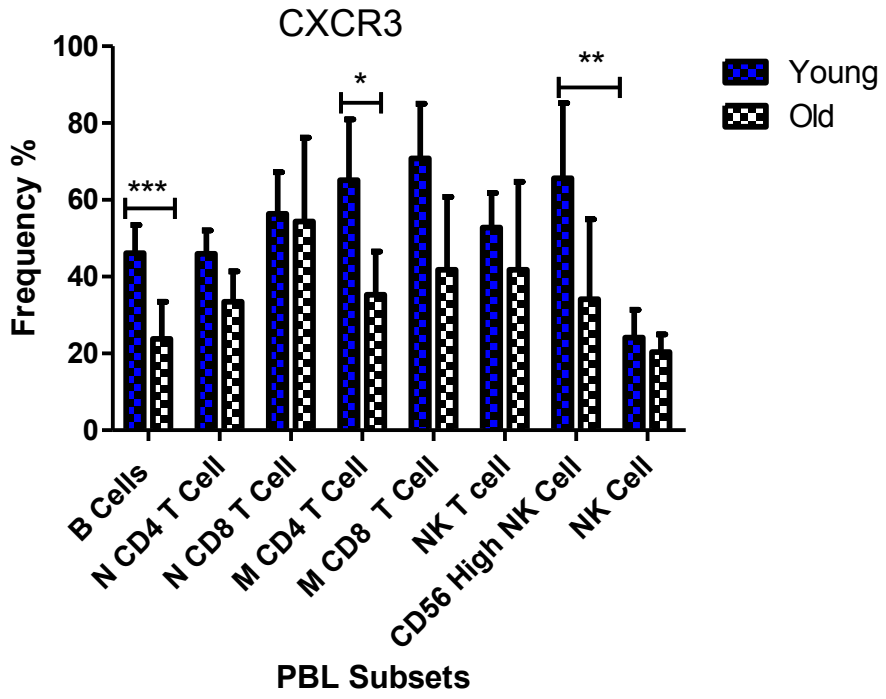

Figure 0-22: **Frequency of CXCR3 receptor on different subsets of migrated PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of CXCR3; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

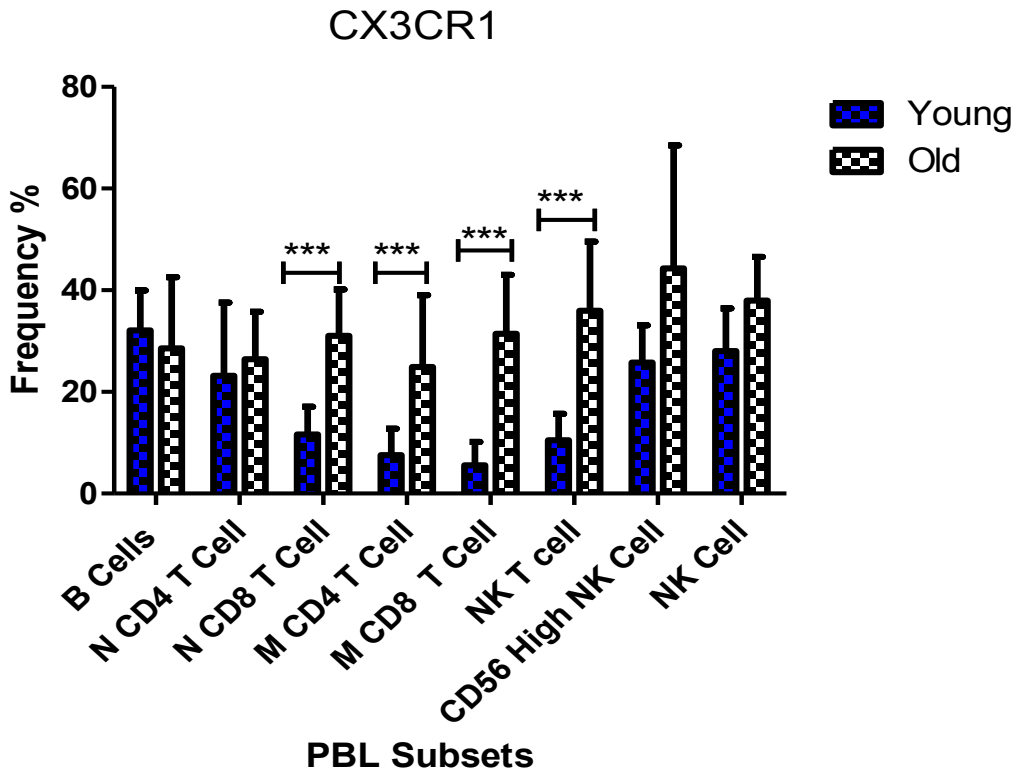

Figure 0-23: **Frequency of CX3CR1 receptor on different subsets of migrated PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of CX3CR1; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression of adhesion molecule receptors on surface adherent PBL from young or old controls:**

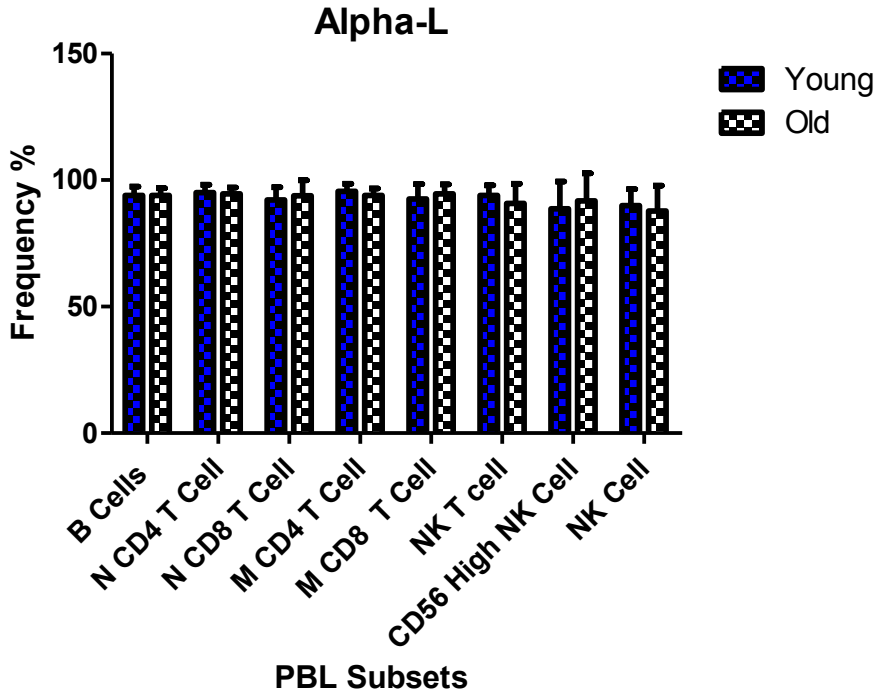

Figure 0-24: **Frequency of Alpha-L receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Alpha-L; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-L receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

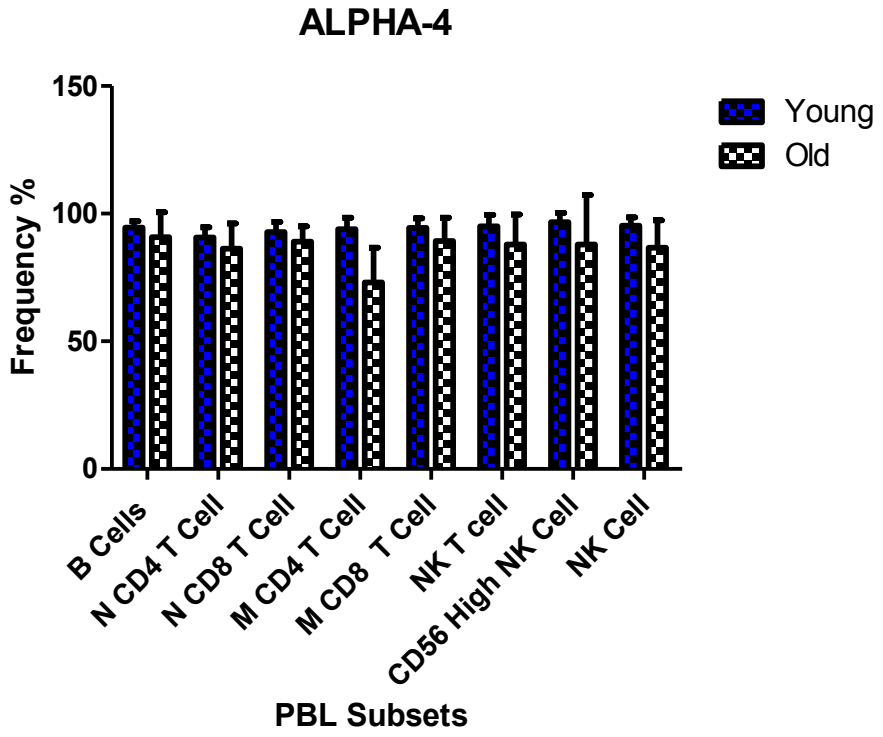

Figure 0-25: **Frequency of Alpha-4 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Alpha-4; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-4 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

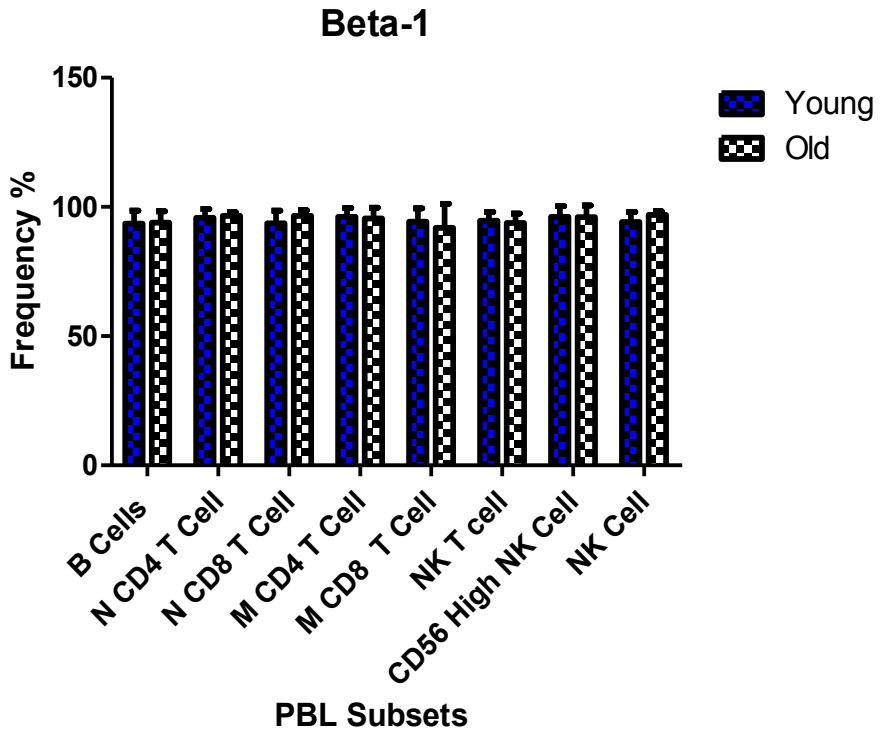

Figure 0-26: **Frequency of Beta-1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Beta-1; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$.

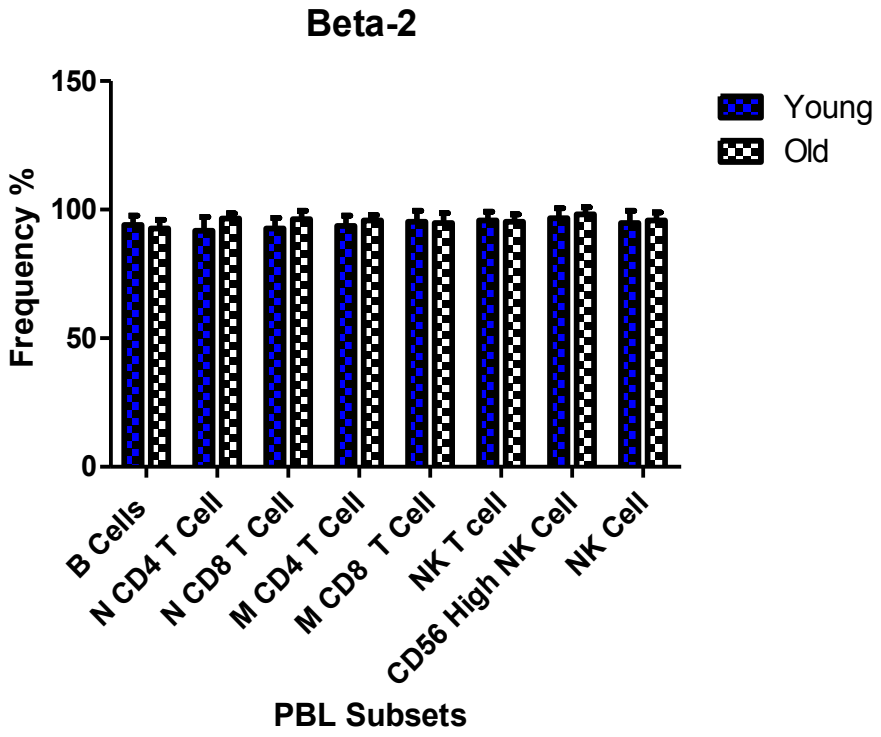

Figure 28: **Frequency of Beta-2 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of Beta-2; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-2 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

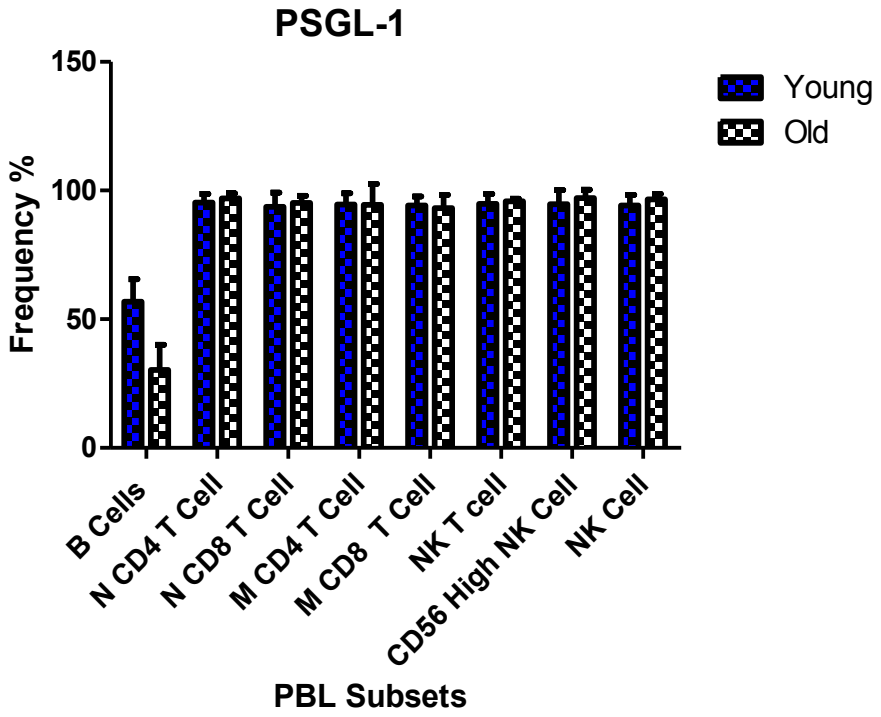

Figure 0-27: **Frequency of PSGL-1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of PSGL-1; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. PSGL-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression of chemokine receptors on surface adherent PBL from young or old controls:**

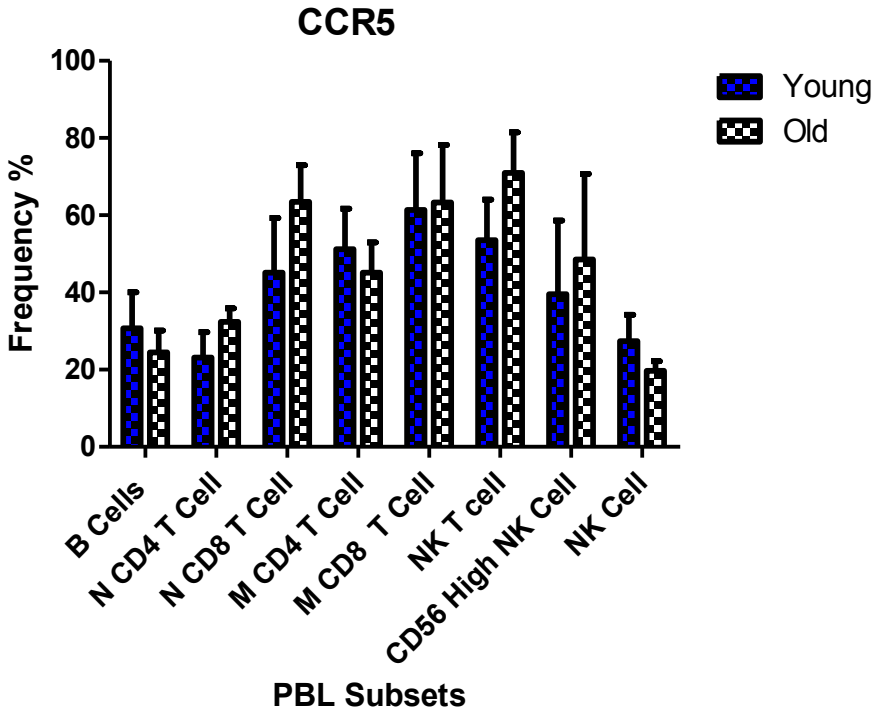

Figure 0-28: **Frequency of CCR5 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of CCR5 receptor; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

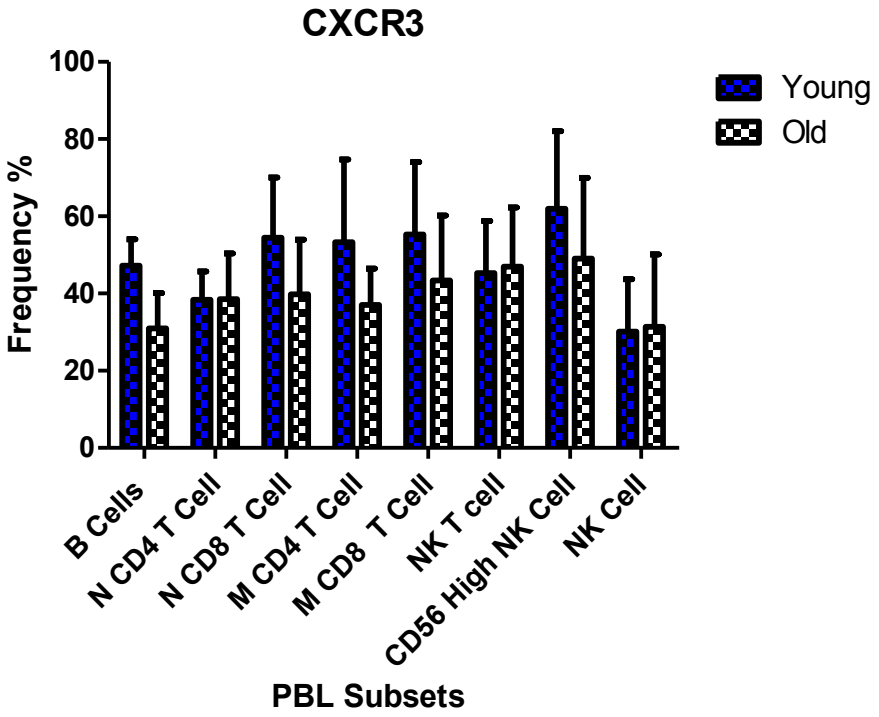

Figure 0-29: **Frequency of CXCR3 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of CXCR3; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

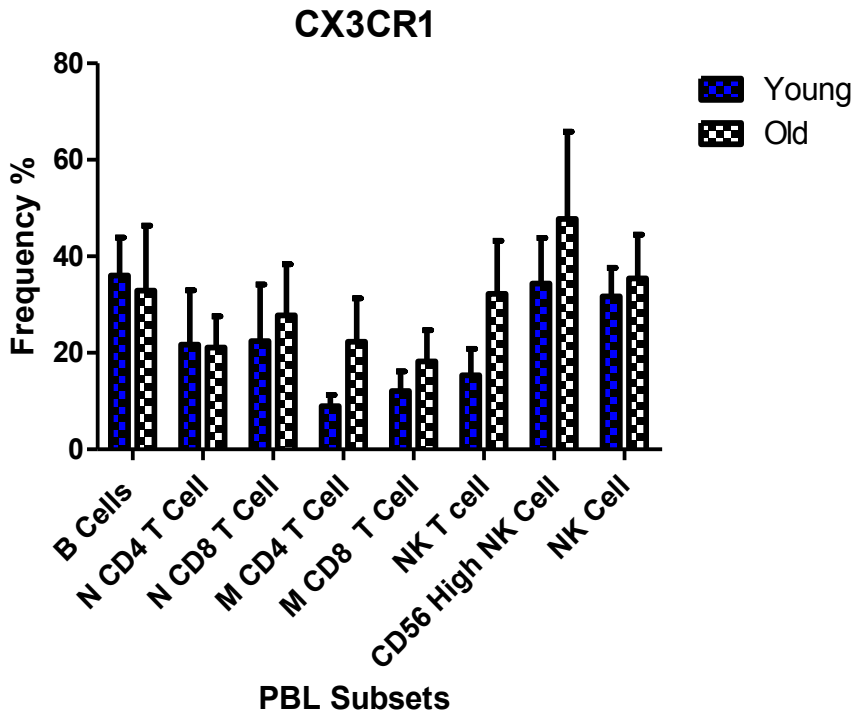

Figure 32: **Frequency of CX3CR1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the frequency of CX3CR1; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression (MFI) of adhesion molecules on surface adherent PBL from young or old controls:**

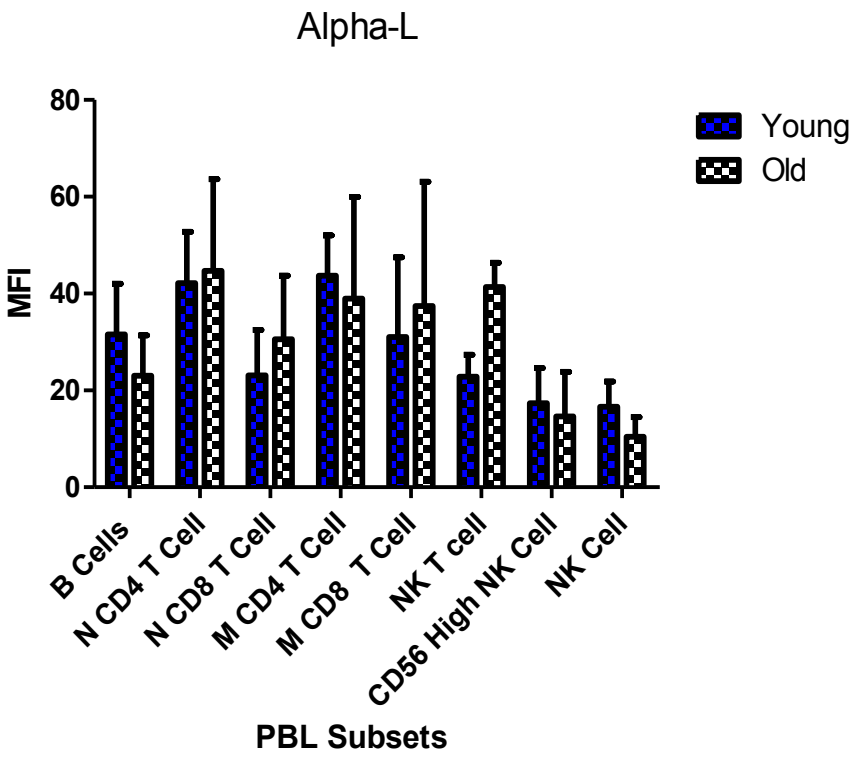

Figure 0-30: **MFI of Alpha-L receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the MFI of Alpha-L; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-L receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

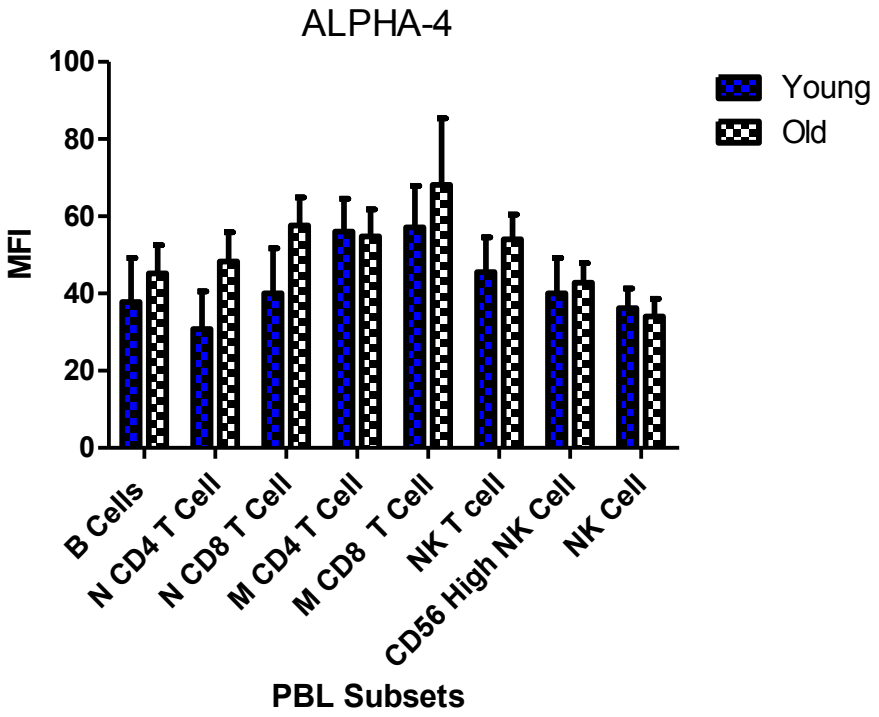

Figure 0-31: **MFI of Alpha-4 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the MFI of Alpha-4; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-4 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

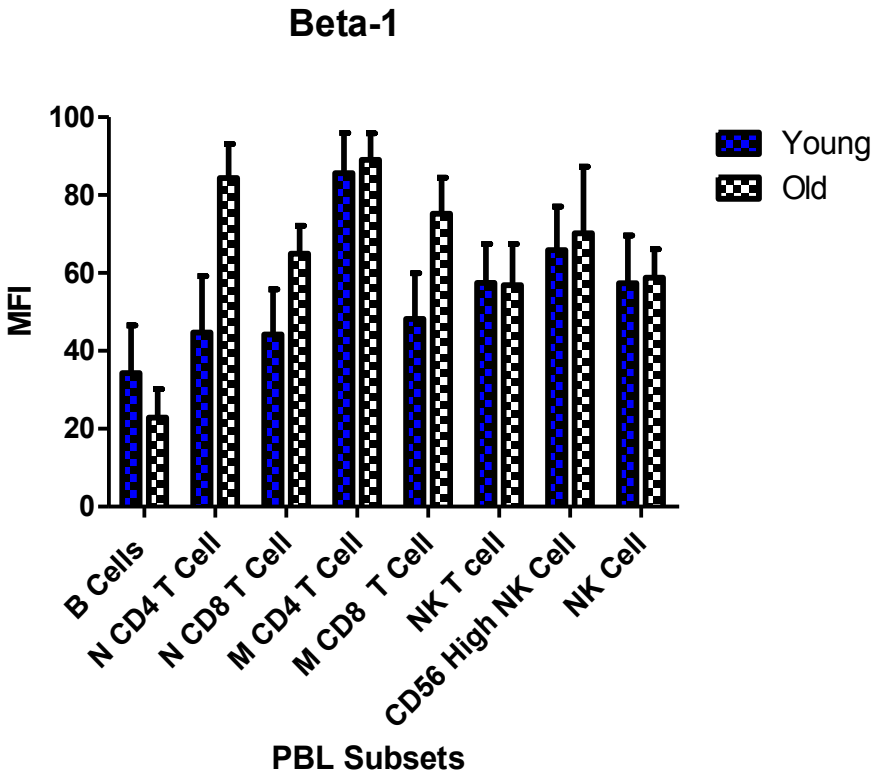

Figure 0-32: **MFI of Beta-1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the MFI of Beta-1; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$.

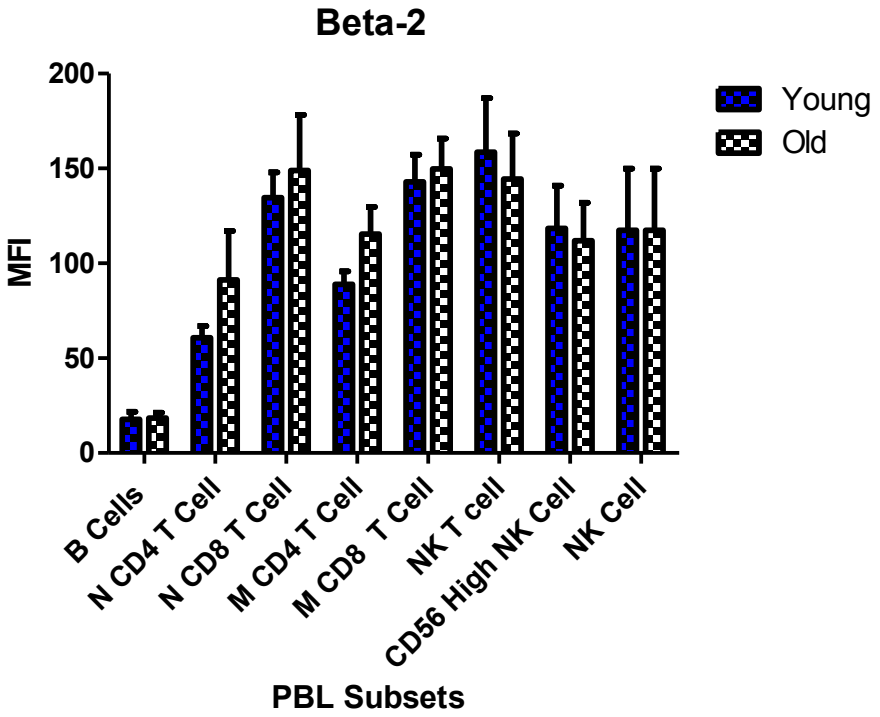

Figure 0-33: **MFI of Beta-2 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the MFI of Beta-2; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$.

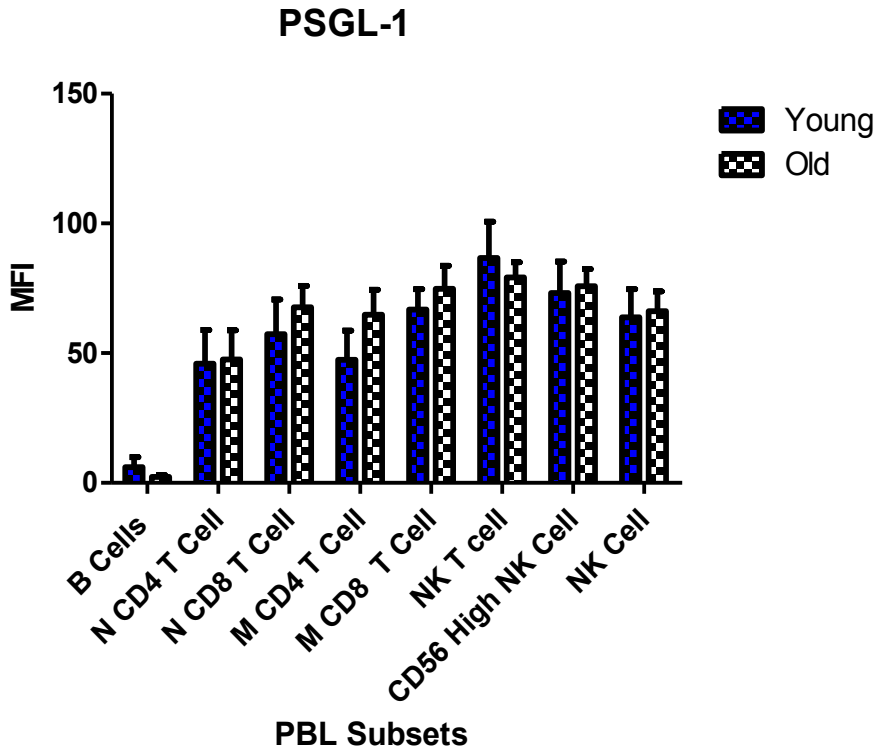

Figure 0-34: **MFI of PSGL-1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the MFI of PSGL-1; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. PSGL-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression (MFI) of chemokine receptors on surface adherent PBL from young or old controls:**

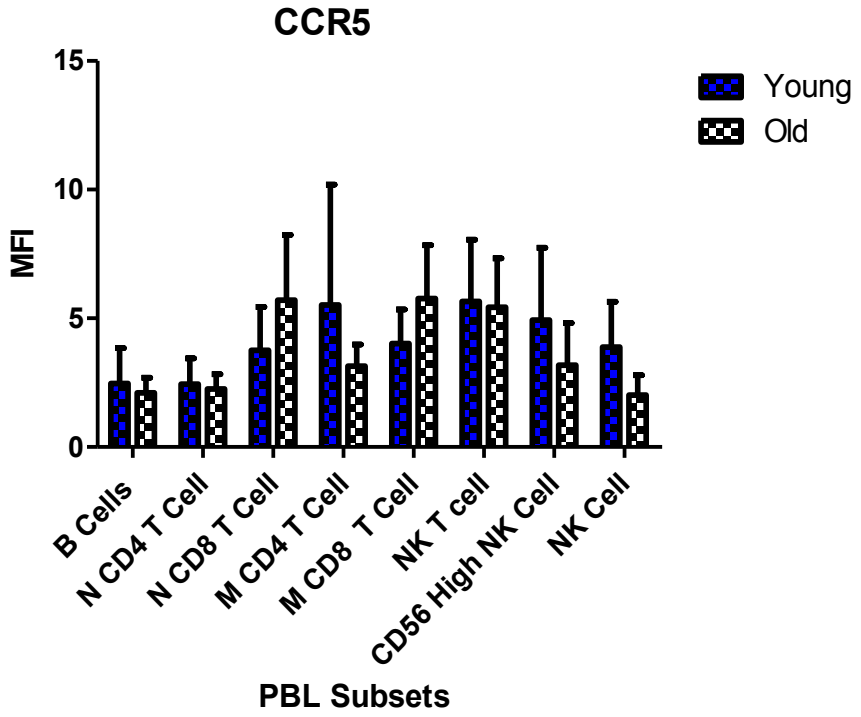

Figure 0-35: **MFI CCR5 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the MFI of CCR5 receptor; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

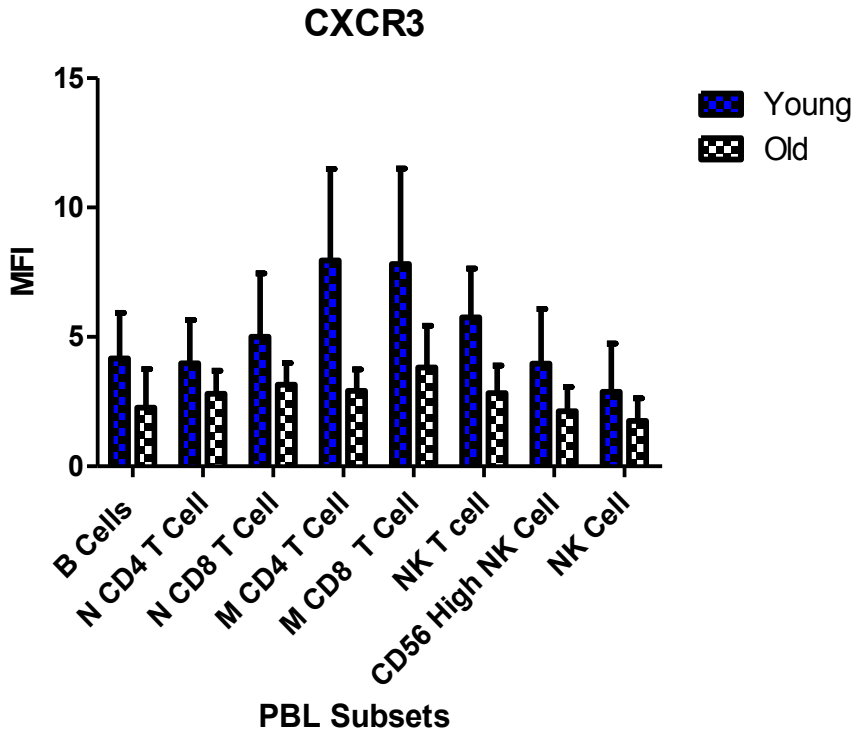

Figure 0-36: **MFI of CXCR3 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the MFI of CXCR3; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

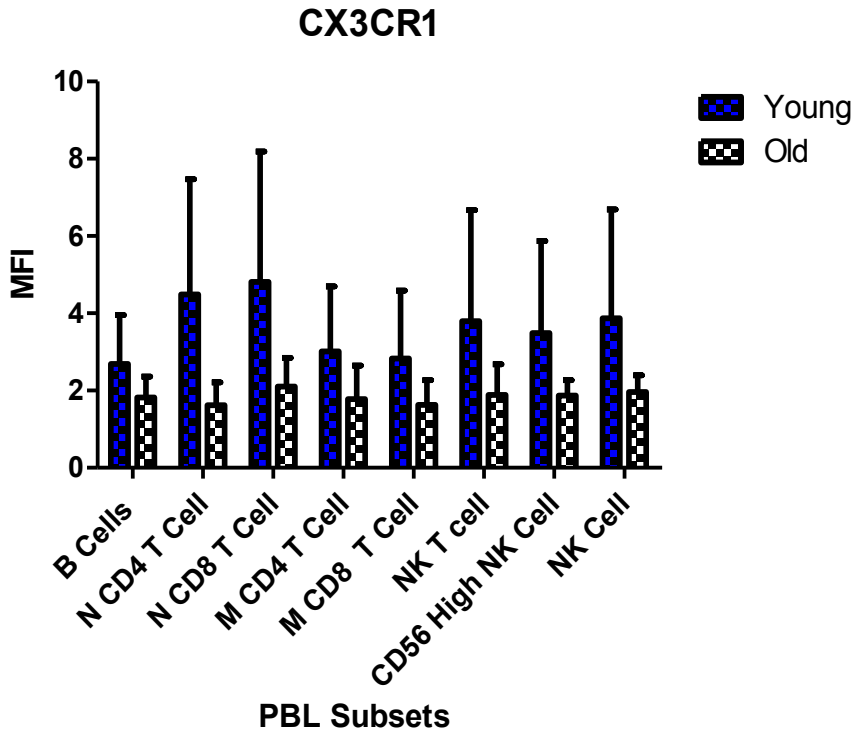

Figure 0-37: **MFI of CX3CR1 receptor on different subsets of PBL isolated from healthy donors.**

Flow cytometry was used to measure the MFI of CX3CR1; Fresh blood was collected from young or old subjects. Young subjects aged from 20-35 whereas old subjects aged 60-75 years old. Subjects were matched sex, ethnicity, and BMI. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, Then, using Phosphate-buffered saline (PBS) surface adherent The PBL was collected gently using pipette. The PBL was collected and stained withe same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

CHAPTER 6: STUDYING THE EFFECT OF ATHEROSCLEROSIS ON CHEMOKINE AND INTEGRIN RECEPTORS EXPRESSION ON PBL SUBSETS:

- **surface expression of adhesion molecules receptors on PBL from patients with atherosclerosis or age matched controls:**

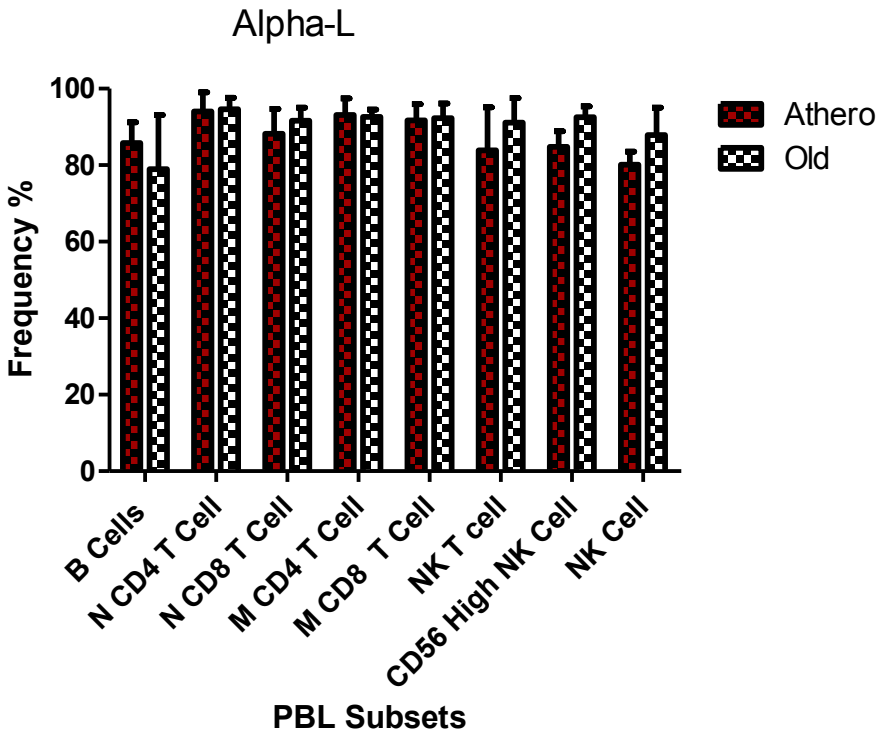

Figure 41: **Frquency of Alpha-L receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the MFI of Alpha-L receptor on fresh blood PBL subsets; Fresh blood was collected from from atherosclerosis patients or age match control subjects. Young subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$)

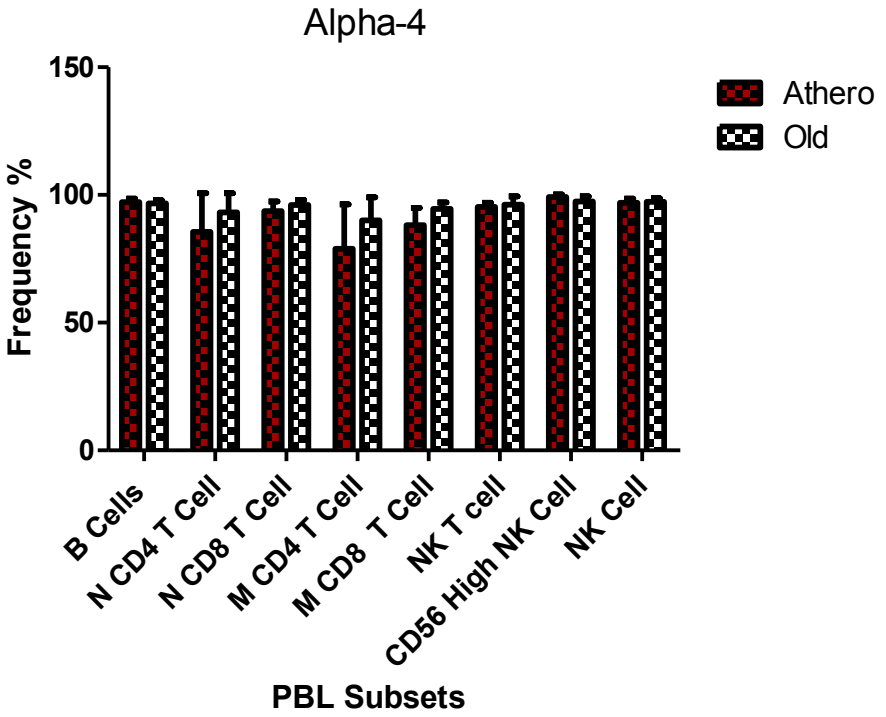

Figure 42: Frequency **of Alpha-4 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the MFI of Alpha-4 receptor on fresh blood PBL subsets; Fresh blood was collected from from atherosclerosis patients or age match control subjects. Young subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-4 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

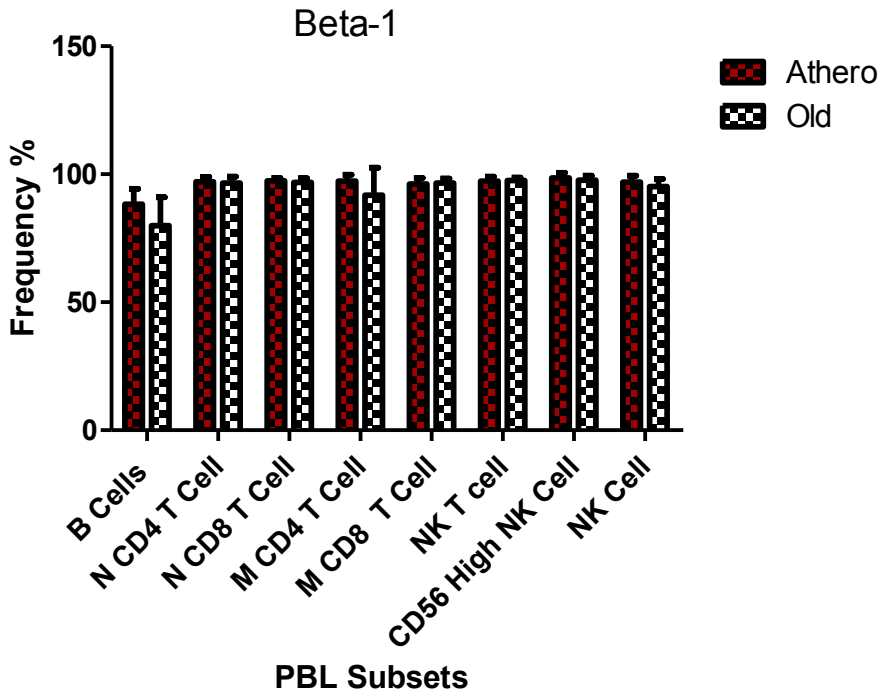

Figure 43: **Frequency of Beta-1 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the frequency of Beta-1 receptor on fresh blood PBL subsets; Fresh blood was collected from from atherosclerosis patients or age match control subjects. Young subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

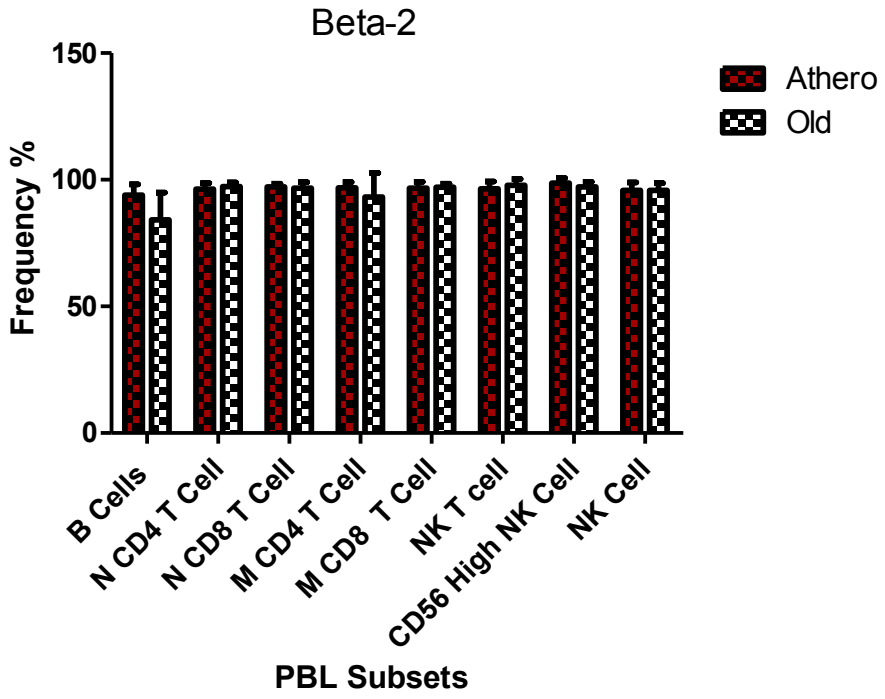

Figure 44: **Frequency of Beta-2 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the frequency of Beta-2 receptor on fresh blood PBL subsets; Fresh blood was collected from from atherosclerosis patients or age match control subjects. Young subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-2 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

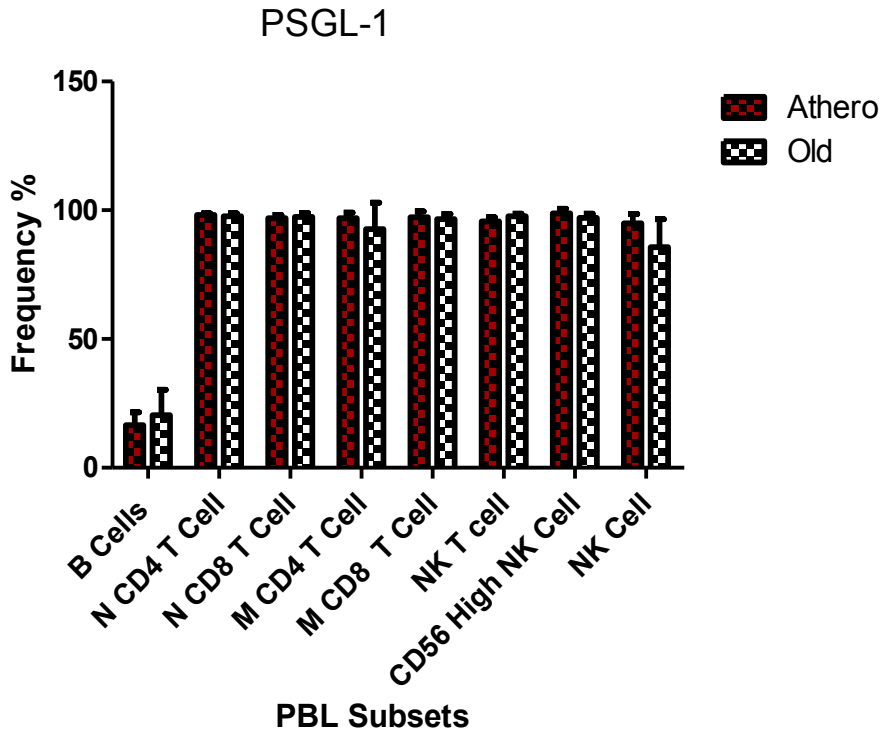

Figure 45: **Frequency of PSGL-1 receptor on different subsets of PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the frequency of PSGL-1 receptor on fresh blood PBL subsets; Fresh blood was collected from from atherosclerosis patients or age match control subjects. Young subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL was isolated from fresh blood samples, stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. PSGL-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression of chemokine receptors on migrated PBL from patients atherosclerosis or age matched controls:**

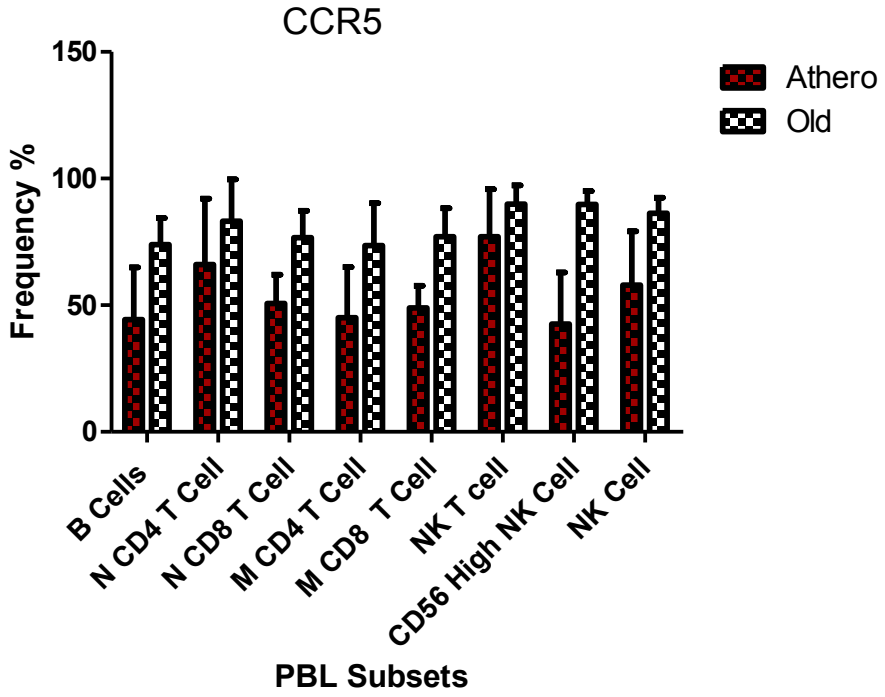

Figure 46: **Frequency of CCR5 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of CCR5 receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

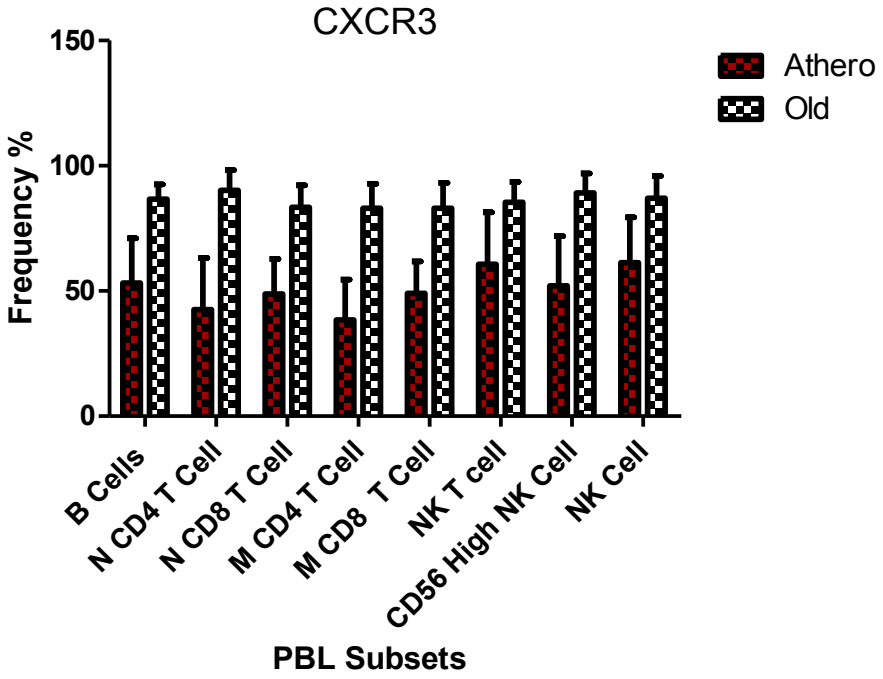

Figure 47: **Frequency of CXCR3 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of CXCR3 receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

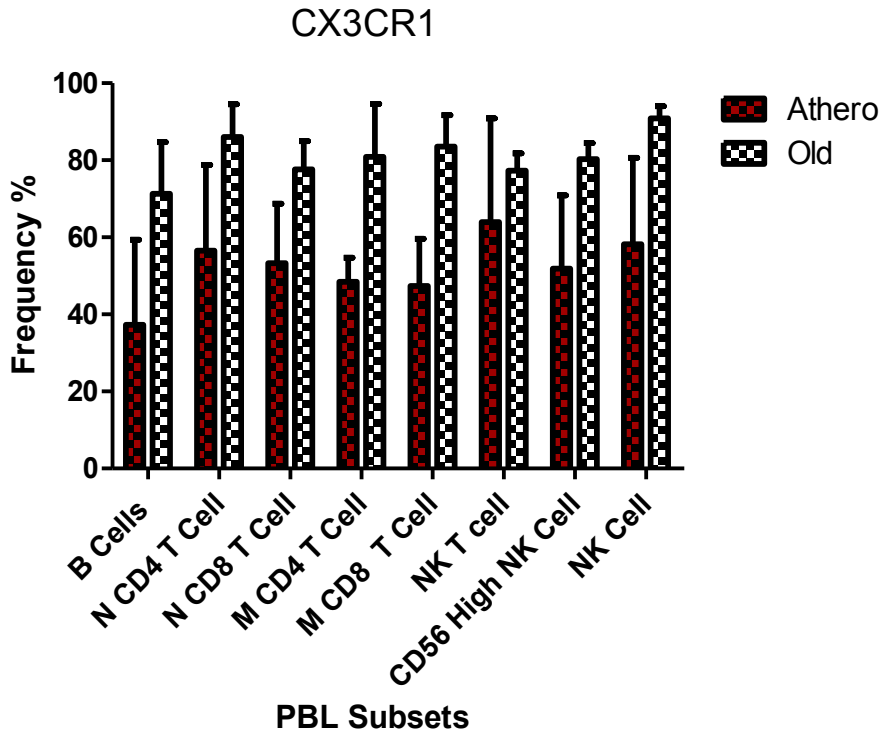

Figure 48: **Frequency of CX3CR1 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of CX3CR1 receptor; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression of adhesion molecules receptors on migrated PBL from patients with atherosclerosis or age matched controls:**

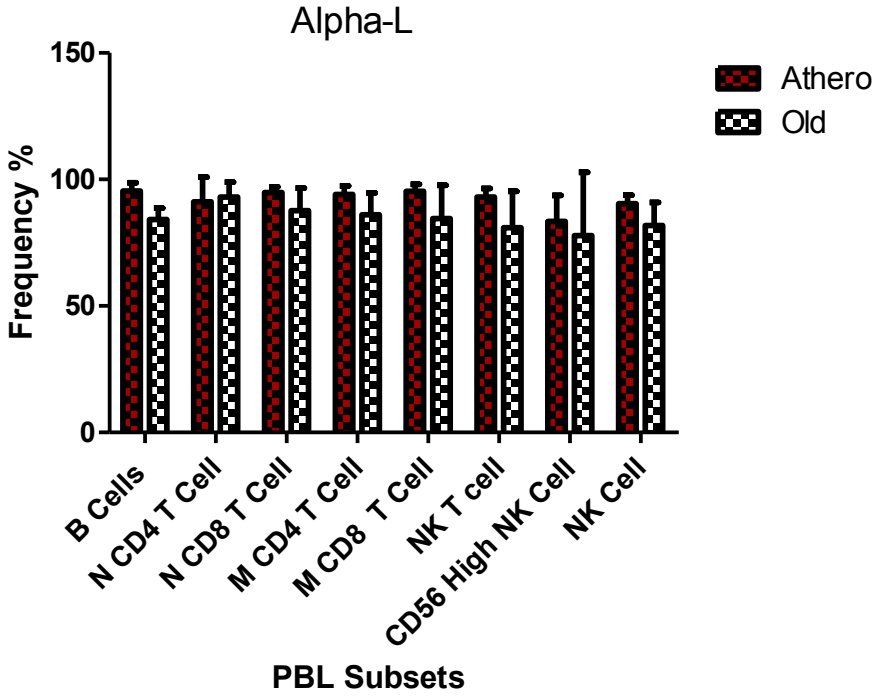

Figure 48: **Frequency of Alpha-L receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of Alpha-L receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-L receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

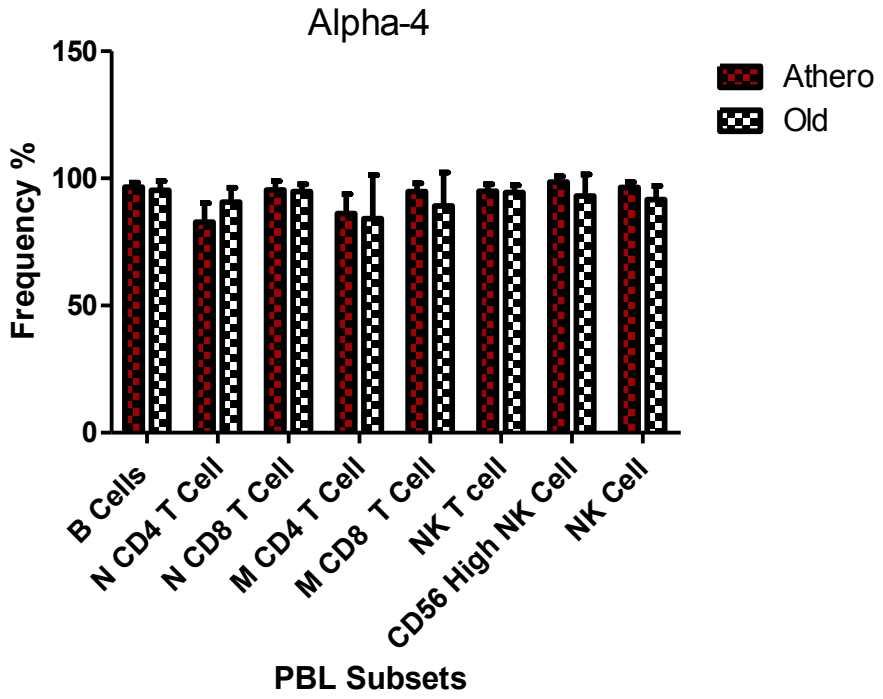

Figure 49: **Frequency of Alpha-4receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of Alpha-4 receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Alpha-4 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

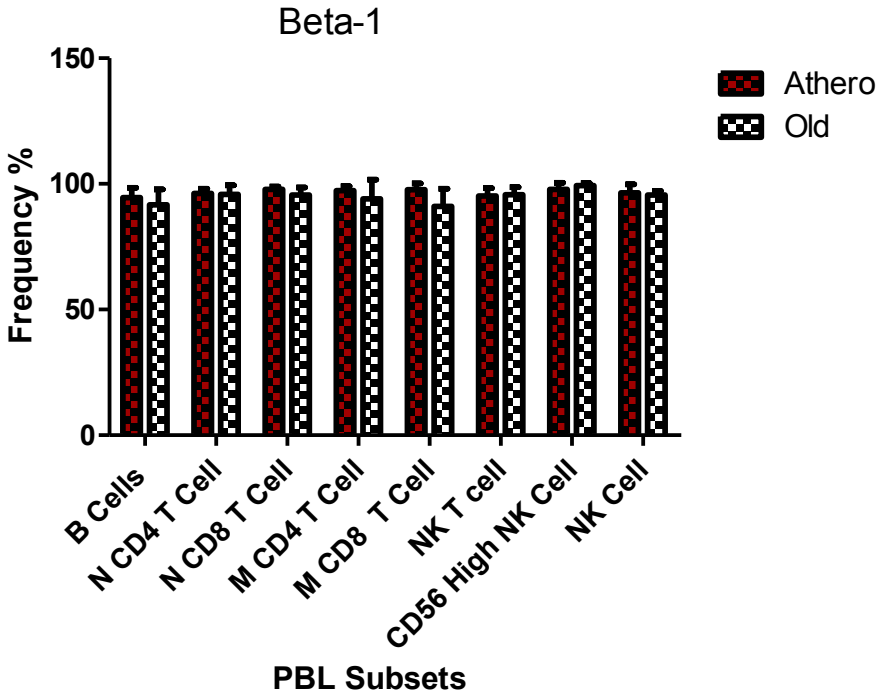

Figure 50: **Frequency of Beta-1 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of Beta-1 receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

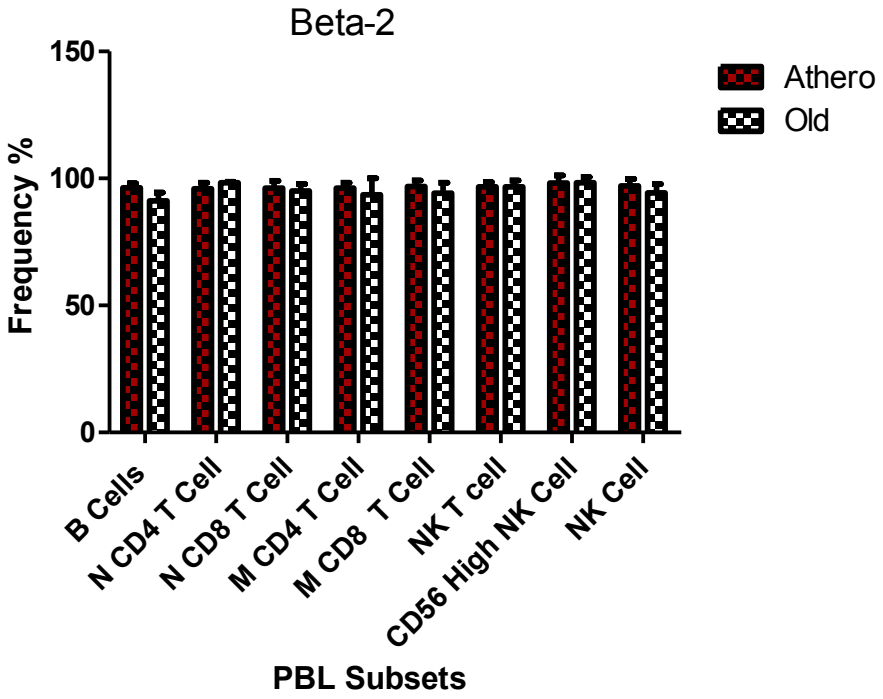

Figure 51: **Frequency of Beta-2 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of Beta-2 receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. Beta-2 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

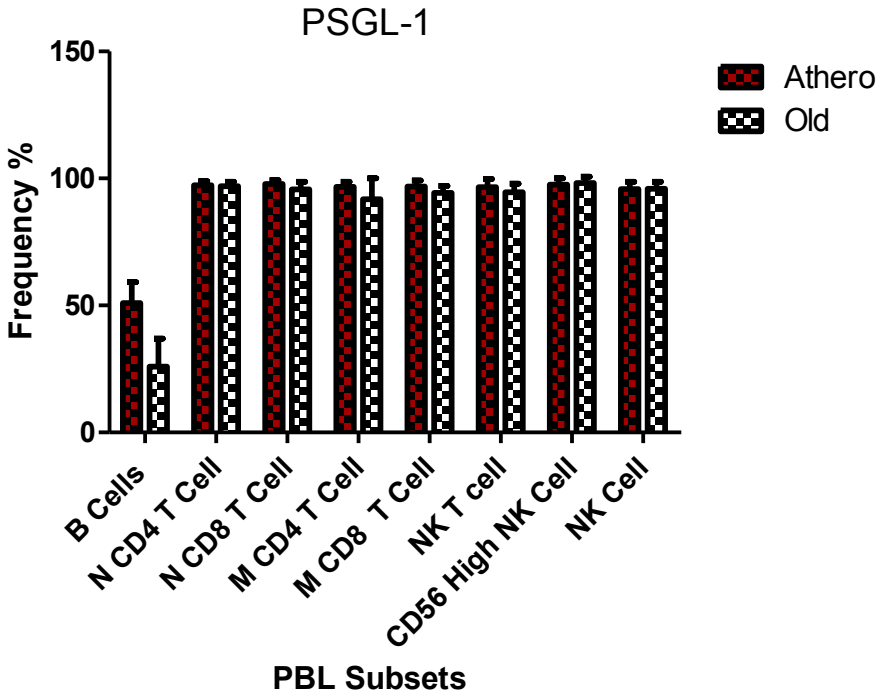

Figure 52: **Frequency of PSGL-L receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of PSGL-1 receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. PSGL-1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

- **Surface expression of chemokine receptors on migrated PBL from atherosclerosis patient or age matched controls.**

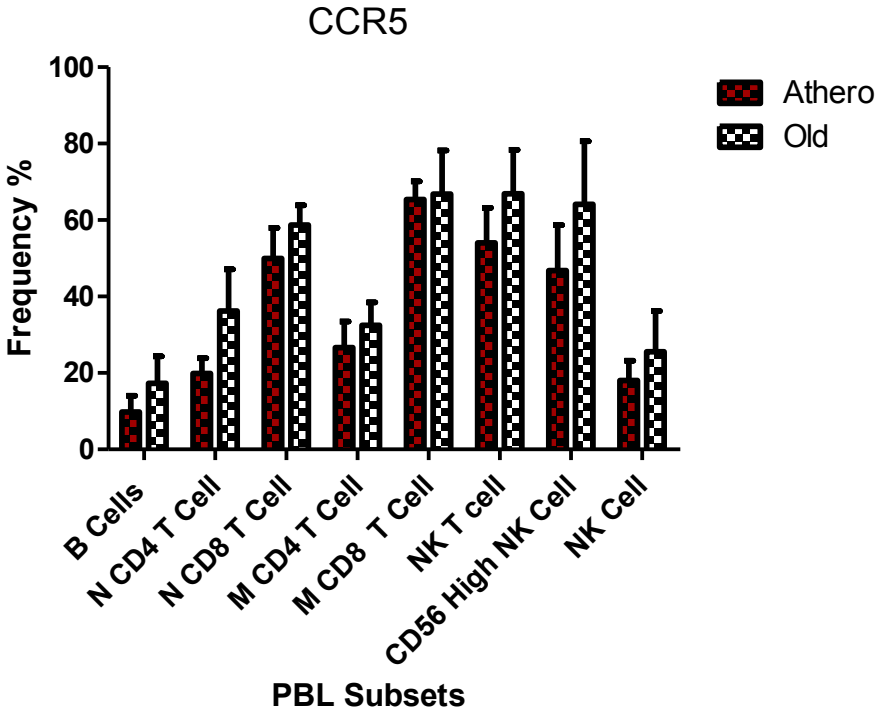

Figure 53: **Frequency of CCR5 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of CCR5 receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CCR5 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

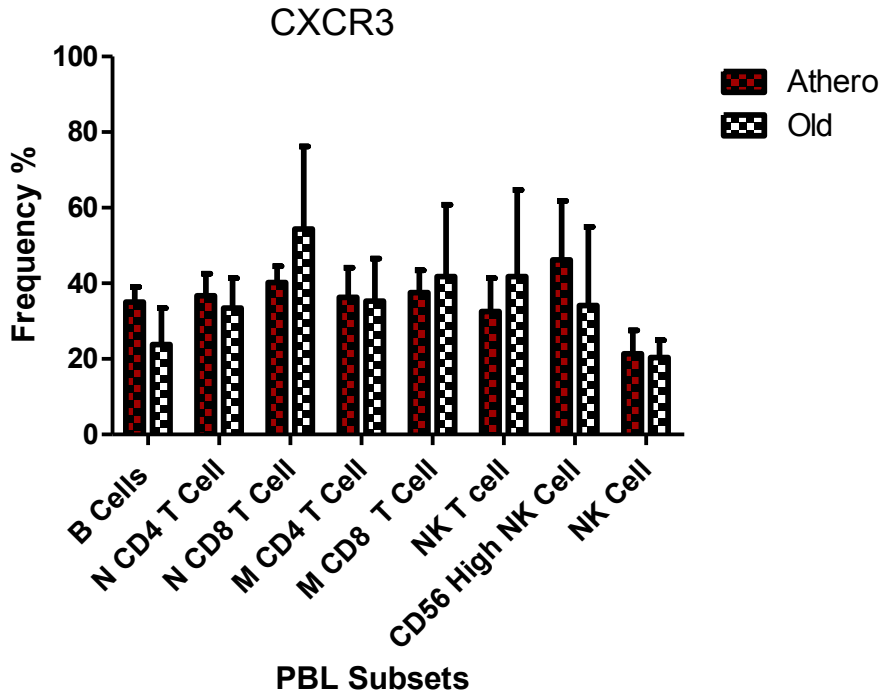

Figure 54: **Frequency of CXCR3 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of CXCR3 receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CXCR3 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).

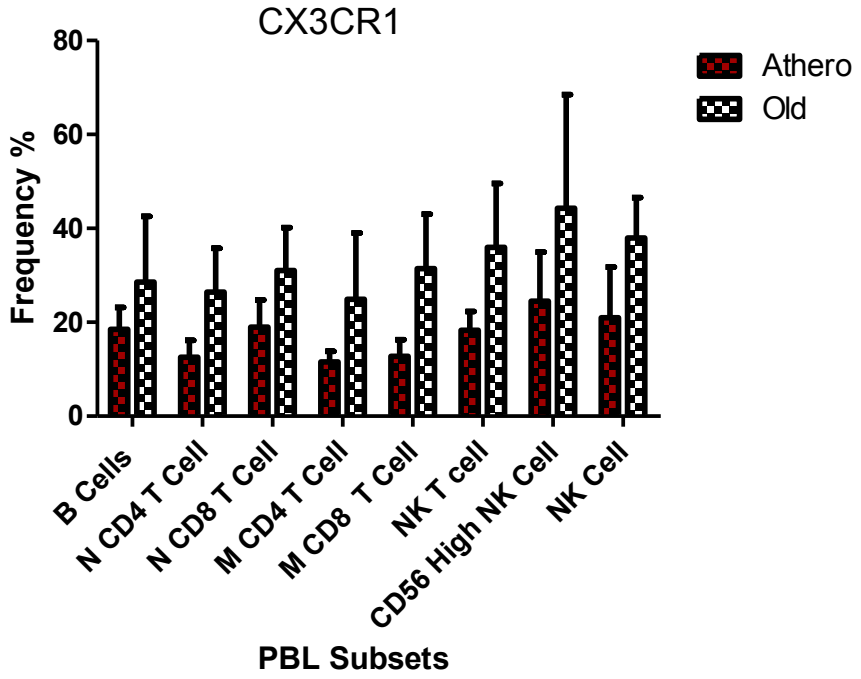

Figure 55: **Frequency of CX3CR1 receptor on different subsets of migrated PBL isolated from atherosclerosis patients or age match control.**

Flow cytometry was used to measure the Frequency of CX3CR1 receptors; Fresh blood was collected from atherosclerosis patients or age match control subjects. Subjects aged from 60-75. Subjects were matched for age, sex, ethnicity, BMI, and Statin usage. PBL collected from transmigration assay where PBL are added on DMEC and incubated for 7 minutes at 37°C. Then, TrypLE was used as cell-dissociation enzymes that make transmigrated PBL floating. The PBL was collected and stained with same antibodies panel. PBL was stained, and gated on their forward/side scatter profile. Direct conjugated antibodies were used for staining. CX3CR1 receptor expression was measured on the basis of the isotype control for each subset. Data is mean ± SEM (N=6) and the differences between groups were determined by using an independent sample t test. The probability values of less than 0.05 were considered significant (* = $P \leq 0.05$, **= $P \leq 0.01$, *** = $P \leq 0.001$).