# THE EFFECT OF $\beta_2$ AGONISTS ON THE IMMUNE FUNCTION

**By**

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A thesis submitted to
University of Birmingham
For the degree of
DOCTOR OF PHILOSOPHY

School of Sport and Exercise Sciences
The University of Birmingham
February 2012

## ABSTRACT

This project investigated the effect of β2-adrenergic receptor (β2-AR) stimulation on the function of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells *in vitro*. Catecholamines have been previously shown to increase efflux of these cells into the blood, but the effects on cell function are unclear. In this thesis three aspects of function have been addressed. The results of the studies presented in this thesis showed that:

(1) β2-adrenergic stimulation by salmeterol reduced the percentage of IFN-γ producing CD4+ and CD8+ T cells activated by Staphylococcus Aureus enterotoxin type B (SEB) superantigen, cytomegalovirus lysate (CMV) or CMV pp65 (pp65) recombinant protein.

(2) salmeterol, at high concentrations, increased rolling behaviour and decreased stationary behaviour of peripheral blood lymphocytes (PBLs) on human microvascular cell line (HMEC-1) and on vascular cell adhesion molecule-1 (VCAM-1), in both flow and static assays.

(3) adrenergic stimulation impaired the activation and cytotoxic function of CD8+ T and NK cells, as indicated by lower expression of CD107a (a marker of CD8+ T and NK cell activation and function) following incubation of peripheral blood mononuclear cells (PBMCs) with human erythromyeloblastoid leukemia (K562) cell line or MHC class I chain-related gene A (MICA*009) transfected Chinese hamster ovary cells (T-CHO) was analysed.

The results presented in this thesis showed that adrenergic stimulation influences a number of cellular functions, such as those related to migration, cytokine production and cytotoxic function. Together the above studies may contribute to our understanding about how stress affects the ability of the cytotoxic cells.

## DEDICATION

Te dashurit e mi, nuk kam fjale, t'ju shpreh falenderimin tim per nje nga mesimet me te rendesishme te jetes, "edukimin". Nuk mjafton nje flete apo nje liber per t'ju pershkruar dashurine e madhe dhe sakrificat tuaja per mua edhe Detin. Tani me vijne ne mendje vecse disa fragmente si per shembull kur vendosnit karamelet tek koka e krevatit tone perpara se te iknit ne pune; kur na mbanit ne krah ndersa na conit tek poliklinika per te bere gjilpera; kur hiqnit xhaketat e na mbulonit ndersa na thante nata e ftohte e Frances gjate emigrimit per ne Angli; kur na mbanit e na shtrengonit fort ne preher sic e beni edhe tani; kur na jepnit forcen edhe kurajon per te vazhduar perpara. Na keni mbrojtur si zogj te vegjel, na keni mbajtur ngrohte, ngrohte ne krahet tuaj, ka qene gjumi me i lumtur. Ne ate gjume te lumtur, kemi enderruar nje enderr te bukur, qe ne nje te ardhme te behemi prinder aq shembullore sa ju keni qene per ne. Do t'ua shperblejme te gjitha sakrificat. Jeni jeta, bota, ajri edhe shpresa jone.

Kjo eshte arritja juaj ne radhe te pare, pastaj e imja.

Ju adhuroj!

Pergjithmone

Femija Juaj.

# ACKNOWLEDGEMENTS

Above all, I want to thank the person who stood beside me during the whole period of the past three years, and on each level: from the participation in the project, through developing my ideas and learning to present them, Dr. Graham Wallace.

Graham, I cannot express enough how valuable it has been to have the door always open to your guidance, help, and expertise. You spent time contemplating my ideas, always coming back with inspiring comments. Knowing them made my life richer and my faith in science and mankind much stronger. This thesis would have not been possible without you.

Likewise I would like to thank Dr. Jos Bosch and Prof. Paul Moss, my supervisors, who hired me for this project. In particular thank you so much Jos, as like Graham your guidance, help and expertise has been brilliant during the 1st year of my thesis. I believe you are an excellent person, and I feel lucky to have created a professional relationship also based on friendship.

A special thank you goes to Dr. Natalie Riddell, Dr. Helen McGettrick, Prof. Gerard Nash for their insightful criticism throughout the duration of this project. I would like to thank Dr. Oliver Goodyear, Dr. Annette Pachnio, and all members of Paul Moss group for their support, high spirit and jollity.

Thanks are also due to all members of the Jos Bosch laboratory, past and present. I would like to especially thank one lab member, Dr. James Turner who has been by my side especially during the first year providing support and encouragement during my stay in Birmingham. Thanks also to my office friends Nick Stanger, Andrew Dewer, and Jo Long, for their kind support. It has been such a pleasure knowing you all. Thanks to all members of the SPORTEX department, Professors, Doctors, and students for support and encouragement throughout this Ph. D. I love all you guys.

Special thanks go to my fiancé Redin, for his love, encouragement and patience. I will always remember you giving me lift to Birmingham on Saturdays or Sundays so that I could carry on with experiments. I will always remember how you stood by my side in the laboratory. Hope you can always remember how I showed you what the red cells looked like in the microscope to make your stay more interesting. I love you very much!

Finally, I would like to thank very much my Dad, Mom, and twin sister for their patience, encouragement and unwavering support through the years. You are my compass. Dad, Mum and Deti, I feel the luckiest person of the world, because I have you.

“Mami-Babi-Deti, keni qene, jeni dhe do te jeni gjithmone drita e syve te mi!”. I devote this Ph. D. to you.

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**LIST OF ABBREVATIONS**

ADCC, antibody dependent cell cytotoxicity

APCs, antigen presenting cells

APES, aminopropyltriethoxysilane

ARI, anger recall interview

autoMACS, automated magnetic cell sorter

BCR, B cell receptor

BSA, bovine serum albumin

cAMP, cyclic adenosine monophosphate

CAMs, cell adhesion molecules

CAs, catecholamines

CCR7, CC-chemokine receptor 7

CD, cluster of differentiation

CD3ζ, CD3 zeta chain

CD40 ligand (CD40L)

CD4TLs, CD4+ T lymphocytes

CD62E, E-selectin

CD62L, L-selectin

CD62P, P-selectin

CD8+αβ T cells, CD8+ alpha-beta T cells

CD8TLs, CD8+ T lymphocytes

CD8TLs, CD8+ T lymphocytes

CD94/NKG2C, C-type lectin surface receptors

CFSE, carboxyfluorescein diacetate succinimidyl ester

CHO, Chinese hamster ovary cells

CI-MPR, cation-independent mannose 6-phosphate

CM, central memory cells

CMV, cytomegalovirus

CNS, central nervous system

CTLA-4, cytotoxic T-lymphocyte antigen-4

CTLs, cytotoxic T lymphocytes

DAP10, DNAX-activating protein of 10 kDa

DAP12, DNAX-activating protein of 12 kDa

DARs, dopamine receptors

DCs, dendritic cells

DNAM-1, DNAX-accessory molecule-1

EBV, Epstein-Barr virus

EDTA, Ethylenediaminetetraacetic acid

EM, effector memory

EMRA, CD45RA+ effector memory

EPI, epinephrine

ERK, extracellular signal-regulated kinase

FACS, fluorescence-activated cell sorting

FasL, Fas ligand

FATAL, fluorometric assessment of T lymphocyte antigen specific lysis assay

FBS, fetal bovine serum

FcεRIγ, Fc epsilon receptor gamma chain

Fc, fragment crystallisable

FCS, fetal calf serum

FcγRIIIA (CD16a), immunoglobulin gamma Fc region receptor III-A

FITC, fluorescein isothiocyanate

FN, fibronectin

GAS, IFN-γ-activation site elements

GPCR, guanine nucleotide binding protein-coupled receptors

Grb2, growth factor receptor-bound protein 2

HCMV, human Cytomegalovirus

HLA, human leukocyte antigen

HMEC-1, human microvascular endothelial cell line-1

HPA axis, hypothalamic-pituarity-adrenal-axes

HS, human serum

ICAM-1, intercellular adhesion molecule

IFNGR1, interferon-γ receptor 1 and 2

IFN-γ, interferon-gamma

IgSF, immunoglobulin superfamily

IL-, interleukin-

IO, ionomycin

IP-10, interferon-inducible protein-10

ITAM, immunoreceptor tyrosine-based activatory motifs

ITIMs, immunoreceptor tyrosine-based inhibitory motifs

Jaks, Janus kinases

JAM-1, junctional adhesion molecule-1

K562, human erythromyeloblastoid leukemia cell line

KIRs, killer cell immunoglobulin-like receptors

KLRG-1, killer cell lectin-like receptor subfamily G member 1

LAD, leukocyte adhesion deficiency syndrome

LC, langerhans cells

LCMV, lymphocytic choriomeningitis virus

LFA-1 is (αL/β2 integrin )(CD11a/CD18), lymphocyte function-associated antigen-1

LILRs, leukocyte immunoglobulin-like receptors

LPAM-1, Lymphocyte Peyer's patch adhesion molecule-1

LPF, lymphocytosis-promoting factor

LPS, lipopolysaccharides

mAb, monoclonal antibody

MAdCAM-1, mucosal addressin cell adhesion molecule-1

MAP, mitogen activated protein

MHC, major histocompatibility complex molecules

MICA*009, MHC class I chain-related gene A

MICs, MHC class I related chains

MLR, mixed lymphocyte reaction

MNC, mononuclear cells

NCR, natural cytotoxicity receptors

NE, norepinephrine

NK cells, natural killer cells

NKG2, natural killer group 2

NKT, natural killer T cells

NRIs, norepinephrine reuptake inhibitors

$Ot_{50}$, the time of onset of action is defined as the time from starting administration of the test agonist to achievement of 50% of response maximum

PAMPs, pathogen-associated molecular patterns

PBL, peripheral blood lymphocytes

PBMCs, peripheral blood mononuclear cells

PBS, phosphate buffer saline

PE, phycoerythrin

PECAM-1 (CD31), platelet/endothelial cell adhesion molecule-1

PGE2, prostaglandin E2

PHA, phytohaemagglutinin

PI3 kinase, phosphatidylinositol-3 kinase

PKA, protein kinase A

PMA, phorbol myristate acetate

PMT, photon multiplier tubes

PRRs, pattern recognition receptors

PSGL-1, P-selectin glycoprotein ligand-1

Rae-1, retinoic acid early transcripts-1

RANTES, regulated on activation of normal T cell expressed and secreted

RPMI, Roswell park memorial institute Medium

Rt, time for offset of action and is defined as the time from stopping the administration of the test agonist to achievement of 50% of the response maximum

SAM axis, sympathetic-adrenal medullary axis

SEB, Staphylococcus Aureus enterotoxin type B

SH-2, Src homology 2

SHP-, Src homology region 2 domain-containing phosphatase-

sLex , sialyl Lewis x

SLO, secondary lymphoid organs

SNS, sympathetic nervous system

SPSS, Statistical Package for the Social Sciences

STAT-1, signal transducer and activation of transcription

T-CHO, transfected Chinese hamster ovary cells

TCR, T cell receptors

TGF-β, transforming growth factor-β

Th-, T-helper-

TLRs, toll like receptors

TLs, T lymphocytes

TNF-, tumor necrosis factor-

TNF-R, tumour necrosis factor receptor

TRAIL, TNF-related apoptosis-inducing ligand

TRAIL-R, TNF-related apoptosis-inducing ligand-receptor

Treg, regulatory T cells

ULBPs, UL16 binding proteins

UoB, University of Birmingham

UoB, University of Birmingham

VCAM-1, vascular cell adhesion molecule-1

VE-cadherin, vascular endothelial-cadherin

VLA-4, very late antigen-4

α4β1 integrin (VLA-4), alpha 4 beta 1 integrin (very late antigen-4 receptor)

β2AR, beta2-adrenergic receptor

β-AR, beta-adrenergic receptor

γδ T cells, gamma-delta T cells

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 The Immune System

The mammalian immune system is a complex system consisting of two different arms - innate and adaptive immunity, each with distinct cell types and functions. The cooperative interactions of these two arms are required for the elimination of infective pathogens with high efficiency (Kawai and Akira, 2009). The innate immune response represents the first line of defence towards invading pathogens and mounts an immediate and non-specific immune response (Sternberg, 2006). In order to be able to sense these microbial infections, the innate sentinel cells (i.e., phagocytes including neutrophils, natural killer cells (NK), macrophages and dendritic cells) express pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs). Since PAMPs are broadly expressed on many different types of pathogens (bacteria, viruses or fungi) but are not present in host cells, PRRs can discriminate between self and non-self cells (Iwasaki and Medzhitov, 2010; Kawai and Akira, 2009). Following binding of PRRs to PAMPs, neutrophils, monocytes (or macrophages once located in the tissue) and dendritic cells engulf and destroy the foreign micro-organism by the process of phagocytosis and induce inflammatory responses. The inflammatory responses are characterized by an increase in local blood flow and increased vascular permeability (i.e. of blood vessels and tissues) to allow the escape of leukocytes from the blood stream to the site of injury and infection. This recruitment of leukocytes to the sites of injury and infection is extremely important in promoting the antigen clearance and wound healing.

### 1.1.1 Introduction to NK Cells

Originally, NK cells were described as large granular lymphocytes that had natural cytotoxicity against tumour cells, viruses, bacteria, parasites, all of which cause stress in affected cells (Anfossi et al., 2006; Bosch et al., 2005a; Cooper et al., 2001; Evans et al., 2011; Loza et al., 2002; Paust and von Andrian, 2011; Vivier et al., 2008). It was not until later that they were recognised as a separate lymphocyte lineage with both cytotoxicity and cytokine effector functions (Vivier et al., 2008).

NK cells represent only a minor fraction of total lymphocytes (2 %-18 %) in peripheral blood of humans and are located throughout lymphoid and non-lymphoid tissues. These front line defenders have the capacity to migrate quickly in and out of tissues enabling them to be among the first immune cells to arrive at sites of tissue damage and infection (Bosch et al., 2005b). The importance of NK cells in human host immunity is clearly observed in patients with isolated genetic deficiencies in NK cells who contract severe infectious diseases despite the presence of functional T cells and B cells (Paust and von Andrian, 2011).

In humans, NK are recognised by the expression of CD56 and CD16 cell surface antigens, in combination with absence of the pan-T cell marker CD3. Whereas, CD56 is expressed on all NK cells, CD16 is not. CD16 is a low affinity fragment crystallisable (Fc) receptor that enables NK to detect antibody-coated target cells and thus to exert antibody dependent cell cytotoxicity (ADCC) (Cooper et al., 2001; Paust and von Andrian, 2011; Vivier et al., 2008), a mechanism of cell-mediated immunity that causes the lysis of target cells coated with antibody, by effector cells with cytolytic activity.

There are two major subsets of NK cells; $CD56^{dim}$ (low expression of CD56) or $CD56^{bright}$ (high expression of CD56) (Bosch et al., 2005a; Cooper et al., 2001; Suzui et al., 2004; Vivier

et al., 2008). These NK populations differ in their trafficking (Evans et al., 2011) and cytotoxic properties (Anfossi et al., 2006). The $CD56^{dim}$ cells compromise 90% of the peripheral blood and spleen NK cells and are cytotoxic related to their high expression levels of perforin. In contrast, most of the $CD56^{bright}$ $CD16^{-}$ NK cells reside in lymph nodes and tonsils, lack perforin, and produce cytokines such as interferon-gamma (IFN-γ) (Vivier et al., 2008). Also, while $CD56^{dim}$ NK cells express high levels of CXCR1 (CXC chemokine receptor 1) and CX3CR1 (CX3C chemokine receptor 1), which are required for their migration to peripheral inflammatory sites, $CD56^{bright}$ NK cells express migration markers that facilitate traffic to secondary lymphoid organs (SLO) such as CCR7 (lymphoid CC-chemokine receptor 7), CXCR3 (CXC chemokine receptor 3) and CD62L (L-selectin). With the inclusion of CD16 human peripheral blood NK cells can be separated into five different subpopulations of NK cells according to the cell surface density (Poli et al., 2009). These are (1) $CD56^{dim}$ $CD16^{bright}$, (2) $CD56^{dim}$ $CD16^{-}$, (3) $CD56^{bright}$ $CD16^{dim}$, (4) $CD56^{bright}$ $CD16^{-}$, (5) $CD56^{-}$ $CD16^{bright}$. Out of these different NK cell subpopulations, the majority of functionally distinct NK cell subsets include $CD56^{dim}$ $CD16^{bright}$ and $CD56^{bright}$ $CD16^{dim/-}$ (Fig.1.1.).

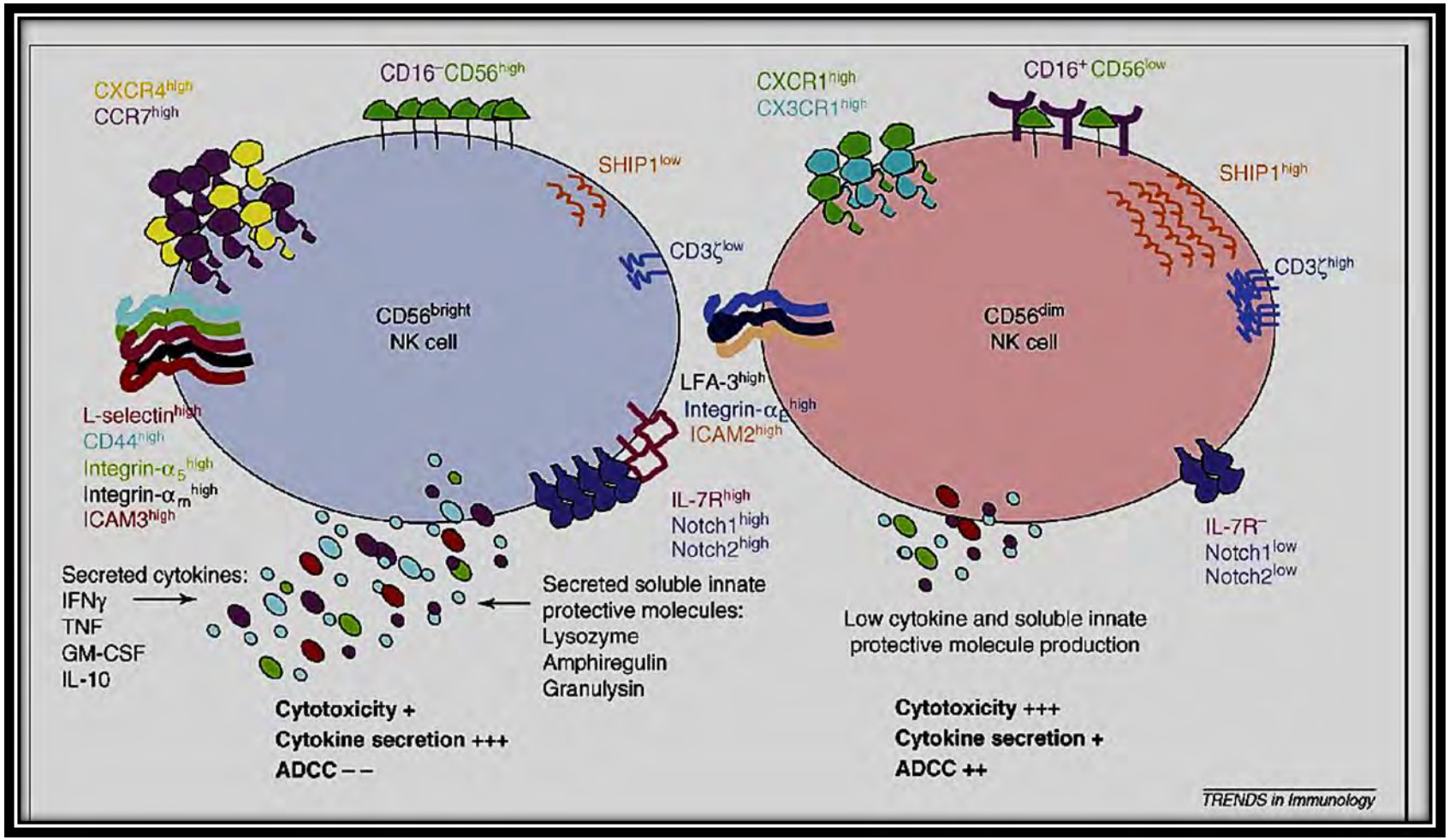



**Figure 1.1. Two main NK cell subsets.** Schematic representation of human $CD56^{dim}$ $CD16^{+}$ and $CD56^{bright}$ $CD16^{-}$ NK cells including their surface phenotype and functional properties. Figure was taken from (Hanna and Mandelboim, 2007).

### 1.1.2 NK Cell Receptors

On the bases of their structural homologies, natural killer cell surface receptors can be divided into two groups. The first group includes killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs). These consist of type 1 transmembrane glycoproteins with two or three extracellular immunoglobulin like domains responsible for ligand recognition (Campbell and Purdy, 2011; Farag et al., 2002; Lanier, 2008; Strong and McFarland, 2004). The second family of human NK receptors includes the C-type lectin family and is characterized by a C-type lectin extracellular domain. One example of the C-type lectin NK receptor is the heterodimeric CD94-NKG2A. This is composed of a (CD94) glycoprotein that is covalently (disulfide) bonded to a chain encoded by a gene of the C-type lectin NKG2 family (Farag et al., 2002; Lanier, 1998; Radaev and Sun, 2003).

### 1.1.3 Inhibitory Receptors

All NK cells undergo a maturation step which serves as an education process which enables NK cells to become competent and recognise self-MHC class 1 molecules through which they become "tolerant to self" or the lack of self-MHC class 1 molecules otherwise referred to as the "missing self" (Fig.1.2). The recognition of the self-MHC class 1 occurs by the specific inhibitory receptors, KIR in humans, equivalent to the lectin-like Ly49 dimers in mice. When NK cells encounter cells with decreased expression of MHC class 1 molecules (i.e. virally-infected or tumour cells), they are no longer controlled by inhibitory signals and NK cell cytotoxicity and cytokine production will be promoted (Anfossi et al., 2006; Vivier et al., 2008).

It is important to note that despite their structural diversity, all inhibitory NK receptors have long cytoplasmic domains with an immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which are directly phosphorylated to induce inhibitory signals (Campbell and Purdy, 2011; Lanier, 2008). More specifically, it is thought that when the ITIM bearing receptors bind their MHC-I ligands, the tyrosine residues in the ITIMs, are phosphorylated by a Src family kinase, and provide docking sites for cytoplasmic phosphatases having a Src homology 2 (SH-2) domain, SHP-1 and SHP-2, which dephosphorylate critical tyrosine phosphoproteins that propagate activation signalling thereby inhibiting the NK cell activation towards target cell (Campbell and Purdy, 2011; Lanier, 2008; Treanor et al., 2006) (Fig.1.3.).

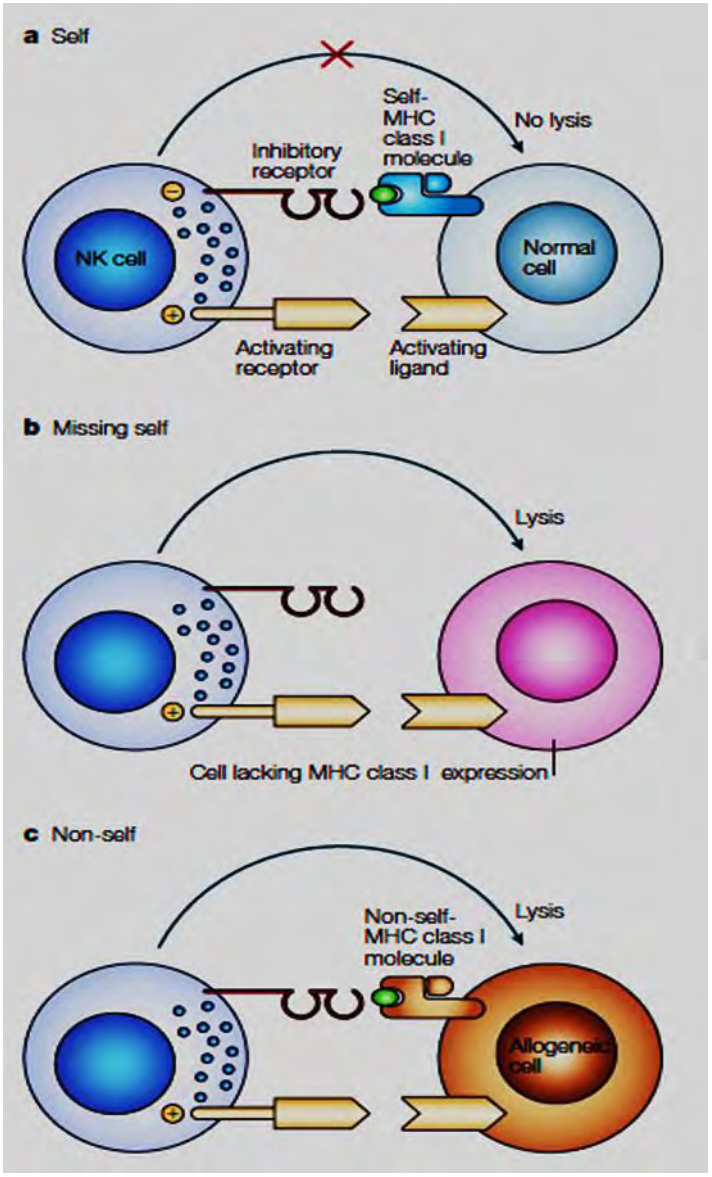



**Figure 1.2. NK cell self-tolerance and the "missing self hypothesis". A-**Upon interaction with a normal target cell, a NK cell might receive activating signals. Despite this, the inhibitory signals from the ligated MHC-binding receptor at the surface of NK cells prevent the autologous target cell lysis, because the target cell expresses the appropriate self-MHC class I alleles. **B-**Viral infection and transformation causes the target cell to lose the expression of MHC class I molecules and thereby results in the disengagement of the MHC-binding inhibitory receptor at the surface of NK cell. With no inhibitory signals received to prevent the autologous target cell lysis, the target cell is perceived by the NK cell to be missing self. **C-**In the case of an allogeneic transplant, NK cells of the host interact with donor target cells that express foreign MHC class I alleles. However, due to the fact that the foreign non-self MHC class I molecules do not engage all of the inhibitory receptors at the surface of a host NK cell, allogeneic cells are lysed by NK cells. Figure was taken from (Kumar and McNerney, 2005).

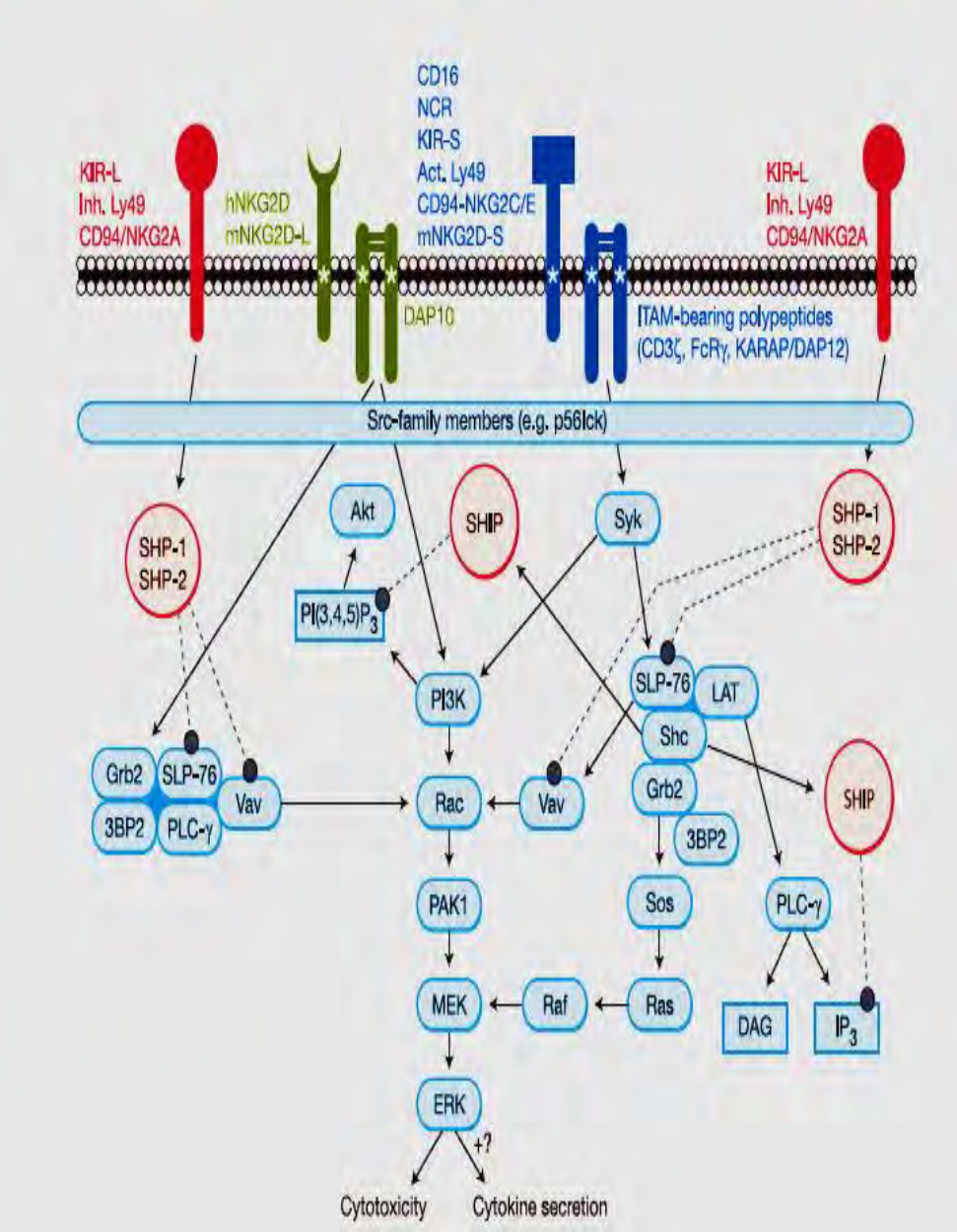



**Figure 1.3. NK cell effector signalling pathways.** ITAM-associated NK cell activating receptors as well as inhibitory receptors are presented. Figure was taken from (Vivier et al., 2004).

### 1.1.4 Activating Receptors

Since receptor inhibition of NK cell function prevents killing of self- MHC class I cells, to be able to exert their cytolytic activity, NK cells require activating signals generated upon effector/target cell interaction (Fauriat et al., 2010; Lanier, 2005; Moretta et al., 2011b; Verheyden and Demanet, 2008).

Several NK cell activating receptors involved in activation of NK-cell effector functions have been identified. These include the natural cytotoxicity receptors, (NCR- NKp46, NKp30, and NKp44) (Moretta et al., 2001a; Moretta et al., 2001b; Moretta et al., 2011a), the C-type lectin surface receptors (CD94/NKG2C; CD94/NKG2E and NKG2D); activatory KIRs (KIR2Ds, KIR3Ds), and the low affinity immunoglobulin gamma Fc region receptor III-A (FcγRIIIA) (Moretta et al., 2001b; Moretta et al., 2011a; Moretta et al., 2011b; Vivier et al., 2004; Vivier et al., 2008). In contrast to the inhibitory receptors, all activating receptors lack ITIMs and carry short cytoplasmic domains, which upon non-covalent association with adapter polypeptides that carry an immuno-receptor tyrosine-based activation motifs (ITAMs), enable the transmission of the activating signals (with exception of NKG2D). NK cells express constitutively several ITAM-bearing adapter proteins including CD3ζ (CD3 zeta chain), FcεRIγ (Fc epsilon receptor gamma chain) and DAP12 (DNAX-activating protein of 12 kDa). In humans, CD3ζ and FcεRIγ associate with NKp30, NKp46, and CD16, whereas DAP12 pairs with several members of activating killer cell immunoglobulin receptors (KIRs); and C-type lectin like receptors, as well as NKp44 (Lanier, 2003; Lopez-Larrea et al., 2008; Vivier et al., 2004).

### 1.1.5 NKG2D

NKG2D is a type II transmembrane-anchored C-lectin-like protein encoded by a gene located on human chromosome 12p13 and mouse chromosome 6. It acts as an activating cell surface receptor and is expressed by NK cells, γδT (gamma-delta T) cells, NKT (Natural killer T) cells, some cytolytic CD8+ αβT (alpha-beta T) cells and CD4+ T cells (Billadeau et al., 2003; Lanier, 2003, 2005, 2008; Radaev and Sun, 2003). The NKG2D receptor is implicated as a key trigger for some forms of NK cell-mediated cytotoxicity and has generated tremendous interest due to the finding that it allows activation of NK cells following recognition of viral infected and transformed cells that have upregulated expression of "stress induced" NKG2D ligands (Billadeau et al., 2003; Lanier, 2003; Radaev and Sun, 2003).

Stress induced ligands recognised by NKG2D include MHC class I related chains (MICs) (i.e. MICA, MICB) and UL16 binding proteins (ULBPs) in humans, and retinoic acid early transcripts-1 (Rae-1), minor histocompatibility antigen H60 and ULBP-like transcript-1 in mice (Ashiru et al., 2009; Billadeau et al., 2003; Endt et al., 2007). MICA and MICB are often expressed in epithelial tumours in lung, breast, kidney, ovary, prostate and colon carcinomas (Bauer et al., 1999; Groh et al., 2006). In mice the anomalous expression of MIC homologue Rae in local tissues, strongly stimulates antitumor immunity leading to tumor rejection by NK and CD8+ T cells, whereas in humans elevated levels of the soluble MICA (resulting from proteolytic shedding of the MICA by tumour cells) correlated with the progression of the prostate cancer (Holdenrieder et al., 2006). Following interaction with its specific ligand, NKG2D associates with a transmembrane–anchored adapter protein (DAP10). DAP10 (which is expressed as a disulfide linked homodimer) has a short (21 amino acids) cytoplasmic domain which contains an YxxM –signalling motif (where x donates any amino acid). Phosphorylation of this motif results in its association with p85 subunit of

phosphatidylinositol-3 kinase (PI3 kinase) and Growth factor receptor-bound protein 2 (Grb2) (Lanier, 2003, 2008) (Fig.1.4.).

The importance of NKG2D in antiviral immunity is best observed in human and mouse cytomegalovirus (CMV) (Lanier, 2005). The human CMV encodes a recombinant UL16 protein, which upon intracellular interaction with NKG2D ligands, prevents their expression on the surface of the infected cells. As result, the susceptibility of the infected cells to NKG2D-dependent NK effector functions are diminished. A second human CMV encoded protein UL142 down modulates the expression of MICA by retaining it in the cis-Golgi apparatus (Ashiru et al., 2009). Recently the importance of NKG2D has also been demonstrated in gastric cancer patients who exhibit decreased expression of this receptor (Saito et al., 2011).

Despite its essential role in antiviral and anti-tumour immunity, ligation of NKG2D receptor alone is not adequate to induce the activation of NK cells. In fact with exception of CD16, the binding of a single activating receptor is not sufficient to trigger the NK cell effector functions. This was clearly demonstrated by Bryceson et al. (2006) who used agonistic antibodies directed against various surface activating receptors and observed that when NKp46, NKG2D, CD2, or DNAM-1 (DNAX-accessory molecule-1) activating receptors were used in isolation, stimulating any of these receptors produced little or no target lysis. In contrast, simultaneous cross-linking of different pairs or combinations of receptors by agonist antibodies, induced a combinative effect on both cytokine production and lytic activity (Bryceson et al., 2006; Lanier, 2008) suggesting that the triggering of NK cell effector functions is a result of several signalling pathways originated from a number of activating receptors (Lanier, 2008; Vivier et al., 2004).

NK cells also utilise other methods to detect and kill the tumour and viral infected cells. NK cells recognise infectious nonself ligands (the CMV-encoded m157) by toll like receptors (TLRs). Indeed, NK cells express several TLRs which induce IFN-γ production and enhance cytotoxicity *in vitro* (Vivier et al., 2008). As stated above, NK cells also detect target cells that are coated with antibody by their expression of their low affinity Fc receptor CD16 (Bosch et al., 2005a; Vivier et al., 2008), $CD56^{dim}$ NK cell have high level expression of KIRs and IL-2 which may control and regulate their cytotoxic properties, the expression of these inhibitory receptors is low or absent in the $CD56^{bright}$ NK cell (Hanna and Mandelboim, 2007; Jacobs et al., 2001). Instead $CD56^{bright}$ NK subset has high expression of CD94/NKG2A inhibitory receptors, which are expressed at low levels in $CD56^{dim}$ NK cell subset. In contrast, both NK subsets express the activatory NKG2D receptor. Therefore most cells are protected by normal expression of MHC class I, while a cell lacking MHC class I will be killed. If cells express both MHC class I and NKG2D ligands it will depend on the balance of signals between stimulation and inhibition of the killing response. Finally, NK cell can be activated through the surface receptors Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL-R) directly on target cells to induce their lysis and apoptosis in a cell-contact dependent manner.

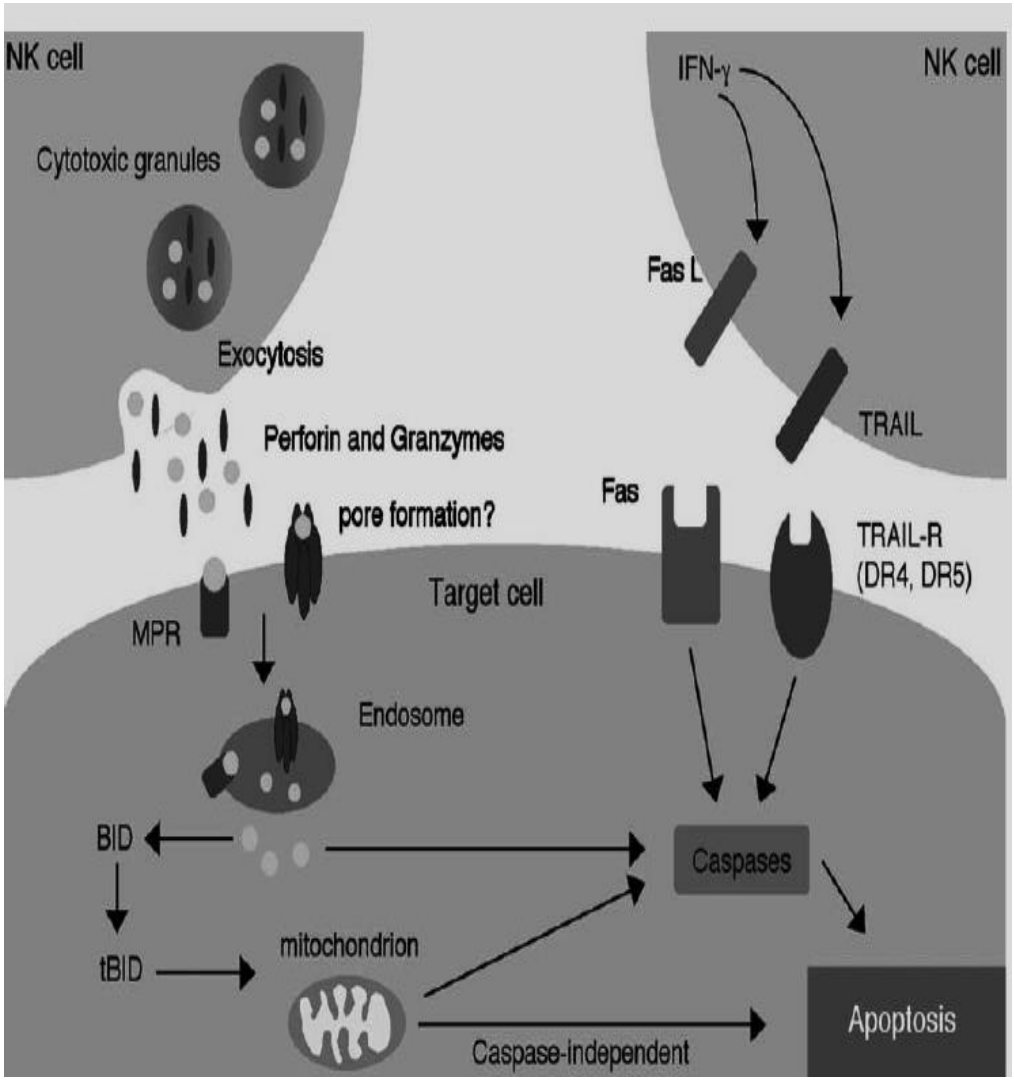



**Figure 1.4. Two pathways of NK cells cytotoxicity.** The elimination of transformed cells occurs through one of the two contact-dependent mechanisms. **A-**Granule exocytosis; following recognition of the target cell, NK cells release the cytotoxic granule contents, perforin and granzymes. Upon entry into target cells cytoplasm, granzymes induce apoptotic cell death either via the activation of cellular caspases or via caspase-independent pathways. **B-**Death receptor engagement; Following the activation of NK cells with cytokines and the triggering of the activation receptors, NK cells express on their surface Fas ligand (FasL), tumor necrosis factor-alpha (TNF-α), and TNF-related apoptosis-inducing ligand (TRAIL).Upon engagement and aggregation of target cell death ligands by their specific receptors (Fas, TNF, TRAIL-R1/TRAIL-R2 respectively), NK cells induce apoptosis of the target cells in a caspase dependent mechanism. Figure was taken from (Smyth et al., 2005).

### 1.1.6 NK Cell Killing

Following recognition of the tumor and virally infected cells, NK cells mediate killing by two major effector functions. Firstly, NK cells release cytotoxic granules such as granzymes and perforin. Perforin is a membrane disrupting protein, which plays an essential role in target-cell apoptosis mediated by cytotoxic T lymphocytes CTLs and NK cells. More specifically, its indispensable role in the apoptosis of viral and transformed cells involves mediating the entry of granzymes into their (target-cell) cytoplasm. Granzymes are a family of structurally related serine proteases, released from the secretory granule contents of the cytotoxic lymphocytes through the process of exocytosis and are responsible for promoting target cell apoptosis. Human NK cells express five different granzymes. These include granzymes A, B, K, H and M. Two models of perforin action have been described. Firstly, that perforin polymerized in response to calcium and placed itself into the membrane of transformed cells, creating a channel which allowed the passage of granzymes (Lieberman, 2003; Motyka et al., 2000). Secondly that granzymes enters the cytosol of the target cells in a perforin independent manner through either interaction with a cell surface receptor(s) (i.e. granzyme B requires the interaction and binding with the cation-independent mannose 6-phosphate/insulin-like growth factor receptor (CI-MPR) to be taken up and induce the apoptosis of target cells) or through the activation of clathrin- and dynamin-dependent endocytosis (which removes perforin and granzymes from the plasma membrane to early endosomes) (Thiery et al., 2010; Trapani et al., 2003).

NK cells express on their surface at least three tumour necrosis factor (TNF)-family death ligands including FasL, TNF-α, and TRAIL. Upon engagement and aggregation of target cell death ligands by their specific receptors (Fas, TNF, TRAIL-R1/TRAIL-R2 respectively) NK cells induce apoptosis of the target cells in a caspase dependent mechanism (Screpanti et al.,

2005; Trapani and Smyth, 2002). The increased susceptibility to virus induced death and tumor development after neutralisation of TRAIL *in vivo* (Takeda et al., 2002), indicates the contribution of death-receptor mediated apoptosis to NK cell cytotoxicity. In addition to conferring cytotoxicity, the production of IFN-γ by the NK cells, also participates to the shaping of the adaptive immune response (Anfossi et al., 2006; Martin-Fontecha et al., 2004). Overall, NK cell activity is regulated by the dynamic balance between signals that either activate or inhibit each-other (Anfossi et al., 2006; Vivier et al., 2008).

### 1.2 The Adaptive Immune Response

Although the innate immune system has evolved to rapidly sense and effect the elimination of a wide range of pathogens, it is not always able to completely eliminate a foreign invader (Bonilla and Oettgen, 2010). This has led to the evolution of adaptive immune system which is a more specialised antigen-specific response. Adaptive immune system responses are primarily generated by B cells and T cells. Following development in the primary lymphoid organs (bone marrow and thymus), B and T lymphocytes traffic to secondary lymphoid organs such as lymph nodes and spleen in order to sample antigens from lymph and blood (Bonilla and Oettgen, 2010). To be able to recognise and interact with pathogens, both B and T cells possess the expression of enormous repertoire of antigen receptors on their surface with a potentially unique specificity for a particular pathogen or protein fragment (a peptide) (Chaplin, 2006). B cell receptors (BCR) interact directly with free antigen, whereas T cell receptors (TCR) can only interact with peptide antigens displayed by the major histocompatibility complex (MHC) molecules expressed by antigen presenting cells (APCs). APCs include dendritic cells, macrophages and B cells. These are specialised samplers of environmental antigens and danger signals (Bonilla and Oettgen, 2010) mainly present as immature APCs in the skin and mucosal sites where pathogen encounter is more likely to

occur. Following antigen recognition during an infection via TLRs for example, APCs phagocytose pathogens and present processed microbial antigens on their surface in combination with MHC class II. Maturation follows as the cells migrate towards lymph nodes, via the lymphatic system, where they present the antigens to migrating T cells by MHC molecules. These T cells, scan the foreign antigen-MHC complex in a such way that if a T lymphocyte has an antigen receptor complimentary to the presented antigen, it will bind to the MHC/peptide complex, and begin to clonally expand giving rise to effector T lymphocytes (including cytotoxic-T cells, T-helper cells, and memory cells). This clonal expansion represents the fundamental basis of acquired immunity providing large number of effector cells to migrate to the original site of infection in order to eliminate it. Upon resolution of the infection, most antigen-specific cells die, and are cleared away by phagocytes. However, a small number of these antigen-specific lymphocytes are retained as long lived memory cells for subsequent encounters with the same antigen (Alberts et al., 2002). Thus one of the crucial characteristics of the adaptive immune system is its ability to retain an "immunological memory", and mount a secondary immune response. Unlike the primary immune response (a response to a pathogen not encountered before by the immune system), which is slow and becomes apparent several days after the initial infection, (during an initial antigen encounter the production of antigen-specific population can take up to 6 days) this secondary immune response is very rapid and efficient (immune response can occur as early as 2 days) and prevents any clinical indications of the re-infection thereby rendering the immunological memory one of the main advantages of the adaptive immune system.

### 1.2.1 T Lymphocyte Populations

T cells can be classified in two different main categories, CD4+ T lymphocytes (CD4+ TLs), otherwise referred to as T-helpers (Th) and CD8+ T lymphocytes (CD8+ TLs) also known as

cytotoxic T lymphocytes (CTLs). The main function of CD4+ TLs is to regulate and assist in active immune responses by releasing cytokines. Cytokines are chemical messengers between the immune cells which play a crucial role in maintaining the homeostasis within the immune system by mediating inflammatory and immune responses. CD4+ TLs can be divided into various different types, with distinct cytokine secretion phenotype giving rise to unique functional characteristics for each type, including Th-1, Th-2 , Th-17 and T regulatory cells (Kaiko et al., 2008). Th-1 are involved in the secretion of IFN-γ, IL-2, and TNF-β, which promote cellular immunity and stimulate the functional activity of CD8+ TLs, and activated macrophages to destroy intracellular bacteria (Elenkov et al., 2008). Th-2 cells primarily secrete IL-4, IL-10, and IL-13 which promote allergic inflammation, up-regulate antibody production and target parasitic organisms (Elenkov et al., 2008; Kaiko et al., 2008). Th-17 represent a distinct class of CD4+ T effector cells that do not fall into either Th-1 or Th-2 category. As their name suggests, Th-17 are involved in production of proinflammatory molecules of the IL-17 family including IL-17A-F. IL-17A-F promotes tissue inflammation in response to extracellular bacteria and fungi, by inducing proinflammatory cytokines and chemokines that attract and activate granulocytes, macrophages and neutrophils (Kurts, 2008). In contrast to the aforementioned T helper subsets, Treg are regulatory T cells which suppress the activity of effector and autoreactive T cells *in vivo* by several mechanisms including production of IL-10 and transforming growth factor-β (TGF-β) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Nelson, 2004; Rudensky, 2011; Sansom and Walker, 2006).

### 1.2.2 Subsets of Human $CD8^+$ T Cells

Nowadays, it is possible to discriminate up to 14 CD8+ TLs cell subsets rendering the CD8+ TLs population indeed very heterogeneous (Chen et al., 2001; Monteiro et al., 2007; Sallusto et al., 2004; Sallusto et al., 1999; van Lier et al., 2003). The different subsets of human

memory CD8+ TLs can be identified on the basis of differential expression of the surface markers and homing receptors both of which are related to their activation and function (Chen et al., 2001). The three main subpopulations of $CD8^+$ T cell compartment include naïve, effector and memory subsets. For example, naïve cells are characterized by the expression of CCR7 (lymph node homing marker) and CD62L (high endothelial venules homing marker) because they continuously travel between the lymph nodes, where the antigen is presented by the APCs. In addition they express co-stimulatory molecules CD27 and CD28 which are important for their activation but do not reacquire markers of effector cells like perforin or Fas ligand as they have never encountered an antigen before and thus do not exhibit effector functions.

Von Andrian and Mackay described function and migration of T cells as "two sides of the same coin". This makes sense when considering the fact that in addition to the migratory potential, the CCR7 marker is also related to the specific functional capacity of that cell. For example $CCR7^-$ cells have a superior ability to produce effector cytokines (i.e. IFN-γ) following stimulation compared to $CCR7^+$ cells. In addition to the markers described above, naïve cells can also be discriminated by their expression of the CD45RA marker (leukocyte common antigen isoform). This is one of the four isoforms of the CD45, with each isoform being characterized by a specific molecular weight. The CD45R marker is unusual as it is converted into the CD45RO isoform after antigenic stimulation of the naïve T cells and then following further stimulation is reversibly re-expressed in $CD45RA^+$ effector memory cells. Since both, naïve cells and $CD45RA^+$ effector memory cells express this marker; $CD45RA^+$ cannot, on its own be used to distinguish between naïve cells and $CD45RA^+$ effector memory cells, but is used in combination with other markers previously described. This is extremely important in terms of distinguishing between two different CD8+ TLs subsets as for example

unlike the naïve cells; $CD45RA^{+}$ effector memory cells lack the surface expression of the co-stimulatory molecules CD27 and CD28 respectively. Like naïve cells, central memory cells, (CM; $CD45RA^{-}$, CCR7, $CD28^{-}$, $CD27^{+}$) which have previously responded to an infection also express CCR7 in order to interact with the APCs in the lymph node, but lack the co stimulatory receptor $CD28^{-}$ and are therefore referred to as the intermediate cells, a name that relates to their position along the linear pathway of differentiation (Appay et al., 2002). Following activation, CM cells differentiate into effector memory (EM; $CD45RA^{-}$, $CCR7^{-}$, $CD28^{-}$, $CD27^{-}$); and further $CD45RA^{+}$ effector memory (EMRA; $CD45RA^{+}$, $CCR7^{-}$, $CD28^{-}$, $CD27^{-}$) (Appay et al., 2002; Chen et al., 2001; Sallusto et al., 2004; Sallusto et al., 1999).

$CD8^{+}$ EM cells have a potent lytic and cytotoxic activity as result of their rapid production of high levels of perforin, granzyme a, IFN-γ and TNF-α cytokines. Referred to as the "late" differentiated cells due to their lack of the CD28 and CD27 co-stimulatory molecules for activation, they show immediate effector functions in the peripheral tissues where the infection takes place. As they are localised in the inflamed tissues, they do not express the lymph node homing marker CCR7, but instead express receptors that allows them to home to inflamed areas. In addition to the general CD8+ TLs population, in the recent years, even the effector memory (EM; $CD45RA^{-}$, $CCR7^{-}$, $CD28^{-}$, $CD27^{-}$) CD8+ TLs population has been described heterogeneous (Monteiro et al., 2007; Romero et al., 2007). By combining the simultaneous analysis of surface markers, the multiparameter flow cytometry revealed four distinct subsets of effector memory cells. These include: $CD28^{+}CD27^{+}$ EM1 and EMRA1 sub-types; $CD28^{-}CD27^{+}$ EM2 and EMRA2 sub-types; $CD28^{-}CD27^{-}$ EM3 and EMRA3 sub-types; and the $CD28^{+}CD27^{-}$ EM4 sub-type, with increasing differentiation and cytolytic activity from EM-1 to EM-4.

### 1.2.3 Viral Infection and CD8+ TLs Differentiation

CD8+ TLs play a crucial role in defense against infected cells and tumors. The understanding of the mechanisms through which the immune system controls such pathological situation, depends on the understanding of mechanisms involved in CD8+ TLs differentiation and the development of distinct memory subsets following an initial encounter with the antigen (Monteiro et al., 2007; Schluns and Lefrancois, 2003). To date, the precise pathways involved in CD8+ TLs differentiation and distinct memory subsets development have been the subject of intense debate and are not completely understood (Schluns and Lefrancois, 2003; van Lier et al., 2003). There are now data indicating that the frequency of antigenic stimulation and environmental cytokines, in particular IL-7 and IL-15 act at each stage of the immune response and are crucial factors to promoting proliferation and survival of CD8+ TLs (Gamadia et al., 2004; Kaech et al., 2003; Schluns and Lefrancois, 2003; Weng et al., 2002). Studies of CD8+ TLs demonstrate that response is related to its infection history, as T cells with specificities for different persistent viruses vary in phenotype and function (Appay et al., 2002; Gamadia et al., 2004; van Lier et al., 2003; Waller et al., 2008). The response to CMV results in an immunological response largely characterised by terminally differentiated-effector-memory subsets ($CCR7^{-}CD27^{-}CD28^{-}CD45RA^{+}$) (Khan et al., 2002; Klenerman and Dunbar, 2008; van Lier et al., 2003). CMV is a major β-herpes virus infecting above 70% of the world's wide population (Fletcher et al., 2005; Gillespie et al., 2000; Khan et al., 2002; Osawa and Singh, 2009). As with any other virus, once CMV enters the cell, it causes a primary infection. However because of the host's inability to eliminate the virus, it will lead to a latent infection whereby the presence of the virus in the host will be permanent throughout the host's lifetime. The CD8+ TLs differentiation phenotype observed following

the CMV infection is thought to occur as result of repeating activation of CMV specific CD8+ TLs due to the intermittent reactivation of CMV in the host's body.

In contrast to infection with CMV, Epstein-Barr virus (EBV) (a latent herpes virus infecting ~90% of population) infection gives rise to an early or intermediate effector-memory phenotype ($CCR7^{-}CD27^{+}CD28^{+}CD45RA^{-}$) (Appay et al., 2002; van Lier et al., 2003), whereas infection with influenza (a virus that does not cause chronic infections) exhibits an early-effector memory phenotype ($CCR7^{+}CD27^{+}CD28^{+}CD45RA^{-}$) (van Lier et al., 2003). The fact that these individual viruses have the ability to induce different responses in the memory and latency phase indicates that the immune system has developed means to establish protection during co-evolution with viruses depending both on the viral load and the biological properties of the virus (van Lier et al., 2003).

## 1.3 STRESS AND IMMUNE FUNCTION

### 1.3.1 The Birth of Psychoneuroimmunology

The term psychoneuroimmunology was coined by Robert Ader (a psychologist) and Nicholas Cohen (an immunologist) who demonstrated that immune system could be classically conditioned (Ader and Cohen, 1975). In an experiment of classical conditioning of rats Ader and Cohen (1975) fed rats with a combination of saccharin-laced water (the conditioned stimulus) and the drug Cytoxan which unconditionally induces nausea and taste aversion, and suppression of the immune system. As a result, mice learned to avoid saccharin. What was considered a surprising discovery reflected the fact that when the experiment was repeated without the injection of drug to reverse the aversion, high proportion of rats died when receiving saccharin alone (Ader and Cohen, 1975). Thus, conditioning of rats was adequate enough to cause the death of high proportions of rats and Ader and Cohen explained this

finding by proposing that rats had been immunosuppressed after receiving the conditioned stimulus. To test this hypothesis, they deliberately immunized conditioned and unconditioned animals, and exposed these and other control groups to the conditioned taste stimulus and measured the amount of antibody produced. The results showed that conditioned rats exposed to the conditioned stimulus were indeed immunosupressed indicating that a signal via the nervous system (taste) was affecting the immune system. This was one of the first scientific experiments that demonstrated that the immune system can be affected by the nervous system (Ader and Cohen, 1975).

The field growth continued and in 1981, David Felten provided one of the first indications of how neuro-immune interaction occurs by discovering a network of nerve fibers innervating the blood vessels as well as cells of the immune system (Felten et al., 1981). These nerves were also found in the thymus and spleen terminating near clusters of lymphocytes, macrophages, and mast cells, which play an important role in controlling the immune function (Felten et al., 1985).

The field of psychoneuroimmunology was further developed in 1985, by neuropharmacologist Candace Pert who revealed that neuropeptide specific receptors are present on both the cell walls of the brain and in the immune system (Pert et al., 1985). He demonstrated that neuropeptides and neurotransmitters act directly upon the immune system showing their close association with emotions and suggesting mechanisms through which emotions and immunology are deeply interdependent (Pert et al., 1985).

Existing advances in psychiatry, immunology, neurology and other integrated disciplines of medicine has promoted enormous growth for psychoneuroimmunology. Besides, it is possible that the mechanisms underlying behaviorally induced alterations of immune function, and

immune alterations inducing behavioral changes, could involve clinical and therapeutic implications that will not be fully appreciated until more is known about the extent of these interrelationships in normal and pathophysiological states.

### 1.3.2 What Is Stress?

The scientific literature defines stress in various ways. One of the most broad psychological definitions has been that stress occurs when an individual perceives that the demands from the environment exceed their adaptive capacity or ability to cope (Chen and Miller, 2007; Cohen et al., 2007; Dhabhar, 2002; Glaser and Kiecolt-Glaser, 2005). A more specific definition of stress, describes it as a process consisting of a series of steps. First, a stressor (1. a stimulus from academic examinations to bereavement) initiates a conscious or sub-conscious reaction in the brain that once perceived (2. stress perception), initiates an emotional (i.e. anxiety and embarrassment), or behavioural, (fight/flight) response (3. stress response) (Chen and Miller, 2007; Dhabhar, 2002). Within this conceptualization of stress, two different types of stress have been distinguished. These are the acute stress and chronic stress. Acute stress represents the reaction to an immediate threat, commonly referred to as the fight or flight response. This type of stress is time-limited in duration lasting for a period of minutes to hours and can come any time through life experiences. Thus, acute stress deals with unexpected situations which may be experienced consciously and unconsciously, real or imagined as a danger thing. Example of common acute stessors include: noise, crowding, isolation, hunger, danger, infection, imagining a threat or remembering a dangerous event. It is important to note that generally once the acute threat has passed, levels of stress hormones return to normal and the individual reaches the relaxation response.

In contrast to the short-lived acute stress response, chronic stress refers to on-going stressful situations eliciting prolonged responses persisting for months, as result of stable life

difficulties. Examples of common chronic stressors include: long term relationship problems, on-going highly pressured work, loneliness and persistant financial worries. Both categories of stress influence the immune function in various ways (Chen and Miller, 2007; Dhabhar, 2002; Glaser and Kiecolt-Glaser, 2005) (see section 1.3.3). The physical changes in response to both types of stress are understood by researchers since in prehistoric times, to be an essential adaptation for meeting natural threats. Thus in modern times, stress response can be an asset for raising levels of performance during critical events such as a competitive sports activity, an important job interview, or in unexpected situations of actual danger or crisis.

### 1.3.3 Acute and Chronic Stress

Acute psychological stress has a number of characteristic effects on human cellular immune system that enhance the innate immune response. Previous animal studies have shown that acute stress is associated with enhanced skin delayed-type hypersensitivity responses, enhanced inflammatory phase of both innate and adaptive immune responses, an increase in the T cell dependent antibody production (mainly IgG), an increase in the leukocyte trafficking in the site of infection, faster wound healing and enhanced vaccination responses (Atanackovic et al., 2006; Dhabhar, 2002; Dhabhar and McEwen, 1997; Silberman et al., 2003). Whereas some immunoenhancement effects of the acute stress may increase the severity of inflammatory (i.e. cardiovascular disease) or autoimmune diseases (arthritis or multiple sclerosis), others could be potentially beneficial as for example in the context of wound healing, vaccination or resistance to infection. Furthermore, the effect of acute stress is also important in preparing the immune system for responding immediately to the immunological challenge (i.e. wounding or infection) imposed by the action of a stressor (a predator) (Atanackovic et al., 2006; Dhabhar, 2002; Dhabhar and McEwen, 1997; Viswanathan et al., 2005; Viswanathan and Dhabhar, 2005).

In contrast to the acute stress which is associated with immunoenhancement, chronic stress induces changes in the immune system that result in immunosuppression which has negative health consequences (Dhabhar and McEwen, 1996). For example caregiving for a spouse with dementia is associated with poor antibody and virus specific T cell responses to influenza virus vaccination and can also significantly inhibit the IgG antibody response to a pneumococcal bacterial vaccine when compared to non-caregiving control (Glaser, 2005; Glaser and Kiecolt-Glaser, 2005; Glaser et al., 2000; Kiecolt-Glaser et al., 1996). Furthermore, evidence has associated chronic stress with slower wound healing (Glaser and Kiecolt-Glaser, 2005; Kiecolt-Glaser et al., 2010; Kiecolt-Glaser et al., 2009; Kiecolt-Glaser et al., 2005) and with increased susceptibility to the common cold and upper respiratory infections thus indicating its potential clinical relevance which possibly reflects the wide emphasis of chronic stress in the literature (Zorrilla et al., 2001).

### 1.3.4 Effect of Acute Stress on Leukocyte Distribution in Blood

Acute psychological stressors causes large, rapid and reversible changes in the distribution of peripheral blood leukocyte subpopulations which may determine the ability of the immune system to induce an antigen specific, cell mediated immune response in vivo and thus perform its surveillance (Benschop et al., 1996b; Bosch et al., 2003; Bosch et al., 2005b; Dhabhar et al., 1996; Silberman et al., 2003). Previous studies have reported that both the psychological and physical acute stress cause a transient increase in lymphocyte numbers termed lymphocytosis (within 10 minutes) (Anane et al., 2009; Anane et al., 2010; Atanackovic et al., 2006; Benschop et al., 1996b; Campbell et al., 2009; Dhabhar et al., 1995; Dimitrov et al., 2010; Silberman et al., 2003; Simpson et al., 2007; Viswanathan and Dhabhar, 2005). The lymphocytosis phenomena is characterised by a large increase (400%) in relative percentages of NK cells and other lymphocytes (150 % in T cells and B cells) as well as an increase in the

absolute number of monocytes and granulocytes. These immunological alterations are catecholamine-mediated (Atanackovic et al., 2006; Benschop et al., 1996b; Dhabhar and McEwen, 1997; Silberman et al., 2003).

Catecholamines (CAs) are neuro-endocrine signalling substances that participate in regulation of the activities of key life support systems, such as that of the immune system. Example of CAs include, epinephrine (EPI), norepinephrine (NE), dopamine etc., which among other things are used to treat cardiac arrest and low blood pressure. CAs are synthesized from the amino acid precursor L-tyrosine and are released from the adrenal medulla and sympathetic nerve terminals into the blood, during stressful stimulations like strenuous exercise, physical trauma, inflammation, and psychological stress. Upon their entry into the blood from the site of release (Kvetnansky et al., 2009) CAs modulate the immune response via receptors expressed on the immune cells (Szelenyi and Vizi, 2007). Indeed CAs, EPI and NE exert their effects by binding to 7 transmembrane spanning G-protein-coupled cell surface receptors termed adrenoreceptors.

The four major types of adrenoreceptors by which the CAs mediate their effects are ($\alpha$1, $\alpha$2, $\beta$1, $\beta$2). These are all expressed on the surface of many different cell types throughout the body. It is now reported that whereas $\alpha$-adrenoreceptors are responsible for the changes on granulocyte trafficking, the changes in lymphocyte trafficking are driven by $\beta$-adrenoreceptors ($\beta$-AR) which mediate endothelial detachment and subsequent re-circulation (Anane et al., 2009a; Anane et al., 2010a; Benschop et al., 1996b; Bosch et al., 2003; Bosch et al., 2005a; Campbell et al., 2009; Dimitrov et al., 2010; Silberman et al., 2003; Viswanathan and Dhabhar, 2005). This is confirmed by the fact that when $\beta$-AR are blocked with propranolol (a nonselective $\beta$-blocker) prior to infusion with EPI, lymphocytosis is

completely inhibited (Benschop et al., 1996b; Gader, 1974). Similarly, administration of β-AR antagonists also abrogates exercise or psychological stress induced lymphocytosis (Benschop et al., 1996a; Benschop et al., 1994a). Studies have indicated that of all β-AR, lymphocytosis is mainly underlined by the involvement and activation of β2AR in particular. For example, adhesion of NK cells to endothelial cells *in vitro* can be reversed in a β2AR specific way and mobilization of NK cells, during catecholamine infusion is inhibited by β2AR selective antagonists (Benschop et al., 1996a; Benschop et al., 1994b; Benschop et al., 1996b).

While all lymphocyte express β2AR on their surface, lymphocytosis in psychological and physical acute stress is largely governed by a selective increase in the cytotoxic lymphocytes, which express high density β2AR on their surface. Of all cytotoxic cells, NK cells have the highest β2AR expression, followed by CD8+ TLs and γδ TLs which display intermediate β2AR expression and ultimately CD4+ TLs and B cells with low β2AR expression (Anane et al., 2009; Anane et al., 2010; Campbell et al., 2009).

Aside from the β2AR expression, the selective mobilisation of leukocytes is also determined by the specific profile of adhesion molecules expressed on the cells as well as the cell's cytotoxic potential. Based on this context, studies have reported that within the cytotoxic lymphocyte category, acute stress mostly mobilises cytotoxic cells that have a great capacity to migrate to sites of infection as well as display a differentiated memory phenotype. These include $CD45RA^{+}$ EMRA effector memory cells (e.g. γδ and CD8+ T cells), cytotoxic cells possessing high tissue migratory potential ($CD11a^{high}$ and $CD62L^{low/neg}$) and cytotoxic cells (e.g. CD8+ TLs) that have shortened telomeres and exhibit high expression of NK receptors KLRG-1 and $CD57^{+}$ (features of replicative senescence) (Anane et al., 2009; Anane et al.,

2010; Bosch et al., 2003; Campbell et al., 2009). It is important to emphasize that is through these important phenotypic characteristics that cytotoxic cells gain the potential to quickly destroy foreign antigens present in the body's peripheral tissue.

### 1.3.5 Possible Mechanisms of Lymphocytosis

To be able to understand what regulates and controls the levels of circulating lymphocytes, there's need for understanding the mechanisms involved in lymphocytosis. Several agents have been utilised to cause an increase in the number of blood lymphocytes count in experimental animals, including heparin and heparinoid compounds which induce the lymphocytosis as a result of mobilisation of cells from lymphoid organs (Rai et al., 1971); partially purified lymphocytosis-promoting factor (LPF) (Adler and Morse, 1973); as well as *Bordetella pertussis* toxin which has been mostly used to investigate lymphocyte physiology (Rai et al., 1971). *Bordetella pertussis* is a gram-negative coccobacillus of the genus *Bordetella,* responsible for the whooping cough. In previous studies *Bordetella pertussis* has been used as a nonspecific immune stimulant to increase the immunological activity in mice (Rai et al., 1971). It has been reported that the significant lymphocytosis induced by *Bordetella pertussis* could involve one or several mechanisms and disrupt the normal lymphocyte circulation pathway, including (1), an increased rate of lymphocytes entry to into the circulation via the efferent lymphatics; (2) a prolonged average residence time of lymphocytes in blood (thus a decrease in the lymphocytes rate of exit from the blood via postcapillary venules); (3) direct entry of lymphocytes into the blood vessels without passing through lymphatics, or in addition to lymphatics; (4) increased new cell production (Morse and Riester, 1967; Rai et al., 1971). Morse and Barron(1970) reported that lymphocytosis induced in mice by *Bordetella pertussis* could not be a result of cellular proliferation of lymphocytes, as the percentage of circulating lymphocytes with nuclear label was the same in

pertussis treated mice and normal untreated mice. These reports support the concept that lymphocytosis is a result of redistribution of lymphocytes between the circulating (blood) and the tissue pool (lymphoid organs) with the thoracic duct serving as the main channel for the lymphocytes traffic between the two pools (Gowans, 1957). The thoracic duct output of lymphocytes in mice during the *Bordetella pertussis* induced lymphocytosis is significantly decreased despite the high level of circulating lymphocytes compared to normal mice (Adler and Morse, 1973; Morse and Barron, 1970). Chigaev et al. (2008) has demonstrated that a possible mechanism for cell de-adhesion involves the downregulation of $\alpha_4\beta_1$-integrin affinity by guanine nucleotide binding protein-coupled receptors (GPCR) on the peripheral blood leukocytes. Dimitrov et al. (2010) suggested that selective mobilisation of cytotoxic leukocytes by EPI involves the disruption of LFA-1 (lymphocyte function- associated antigen-1; equivalent to CD11a) and CX3CR1 binding to endothelium. LFA-1 (CD11a) and CX3CR1 are highly expressed on the cytotoxic effector function cells, γ/δ T cells, NK cells, and these cytotoxic cells show highest adherence to activated endothelium *in vitro* and rapid release in response to EPI.

### 1.3.6 Neuroendocrine Interaction with Immune System

The brain and the immune system are the two major adaptive systems of the body. Although the immune system is regarded as autonomous, the last two to three decades have provided evidence that the central nervous system (CNS) receives messages from the immune system and *vice versa* messages from the brain modulate immune functions. This has led to the emergence of the field of psychoneuroimmunology in which the brain and the immune system "talk to each other" a process essential to maintaining homeostasis (Black, 1994; Elenkov, 2008; Ziemssen et al., 2003). The two major pathways through which the immune system can be altered are the hypothalamic-pituarity-adrenal-axes (HPA axis) and the sympathetic-

adrenal medullary axis (SAM axis) (Black, 1994; Padgett and Glaser, 2003). Lymphocytes, monocytes or macrophages are directly influenced by the HPA and SAM axis because they display receptors on their surface for many neuroendocrine products of these axis including cortisol and CAs, which are released during stress (Padgett and Glaser, 2003).

In effect, CAs EPI and NE represent the major stress hormones and influence various aspects of the immune responses (Elenkov et al., 2000a). These include proliferation of B and T cells, production of cytokines, production of antibodies, antigen presentation, macrophage activity, cytotoxicity of NK and T cells, chemotaxis of monocytes and neutrophils as well as lymphocyte traffic and circulation (Elenkov et al., 2000a; Madden et al., 1995; Padgett and Glaser, 2003; Wiegers et al., 1995; Yang and Glaser, 2000).

## 1.4 Effect of Catecholamines on the Immune Responses

### 1.4.1 Proliferation of Lymphocytes

Previous studies have shown that CAs or βAR agonists inhibit the proliferation of T cells *in vitro*. For example, stimulation of T cells with isoproteronol, (βAR agonist) increases cyclic adenosine monophosphate (cAMP) (a second messenger involved in the signal transduction) in lymphocytes. Elelvated cAMP is associated with inhibition of T cell proliferation (Bartik et al., 1994; Elenkov, 2008; Elenkov et al., 2000a). Among the T cells populations, the proliferative response of $CD8^+$ T cells is inhibited at a greater extent by cAMP than the $CD4^+$ T cells. This is thought to occur as results of $CD8^+$ T receptors having higher numbers of β-AR expressed on their surface (Bartik et al., 1993). In addition, the elevation of the cAMP also inhibits IL-2 secretion (a cytokine that promotes T-cell proliferation) by T cells (Bartik et al., 1994; Bartik et al., 1993).

Another mechanism, by which CAs might inhibit T-cell proliferation, is through modulation of $K^{+-}$ channel gating. These channels represent the dominant ion channels in lymphocytes and are involved in the processes of lymphocyte activation and proliferation (Elenkov et al., 2000b). NE and isoproterenol inactivate $K^{+}$ channels of human $CD8^{+}$ peripheral lymphocytes and rat thymocytes (Elenkov, 2008; Vizi et al., 1995).

### 1.4.2 Catecholamines and the TH-1/TH-2 Balance

CAs inhibit type-1/pro-inflammatory cytokine production. Both EPI and NE inhibit Th-1 responses by suppressing the production of IL-12 (major inducer of Th-1 responses) from monocytes and dendritic cells through stimulation of the β2AR (Elenkov et al., 1996). It is thought that the inhibition of IL-12 may re-present one of the major mechanisms by which CAs affect the Th-1/Th-2 balance, as IL-12 is a potent enhancer of IFN-γ synthesis by Th-1, and inhibitor of IL-4 synthesis by Th-2 cells (Elenkov et al., 2008; Elenkov et al., 1996; Panina-Bordignon et al., 1997). Moreover, EPI, NE and β2AR agonists inhibit the production of TNF-α by monocytes, microglial cells, as well as result in the indirect inhibition of IL-1 via inhibition of TNF-α and potentiation of IL-10 production (Hetier et al., 1991; Nakamura et al., 1998; Severn et al., 1992).

In contrast to their effects on type 1 pro-inflammatory cytokine production, CAs upregulate the production of regulatory cytokines by APCs including the production of IL-10, (an effect that is β2AR mediated and cAMP dependent) induced by lipopolysaccharides (LPS) in human monocytes or mouse peritoneal macrophages. Similarly, the massive release of CAs during an acute brain trauma, results in secretion of substantial amounts of systemic IL-10 (Elenkov, 2008; Elenkov et al., 1996; van der Poll et al., 1996).

### 1.4.3 Effect of Catecholamines on NK-cell Activity

*In vitro* studies show that EPI and isoproterenol elevate cAMP about 2.5 fold and induce an inhibition of NK cell activity (Elenkov et al., 2000b; Hellstrand and Hermodsson, 1989; Whalen and Bankhurst, 1990). The effect is blocked by propranolol (a beta blocker) and is mimicked by terbutaline, (a β2AR agonist) indicating the involvement of β2AR in this process (Elenkov et al., 2000b; Hellstrand and Hermodsson, 1989; Whalen and Bankhurst, 1990). In support of these findings when a β2AR agonist is administered *in vivo*, NK activity is suppressed an effect blocked by nadolol (a peripherally acting βAR antagonist) (Katafuchi et al., 1993a; Katafuchi et al., 1993b; Shakhar and Ben-Eliyahu, 1998). In patients with heart failure, (a disease characterized by chronically high levels of plasma NE), NK cells show an anergic cytotoxic low response to the stimulation with IL-2 and IFN-γ (Vredevoe et al., 1995). Thus CAs may also inhibit NK cells activity indirectly through the suppression of Th1-type cytokines, and particularly through the potent inhibition of IL-12 and IFN-γ production which are essential for promoting NK activity (Elenkov et al., 2000). NK cells are the most "sensitive" cells to the suppressive effect of stress. The reason why we observe the potent suppressive effect of CAs on the NK cell activity might be due to the fact that NK cells probably possess the highest number of β2AR among lymphoid cells (Irwin, 1994).

### 1.4.4 Catecholamines and T-Cytotoxic Activity

There are only few observations on the effect of CAs on CD8+ TLs cytotoxicity. Studies have shown that EPI, NE and isoproterenol inhibit the *in vitro* generation of anti MOPC tumor (a murine myeloma tumour) cytotoxicity by splenic T cytotoxic lymphocytes (Cook-Mills et al., 1995; Elenkov et al., 2000b). Furthermore, increasing cAMP with either a cAMP analogue or the βAR agonist metaproteronol significantly inhibits the *in vitro* development of memory T cytotoxic activity in mice (Bender et al., 1993; Elenkov et al., 2000b).

### 1.4.5 Salmeterol

Salmeterol is a potent selective β2AR agonist that exhibits a long duration of action on airways smooth muscle *in vitro* but it is also a long acting bronchodilator *in vivo* (Ball et al., 1991; Butchers et al., 1991; Johnson, 1990; Nials et al., 1993).

It has been reported that the sustained activity of salmeterol does not result from the irreversible binding to β2AR. The lipophilic N-substituent "tail" of salmeterol associates with an "exo-site" which holds salmeterol in a such position that allows its phenylethanolamine "head" to continuously associate/disassociate with, and activate/inactivate the β2AR (Ball et al., 1991; Nials et al., 1993). Another possible explanation for the long duration effect of salmeterol could result in part from the lipid solubility of the compound (Ball et al., 1991).

A marked difference between salmeterol and other βAR agonists is the slower rates of action onset and offset. Whilst isoprenaline (a non-selective βAR agonist) and salbutamol (a β2AR agonist) have a rapid action onset ($Ot_{50}$ < 3 minutes) salmeterol has an onset of action of $Ot_{50}$ < 27.6 minutes) ($Ot_{50}$ refers to the time of onset of action and is defined as the time from starting the administration of the test agonist to achievement of 50% of response maximum). Moreover, whereas both isoprenaline and salbutamol are short acting ($Rt_{50}$ < 7 minutes), the effect of salmeterol can last in excess of 8 hours (Nials et al., 1993). (Rt refers to the time for offset of action and is defined as the time from stopping the administration of the test agonist to achievement of 50% of the response maximum). Furthermore, clinical preliminary data has also indicated salmeterol to exhibit long lasting bronchodilator activity (lasting up to 12 hours) following aerosol administration to both normal and asthmatic subjects (Ball et al., 1991). However, similarly to isoprenaline, the rate of reversal of salmeterol response by β-antagonists propranolol, pindolol, ICI118, 551 and atenolol was rapid, requiring ~11 minutes or less to reach 50% reversal of agonist activity (Nials et al., 1993).

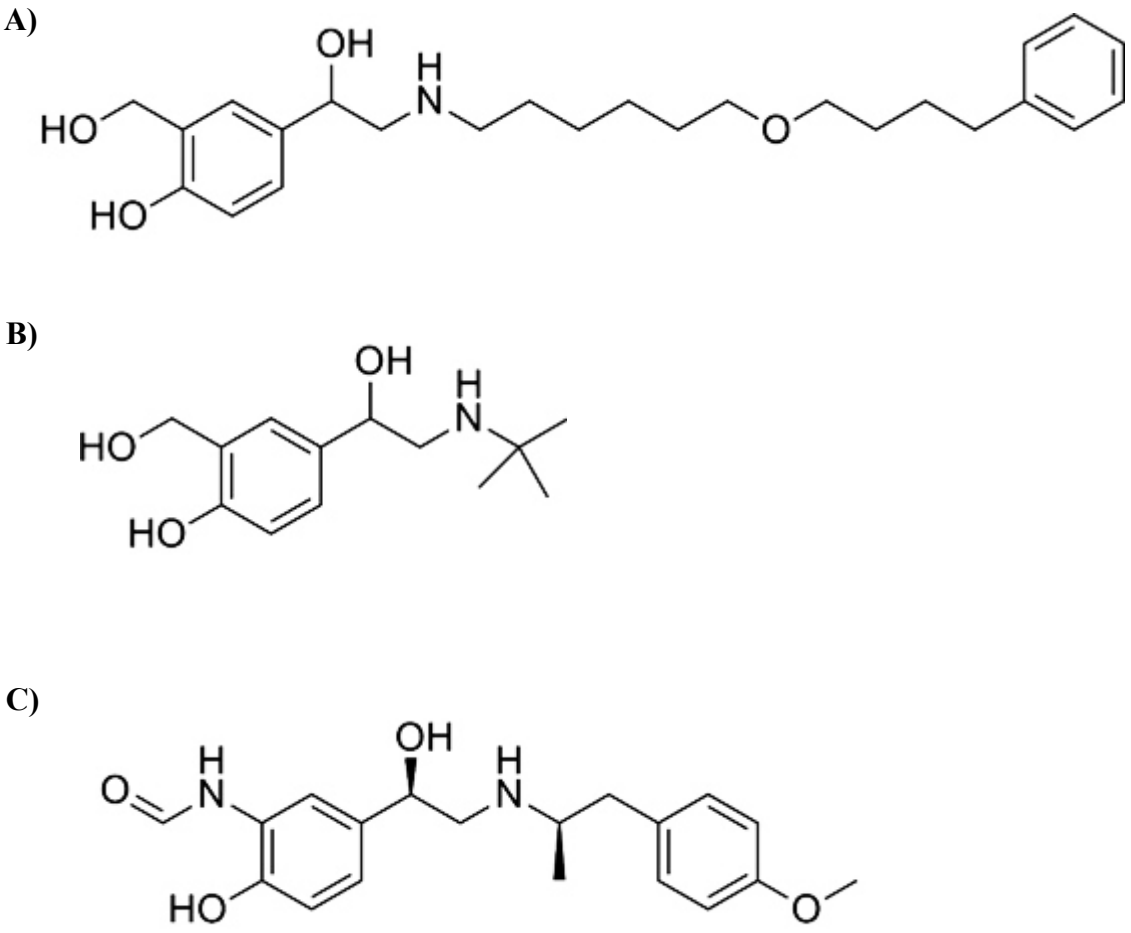



**Figure 1.5. Structure of salmeterol (A), salbutamol (B) and formoterol (C).** β-Agonists have an asymmetric centre due to the presence of β-OH group. The asymmetric centre leads to the molecule existing as a pair of optical isomers known as R and S enantiomers in a racemic mixture. The enantiomers are stereospecific. **S**almeterol (A) has an aryl alkyl group with a chain length of 11-atoms from the amine. This bulkiness renders the compound more lipophilic and it also makes it more β2-receptor selective. Salbutamol (B) is also more selective for β2-receptors with the tertiary butyl group in salbutamol being responsible for its selectivity. Fenoterol (C), is a short acting β2-AR agonist and moderately lipophilic. It has two asymmetric centres, producing four enantiomers. Figure was taken from (Shan et al., 2012).

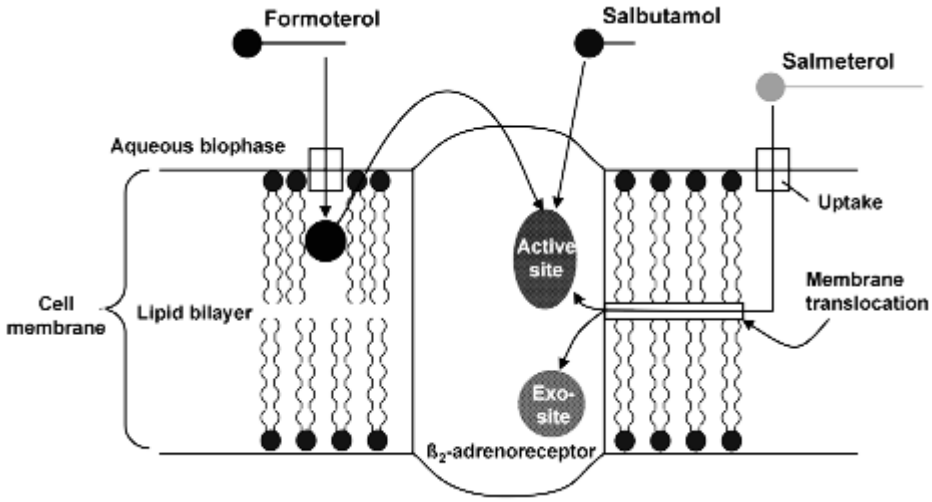



**Figure 1.6. A representative model for the interaction of salmeterol, salbutamol, and formoterol with the β2-AR.** Salmeterol is >10 000 times more lipophilic than salbutamol. Salmeterol partitions rapidly (<1 min) into the cell membrane. It then diffuses laterally in order to approach the active site of the β2-AR in the membrane. This process is slow (>30 min). The nature of salbutamol is hydrophilic. It enters the active site of the β2-AR directly from the extracellular aqueous compartment. Salbutamol has a limited residency time at the receptor active site resulting in a short duration of action (4-6 h). Formoterol is moderately lipophilic. It is taken into the cell membrane in the form of a depot, in a way that it progressively leaches out from the depot to interact with the active site of the β2-AR. The size of the depot is directly proportional to the concentration or dose of formoterol applied. Figure was taken from (Johnson, 2001).

## 1.5 Cell Migration

### 1.5.1 Leukocyte Extravasation during Inflammation

Due their migratory properties, white blood cells are recruited rapidly at sites of injury and infection to allow efficient surveillance of tissues for infectious pathogens. The recruitment process initiates in postcapillary venules which display a high intravascular shear force of up to ~30 dynes/cm$^2$ (Butcher and Picker, 1996). This process is responsible for allowing leukocytes to exit the blood, migrate across the vascular endothelial cells, and enter the surrounding tissue (Fig.1.7.). The recruitment process is a "multistep paradigm" split into five successive steps. Initially, flowing leukocytes are marginated to the vessel wall. It is important to note that this process will only occur if the endothelium has been activated by chemical mediators of inflammation such as TNF-α, IFN-γ, and IL-1β. These mediators alter the receptor repertoire on the endothelium, e.g., upregulation of some selectins and members of the immunoglobulin superfamily (IgSF) (Fabbri et al., 1999; Francischetti et al., 2010; Springer, 1995). It is these molecular signals or "area codes" which are displayed on endothelium that will control the immigration of lymphocytes to inflamed tissues (Alon et al., 1995; Middleton et al., 2002; Springer, 1995).

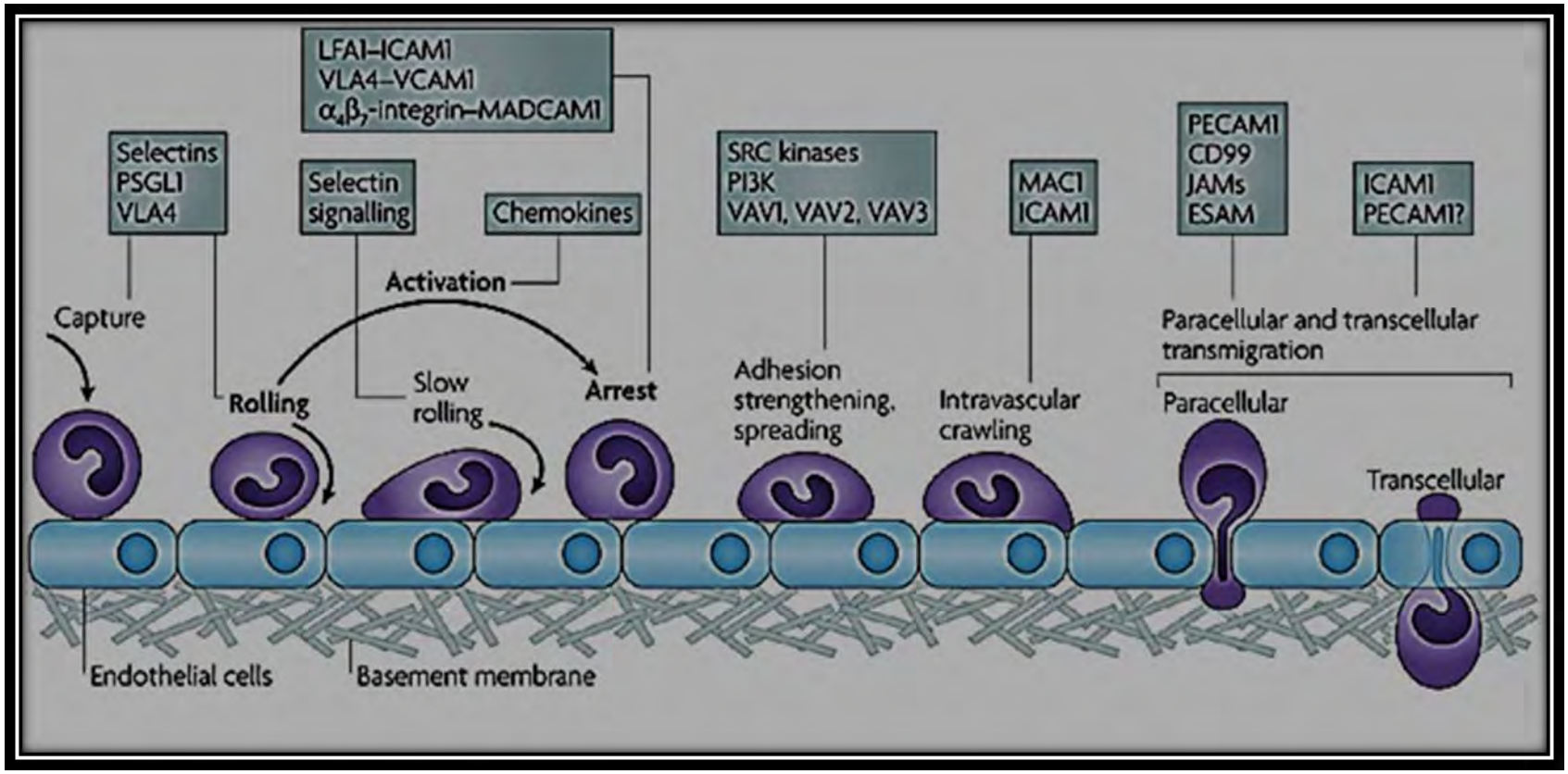



**Figure 1.7. Schematic representation of stages involved in leukocyte recruitment and migration into tissue.**
Adapted from (Ley et al., 2007).

### 1.5.2 The Multi-Step Paradigm - Capture and Rolling

In simple terms, margination is referred to as the transport of leukocytes to the endothelium wall. The flowing of blood in the body, applies a shear stress (blood velocity) to the endothelium and the leukocytes in the circulation. Under inflammatory conditions when the endothelium is activated, the blood stream facilitates the contact of leukocytes with the vessel. An *in vitro* study that involved perfusing whole blood through a verticular glass capillary (microslide) showed margination to be regulated by increasing the haematocrit and by erythrocytes aggregation, as both of these augmented the number of rolling leukocytes (Abbitt and Nash, 2003).

Once in close contact with the vessel, selectins expressed upon the activated endothelium lining the vessel will capture the leukocytes. The selectins are membrane glycoproteins with an amino terminal C-type lectin domain which bind specific carbohydrate with terminal components that include α2,3-linkedsialic acid and α1,3 linked fructose groups symbolized by the sialyl Lewis x ($sLe^x$) determinant on their respective ligands (see appendix; Table A.2) (McEver, 2002). These glycoproteins are expressed exclusively by bone-marrow derived cells and endothelial cells (Marelli-Berg et al., 2008). The selectin family consists of three closely related cell surface molecules. These include L-selectin (CD62L) which is expressed on leukocytes; P-selectin (CD62P) expressed on platelets, and E-selectin (CD62E) expressed on vascular endothelium. L-selectin is expressed on all circulating leukocytes such as neutrophils and T cells and plays an important role in mediating the binding of lymphocytes to the endothelium present in post capillary venules of lymph nodes and leukocyte rolling on vascular endothelium at sites of tissue, injury or inflammation (Lawrence et al., 1994; Ley et al., 1995; Rosen, 2006; Tedder et al., 1995). Upon leukocyte activation, L-selectin is shed (Jung and Dailey, 1990). E selectin is induced on the vascular endothelial cells by cytokines,

therefore its expression is limited to inflamed tissues (Springer, 1994). P-selectin is constitutively stored in Weibel-Palade bodies of endothelial cells and in alpha granules of platelets. Once it is activated by the thrombogenic and inflammatory mediators, P-selectin is mobilized to the cell surface and mediates adhesion of neutrophils and monocytes at sites of injury (Gibson et al., 1995; Lawrence et al., 1994; Lawrence and Springer, 1991; Tedder et al., 1995).

In addition to *in vivo* studies, *in vitro* studies have also provided insights on selectin functions. It has been shown that purified CD62P and CD62E selectins support neutrophil capture from flow (Abbitt and Nash, 2001; Lawrence and Springer, 1991, 1993). This mainly occurs as a result of interactions of selectins with P-selectin glycoprotein ligand-1(PSGL-1) expressed on the surface of all blood neutrophils, monocytes and lymphocytes (Alon et al., 1994; Asa et al., 1995; McEver, 2002; Tedder et al., 1995).

Rolling follows the capture or contact of the circulating leukocytes with the luminal aspect of inflamed endothelium lining vessel wall. This involves separable contact formation "tethering" which allows the lymphocytes to roll on the surface of vessel wall in the direction of the blood flow (Springer, 1994). CD62P but not CD62E selectin supports T cell rolling in activated endothelium (Luscinskas et al., 1995). Interestingly, leukocyte rolling has been challenged by findings that some integrins, can support leukocyte rolling under reduced psychological shear rates. In fact, $\alpha_4\beta_1$ integrin (also denoted as VLA-4; very late antigen-4) can support T cell rolling on purified vascular cell adhesion molecule-1 (VCAM-1), purified mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and activated endothelium in vitro (Alon et al., 1995; Berlin et al., 1995; Johnston et al., 1996; Ley and Tedder, 1995; Luscinskas et al., 1995; Tedder et al., 1995).

Furthermore, $\alpha_4\beta_7$ integrin (also known as LPAM-1; Lymphocyte Peyer's patch adhesion molecule-1) supports T cell rolling on purified MAdCAM-1 and lamina propria in situ (Berlin et al., 1995). In contrast to selectin mediated rolling that occurs in high shear rates, integrin-dependent rolling is less efficient and is limited to lower shear rates (Ley and Tedder, 1995). The importance of selectins is highlighted in leukocyte adhesion deficiency syndrome type 2 (LAD II) (Moser et al., 2009). In this syndrome defects in the glycosylation of the selectin ligands impairs neutrophil capture, resulting in immunodeficiency and re-current infections.

### 1.5.3 Activation and Firm Adhesion

Initial capture of lymphocytes is followed by activation of the lymphocytes by surface presented chemokines. Chemokines are chemotactic cytokines, produced during endothelial cell activation, that bind to their respective receptors expressed on the rolling leukocytes activating integrin adhesiveness and directing leukocyte migration (Chigaev et al., 2008; McGettrick et al., 2009; Springer, 1994). Integrins are a large family of cell surface receptors that mediate cell-cell interaction and cell matrix interactions in diverse biological settings (Chen et al., 1999). Their structure consists of a transmembrane molecule that contain a non-covalently associated α and β subunit with characteristic structural motifs (Fig.1.8.). Several integrins are important in the interaction of leukocytes with endothelial cells. Their primary ligands include matrix proteins and members of the IgSF as described in Table A.3. (see appendix). Since integrins exist in three different affinity states: low, intermediate and high (Mould and Humphries, 2004), one of the ways in which chemokines are understood to trigger integrin activation includes conformational changes of the heterodimer in the integrin ligand binding site. This causes the integrin to shift from the low affinity state to a high affinity state (Laudanna et al., 2002).

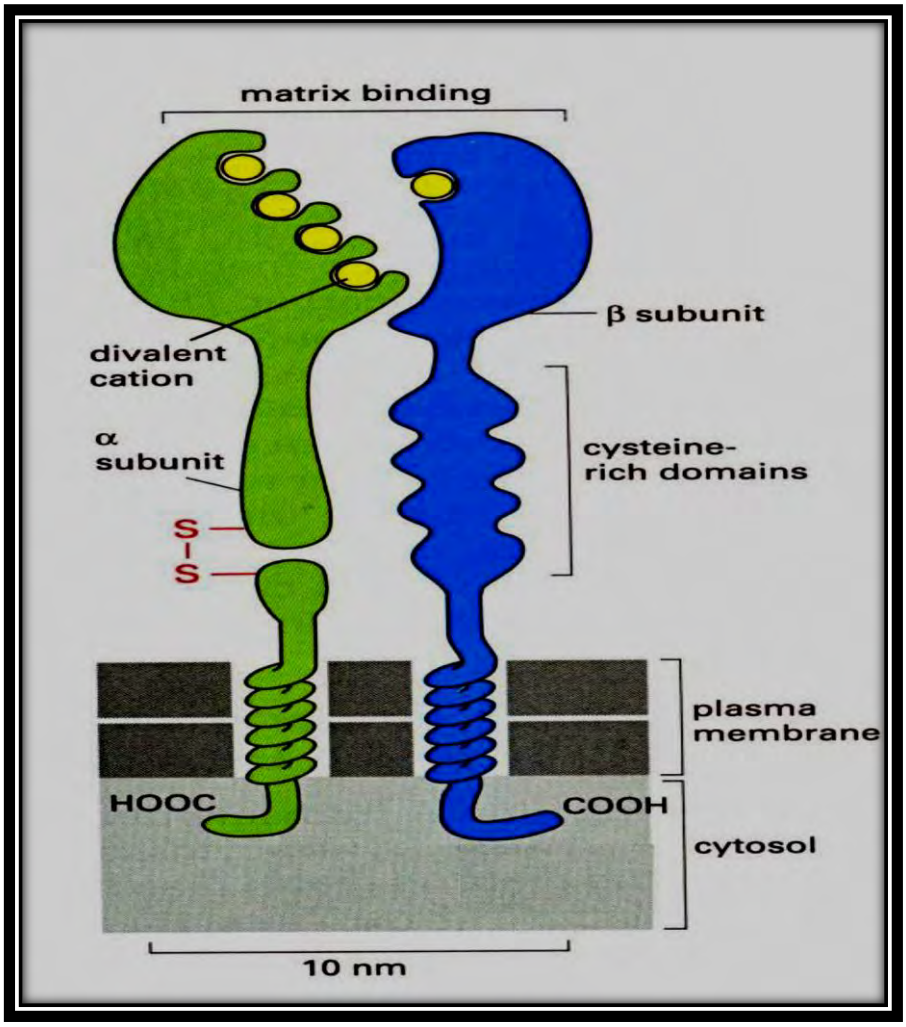



**Figure 1.8. Integrin structure.** Integrins are heterodimeric cell surface receptors. Each integrin is formed by the non covalent association of α and β subunits with characteristic structural motifs. Both α and β subunits consist of large extracellular domains, small transmembrane domains, and short cytoplasmic domains. The extracellular domains serve to bind extracellular ligands, such as fibronectin (FN); whereas the short cytoplasmic domains, bind intracellular anchor proteins providing a link between the extracellular environment and the cytoskeleton. The α and β subunits of integrins differ in the number of extracellular domain binding sites. The extracellular domain of the α subunit contains four divalent-cation binding sites, whereas the extracellular domain of the β subunit contains a single divalent-cation-binding site and a cysteine-rich region (Alberts et al., 2002).

### 1.5.4 Transmigration

The final step in the leukocyte recruitment pathway is transmigration. Transmigration plays a crucial role in maintaining immune surveillance and mounting rapid inflammatory responses to invading pathogens (Adams and Nash, 1996). It is thus considered to be a key event in host defense (Nourshargh and Marelli-Berg, 2005). Migration is dependent on two factors, regulation of integrin adhesivity and actin polymerization (Adams and Nash, 1996; Sheikh et al., 1997). Leukocyte endothelium migration also involves a group of proteins such as platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) CD99, VE-cadherin and junctional adhesion molecule-1 (JAM-1) (Luscinskas et al., 2002a, b; Nourshargh and Marelli-Berg, 2005).

Leukocytes migration occurs through junctions between adjacent endothelial cells (paracellular route) or through the body of the endothelial cells (transcellular route). During this process of migration the flow forces act upon the cells continuously, allowing the cells to release and rebind as they move. The cells first migrate into the subendothelial space, where they must penetrate the underlying basement membrane to be able to move through underlying stromal cells and structures towards the site of infection. A chemotactic gradient guides the migration of leukocytes.

Considering the importance and complexity of migration, it is unlikely that a single type of receptor-ligand interaction could have the required physical characteristics to support all stages of this process in a controlled manner (Adams and Nash, 1996). Instead, a number of adhesion molecules and chemokines are involved in this process. It is important to note that there are variations in adhesion receptors and chemokines utilised by different cell types, such as neutrophils, and T cells. Despite this, the leukocyte recruitment pathway is similar for all leukocytes.

### 1.5.5 Transmigration of T cells

Lymphocyte extravasation is a highly regulated process. Subpopulations of lymphocytes emigrate at specific sites according to their characteristic leukocyte repertoire. Studies in both mice and humans indicate that memory $CD4^+$ T cells bearing $\alpha_4\beta_7$ but lacking CD62L would potentially be capable of entering the gut mucosal tissues (mesenteric lymph nodes, Peyer's patches, and lamina propria) which express MAdCAM-1 (Bradley and Watson, 1996). On the other hand, a subset of memory T cells expressing high levels of $\alpha_4\beta_1$ migrate preferentially to nongastrointestinal, extralymphoid, inflammatory sites, in which VCAM-1 is expressed (Rose et al., 2001).

It has been shown that memory T cells have increased levels of $\alpha_L/\beta_2$ (LFA-1; lymphocyte function-associated antigen-1) and $\alpha_4\beta_1$ integrins compared with naïve cells (Johnston et al., 1996; Nakajima et al., 1994). Therefore in addition to induction of VCAM-1 and intercellular adhesion molecule (ICAM-1) on endothelium, the increased expression of $\alpha_L/\beta_2$ and $\alpha_4\beta_1$ integrins on memory T cells might also contribute to the T cell infiltration into the airways. Indeed, memory T cells show stronger adhesion to cytokine stimulated cultured endothelial cells than naïve cells (Nakajima et al., 1994).

Regulation of leukocyte trafficking is controlled by the interaction of cell surface adhesion molecules between leukocytes (i.e. integrin abundance) and vascular endothelial cells (counter ligand density) (Nakajima et al., 1994; Rose et al., 2001). Recent studies have suggested that $\alpha_4\beta_1$ alone can initiate the lymphocyte endothelial cell interactions as well as mediate cell adhesion (Bradley and Watson, 1996). For example, leukocyte rolling and adhesion within mesenteric postcapillary venules in chronic vasculitis (model of inflammation) is dependent on $\alpha_4\beta_1$ integrin (Johnston et al., 2000). Moreover, other in vivo

studies have shown that the interaction of $\alpha_4\beta_1$ ligand (VCAM-1) with $\alpha_4\beta_1$ integrin (VLA-4) plays a predominant role in controlling antigen induced T cell recruitment into the tissue and that induction of VCAM-1 expression on the endothelium at the allergic inflammatory sites, regulates this T cell recruitment. This is because the in vivo blocking of VCAM-1 and $\alpha_4\beta_1$ integrin (VLA-4) inhibits the antigen induced $CD4^+$ and $CD8^+$ T cell infiltration into the mouse trachea more potent than that of ICAM-1 and $\alpha_L/\beta_2$ (LFA-1) (Nakajima et al., 1994). The crucial role of integrins in T cell migration can also be observed in and experimental model of arthritis in rats. These studies show that monoclonal antibodies (mAb) which block $\alpha_4$ (CD49d) and $\beta_2$ (CD18) integrins, inhibit T cell tissue-migration and hereby reduce the severity of arthritis (Issekutz et al., 1996).

It is important to note that the functions of integrins are dependent on integrin activation. This is because when integrins activate, they undergo a conformational change within a few minutes that increases integrins adhesiveness to ligand without affecting the quantity of surface-expressed receptors. However, increased integrin affinity is also a result of altered interaction with cytoskeleton.

**1.6 Aims of this Thesis:**

1. Previous studies have shown that central nervous system activation, (e.g., during psychological stress) affect CTL function. One of the hallmarks of such activity is the release of the catecholamines epinephrine and norepinephrine, which act on β2-adrenergic receptors expressed on lymphocytes. The first aim of this project is to determine the effects of β2-adrenergic stimulation on the production of IFN-γ by CTLs. These analyses focus on CD8+ T and CD4+ T cells.

2. The effect of stress on the cytotoxic ability of CD8+ T and NK cells remains not well-understood. The second aim was therefore to investigate the effect of epinephrine and salmeterol (a $\beta_2$-adrenergic agonist), on the cytotoxic capacity of CD8+ T and NK cells in vitro. This was studied using a novel assay that measures cytotoxic degranulation.
3. Studies have documented that the selective recruitment of the leukocytes to inflamed tissues is determined by a specific match of adhesion molecules expressed on the cells and on endothelial cell surface (e.g.,VCAM-1, ICAM-1, E- or P-selectin). The final aim was to investigate the effect of different concentrations of salmeterol (β2 agonist) and EPI on lymphocytes recruitment to isolated vascular cell adhesion molecule (VCAM-1) coated microslides and onto endothelial cells. PBL Adhesion, Migration, Rolling Velocity, Transmigration and Migration velocity to the substrates were examined.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Cytokine Stimulation Assay

### 2.1.1 Study Subjects

Healthy participants were recruited from community volunteers and staff and students attending the University of Birmingham (UoB), UK. Each participant gave informed consent for participation in the study and study protocols were approved by the appropriate institutional review board (UoB).

### 2.1.2 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Whole blood from healthy CMV seropositive individuals (CMV status of donors was determined using tetramer staining tested by the Moss group) was collected in tubes coated with Lithium Heparin (anti-coagulant; 1.6 mg/ml; from BD Biosciences, UK). All blood samples from seated individuals were taken in standardized method between 9 am and 11 am. None of the individuals were on any therapy. The whole blood was gently layered onto a Histopaque gradient (2.5 ml Histopaque 1077; from Sigma-Aldrich, UK) and centrifuged for 40 minutes at 1200 x g, at room temperature. Peripheral blood mononuclear cells (PBMCs) sedimented above the diluted blood at the Histopaque interface. The PBMCs layer was collected and transferred into 50 ml Falcon tubes (BD Biosciences,UK). PBMCs were washed twice in Roswell Park Memorial Institute Medium (RPMI 1640, from Sigma-Aldrich, UK) + human serum (HS) (1%) (which was heat inactivated and pooled; a gift from Dr. Oliver Goodyear, Cancer Studies Department, University of Birmingham, UK) for 8 minutes by centrifugation at 500 x g at room temperature. Following washing, PBMCs were re-

suspended in RPMI+HS (10%) and were cultured overnight in an incubator set at 37ºC, 5 % $CO_2$ in air.

### 2.1.3 Stimulation Assay

The next morning, PBMCs were removed from the incubator and were centrifuged at 500 x g, 4 ºC, for 5 minutes. Subsequently, PBMCs were washed with MACS Buffer (containing Phosphate Buffer Saline (PBS); Bovine Serum Albumin (0.5% BSA) and Ethylenediaminetetraacetic acid (2mM EDTA); from Miltenyi Biotec, UK). Following washing, cells were counted and transferred into 4 ml FACS tubes (BD, Bioscience, UK) with each FACS tubes containing 10 x $10^6$ PBMCs. Cells were washed again and the purified peripheral blood lymphocytes (PBL) pellet was re-suspended in 60 ul of MACS Buffer at a final concentration of $10x10^6$ cells. To separate CD4+ T and CD8+ T cells, anti-human CD4 antibody directly labelled microbeads and anti-human CD8 antibody directly labelled microbeads (Miltenyi-Biotec, UK) were added to $10x10^6$ PBMCs, mixed well and incubated for 15 minutes in the refrigerator (2-8 ºC). A fraction ($\sim 5x10^{4)}$ of PBMCs were not labelled with CD4 or CD8 microbeads and were used as control (prior to sorting). Following incubation of PBMCs with microbeads, cells were washed by adding 2 ml of MACS Buffer per $10^6$ cells and centrifuged at 500 x g for 5 minutes at 4ºC. The supernatant was very carefully decanted and the pellet was gently dislodged by tickling the tube without causing air bubbles. Cells were subsequently resuspended in 500 µl of MACS Buffer, ready for separation.

Magnetic separation was carried with autoMACS$^{TM}$ Separator (Miltenyi Biotec, UK). AutoMACS is an automated magnetic separation machine that can be programmed to enrich or deplete the desired cells. Prior to separation, the instrument was prepared and primed (according to the autoMACS™ instructions). The FACS tube containing the sample was

applied and placed at the uptake port. Other FACS tubes for collecting the unlabelled and labelled (CD4+ and CD8+ T) cell fractions were also provided and placed at port neg 1 and port pos 1 respectively. Using the autoMACS cell sorter, separation Deplete05 programme was chosen, which allows the collection of negative fraction from outlet port neg1. (In the initial experiments different separation programs, like Deplete and Posseld, were also tested on the autoMACS$^{TM}$ Separator; Deplete05 proved to give both a high yield and purity).

Following separation, the sorted cells were collected. The labelled CD4+ and CD8+ T cells (positive fraction) were collected into one tube and the non-labelled non-T cells (i.e. PBMCs-T cells; negative fraction) into another. Cells were topped up with MACS Buffer and centrifuged at 500 x g, 4ºC for 5 minutes. The supernatant was carefully decanted and the sorted fractions were re-suspended in the appropriate media. Depending on the experiment, the fraction of cells not to be treated with salmeterol (long-acting β2 agonist; Sigma-Aldrich, UK; catalogue number: S5068) were resuspended in 500 µl of RPMI 1640 + HS (10%). The fraction of cells to be treated with salmeterol were resuspended in 500 µl of RPMI 1640 to make a final concentration of 50 µM salmeterol. (This is because salmeterol is a lipophilic β2-AR agonist and the binding of salmeterol to serum proteins present in the incubation medium, may strongly diminish the free drug concentration; (Borger et al., 1998)). Cells in both positive and negative fraction were counted with trypan blue dye (from Sigma-Aldrich, UK).

### 2.1.4 Antigen Stimulation of the Negative (PBMCs-T cells) Fraction

The -ve fraction (PBMCs-T cells) were resuspended to 1 x $10^6$ cells in 100 µl of RPMI 1640 + HS (10%). 100 µl of the negative fraction were added to corresponding sterile-FACS tubes. 1 x $10^6$ PBMCs-T cells were stimulated with either: 20 µl of Staphylococcal Enterotoxin B/500 µl RPMI (SEB, final concentration 500 ng/ml; from Cancer Studies, University of

Birmingham, UK) and 5 µl (final concentration 1 µg/ml) of anti-CD28/CD49d monoclonal antibody (from BD Biosciences, UK) ; or 30 µl of CMV lysate (strain AD169) per 500 µl of RPMI (a gift from Dr. Annette Pachnio, Cancer Studies, University of Birmingham, UK) and 5 µl (1 µg/ml) of anti-CD28/CD49d monoclonal antibody; or 10 µl of CMV pp65 (65 kDa lower matrix phosphoproteins; referred to as pp65) recombinant protein / 500 µl RPMI (final concentration at 1 µg/ml per individual peptide; from Miltenyi Biotec, UK) and 5 µl (1ug/ml) of anti-CD28/CD49d monoclonal antibody. In addition each experiment had a negative control, where one tube was left unstimulated. Following addition of the SEB or CMV lysate or pp65 respectively, tubes were vortexed and cells were incubated at 37 ºC, 5 % $CO_2$ for 1 hour and 30 minutes.

### 2.1.5 Salmeterol Treatment of the Positive Fraction (CD4+ and CD8+ T cells)

Following counting, T cells were split equally among tubes to be stimulated with salmeterol and tubes to not. The volume in each tube containing the positive fraction was made to 500 µl of RPMI without HS. 25 µl of salmeterol (stock concentration of 1mM) or 2.5 µl of salmeterol (1mM) was added to T cell tubes to be treated with salmeterol, (depending on the experiment) to make a final concentration of 50 µM and 5 µM salmeterol respectively. Following addition of salmeterol, cells were mixed and both treated and untreated T cells were incubated at 37ºC, 5 % $CO_2$ for 30 minutes. After incubation, T cells were washed twice with MACS Buffer. Following the second wash, T cells were re-suspended in RPMI + HS (10%) and counted for a second time. Equal number of T cells were added to corresponding antigen stimulated PBMC tubes (containing PBMCs without (-) T cells; i.e. mostly APCs like B cells and monocytes). T cells were incubated with PBMCs-T cells (for ease from here on denoted as APCs) at 37ºC, 5% $CO_2$ for 1 hour before adding 1 µl of monensin (final concentration at 5 µg/ml; from Sigma-Aldrich, UK). Following the addition of monensin cells

were incubated for an additional 5 hours at 37ºC, 5 % $CO_2$. The same principle was carried out when APCs were treated with salmeterol. Salmeterol treatment of the APCs was carried as described in section 2.1.5 when positive fractions were treated with salmeterol. Similarly to APCs, described in section 2.4, T cells were not treated with salmeterol. However in contrast to the above procedure, since CMV lysate and pp65 require antigen presentation to T cells by APCs; CMV lysate or pp65 or SEB were only added following incubation of APCs with T cells. Cells were left to incubate for 1 hour 30 minutes, after which monensin (5 µg/ml) was added and cells were incubated for an additional 5 hours at 37ºC, 5 % $CO_2$.

### 2.1.6 Salmeterol Treatment of Total PBMCs

PBMCs were counted and re-suspended into 50 ml Falcon tube to ~2 x $10^6$ cells per 200 µl of RPMI without HS. Subsequently, this suspension was transferred into sterile FACS tubes to be stimulated or not with salmeterol. The volume in each tube containing PBMCs was increased to 500 µl of RPMI without HS. 25 µl of salmeterol (1 mM) or 2.5 µl of salmeterol (1 mM) were added to PBMCs to make a final concentration of 25 and 50 µM of salmeterol respectively. No salmeterol was added in the control condition. Subsequently, cells were mixed and both treated and untreated tubes containing PBMCs, were incubated at 37ºC, 5% $CO_2$ for 30 minutes. After this incubation, PBMCs were washed twice with MACS Buffer. The volume in each FACS tube was made to 500 µl with RPMI + HS 10%. Subsequently, PBMCs were stimulated with either 500 ng/ml SEB and 1 µg/ml of anti-CD28/CD49d monoclonal antibody; or 30 µl of CMV lysate/500 µl RPMI and 1 ug/ml of anti-CD28-CD49d monoclonal antibody; or 10 µl of pp65/500 µl RPMI (1 µg/ml per individual peptide) and (1 µg/ml) of anti-CD28-CD49d monoclonal antibody, depending on the appropriate corresponding labelled FACS tube. In addition each experiment had a negative control, where one tube was left unstimulated to control for spontaneous production of IFN-γ cytokines.

Following stimulation, PBMCs were incubated for 1 hour and 30 minutes at 37ºC, 5% $CO_2$, after which (5 µg/ml) of monensin was added to inhibit extracellular secretion of cytokines, and the antigen stimulated PBMCs were incubated for an additional 5 hours at 37ºC, 5% $CO_2$. The cells were then washed twice with MACS Buffer and stained with a cocktail of fluorescent-labelled monoclonal antibodies: CD3-PerCP, CD4-APC, CD8-APC-Cy7 (Becton-Dickinson, UK) for 30 minutes at 4°C in the dark. At this stage, cells were washed with MACS Buffer and centrifuged to remove unbound antibodies and fixed with 1.5 ml FACS lysing solution (BD, Biosciences, UK) diluted to a 2x with same volume of double distilled (dd) $H_2O$ as per manufacturer's instructions. Samples were vigorously stirred for 10 seconds then incubated at room temperature for 10 minutes. Following incubation, cells were washed as above and centrifuged at 500 x g for 5 minutes at 4ºC. The supernatant was carefully decanted and cells were then permeabilized by resuspending in 0.5 ml FACS permeabilizing solution (BD, Biosciences, UK) diluted to a 2x concentration with d.d.$H_2O$. Samples were vigorously vortexed for at least 10 seconds then incubated at room temperature for 10 minutes. Cells were washed as above and stained with 10 µl of intracellular IFN-γ-PE antibody (from Becton-Dickinson, UK) incubated for 30 minutes at room temperature, in dark. Following staining, cells were checked for viability by haemocytometer and subsequently were washed with MACS Buffer (as described above) and centrifuged to remove unspecific binding of the antibodies to PBMCs. The remaining cell pellet was re-suspended in 100 µl 2% paraformaldehyde (Sigma-Aldrich, UK), and stored in the dark at 4°C until analyses. Gated lymphocytes were acquired from each preparation. A total of ~$2x10^5$-$7x10^5$ events were acquired using a flow cytometer (FACS-Canto II, Becton Dickinson, UK) and analysed using FlowJo software (Treestar Inc, Ashland, OR, USA). Cells that fell within the lymphocyte population were gated and analysed. The CD3+/CD4+ (CD4+

T), and CD3+/CD8+ (CD8+ T) cell population was also gated (within the lymphocyte population). The percentage of IFN-γ from the T cells was noted and compared to the unstimulated control and between different samples.

### 2.1.7 The Purity of Positive (CD4+ and CD8+ T cell) and Negative (PBMCs minus T cells) Fraction

To check for the purity of the positive (CD4+ and CD8+ T cells) and the negative fraction (PBMCs-T cells), 5 µl of (each of the fluorochrome-conjugated antibody, CD3-PERCP, CD4-APC, CD8-APC-CY7 were added to 20 µl (~$5x10^4$) of PBMCs, as well as to the positive and negative fraction cells respectively. Cells were incubated for 30 minutes on ice and were subsequently washed twice with RPMI 1640 + BSA (1%). The purity of the negative and positive fraction cells was evaluated by flow-cytometry**.** A total of ~$2 \times 10^4$-$5 \times 10^4$ events were acquired and analysed using FlowJo software. The CD4+ T and CD8+ T population was gated (within the gated lymphocyte population) and the percentage of purity of the positive and negative fraction cells was determined. The purity of sorted populations was always higher than 90%.

### 2.1.8 Multi-colour Flow Cytometry

*Principles and Compensation*

Flow Cytometry uses a beam of laser light to analyse the physical and chemical characteristics of cells, or other biological particles. The flow cytometry use is based on the principle that the beam of laser light is scattered and the fluorochromes (i.e. fluorescent dyes) become excited as each cell intercept the laser. The emitted light is collected and depending on its wavelength prisms and filters act to direct it to different photon multiplier tubes (PMT). PMT then converts the collected light into electrical signals. Scattered light is represented as the forward scatter (which measures the size of the cells) and the side scatter (which measures

the granularity of the cells). Based on forward and side scatter, gates were drawn (see chapter 3, Figure 3.4.A.). The voltages were set so that the negative controls were displayed within the first decade. Anything above the first decade was considered positive. This calibration is only sufficient for single-colour flow cytometry and not for the multi-colour flow cytometry. In contrast, when using multi-colour flow cytometry, compensation is extremely important. This is because in multi-colour flow cytometry, there is an inherent overlap in the emission spectra of different dyes. This overlap in the emission spectra of different dyes must be compensated. For compensation, samples stained with individual fluorochromes are run. One example to indicate importance of compensation is observed in fluorochromes FITC and PE. There is spectral overlap between the fluorochromes FITC and PE. This means that light detected by FL1 (FITC detector) and FL2 (PE detector) contains light from the other dye. The intensity of FITC emission detected by FL2 can be regarded as "excess fluorescence". This "excess fluorescence" gives a false positive PE signal. To correct for such overlap, and thus false positive signals, a percentage of the total FITC signal generated in FL1 (FITC interference) is subtracted from the total PE signal generated in FL2. In the same way, to compensate for PE leakage into FL1, a percentage of the total PE signal generated in FL2 (PE interference) is subtracted from the total FITC signal generated in FL1 (Rahman et al., 2006). Here, the flow cytometer (FACS-Canto II, Becton Dickinson, UK) was used from Medical School, (UoB).

### 2.1.9 Cell Counts Using a Haemocytometer

### 2.1.10 Preparing Haemocytometer

The dry Neubauer haemocytometer (Fisher, UK) was cleaned using 70% (v/v) ethanol/d$H_2O$ ethanol. The cover slip was carefully affixed on top of the counting chamber, making sure that both sides were resting on the raised area. The haemocytometer was then placed on the stage

of the Leica CME (compound microscope education) light microscope (Leica Microsystems, UK).

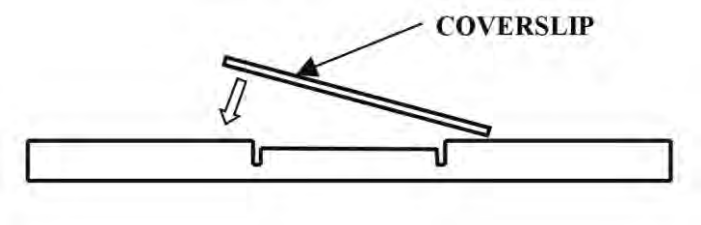



**Figure 2.1. Preparing haemocytometer.** Figure was taken from (Mishell and Shigii, 1980).

### 2.1.11 Preparing Cell Suspension

PBMCs were re-suspended in a volume of 10 ml into a 15 ml Falcon tubes. Cell suspension to be counted was well mixed using a serological pipette (Appleton Woods, UK). Subsequently 1 ml of cell suspension was drawn using a serological pipette and placed in a 2 ml Eppendorf tube. Using a 100 µl pipette, the cells in this sample were again gently mixed. 100 µl of cell suspension was drawn out and placed into a new Eppendorf tube. Subsequently 100 µl of trypan blue dye was added and mixed gently with this cell suspension.

### 2.1.12 Counting

10 µl of cell suspension containing trypan blue were drawn up using the 10 µl Gilson pipette (Anachem, UK).The haemocytometer was carefully filled by resting the end of the Gilson tip at the edge of the chambers (filling notch) taking care not to overfill. The liquid was drawn out of the pipette and into the chamber.

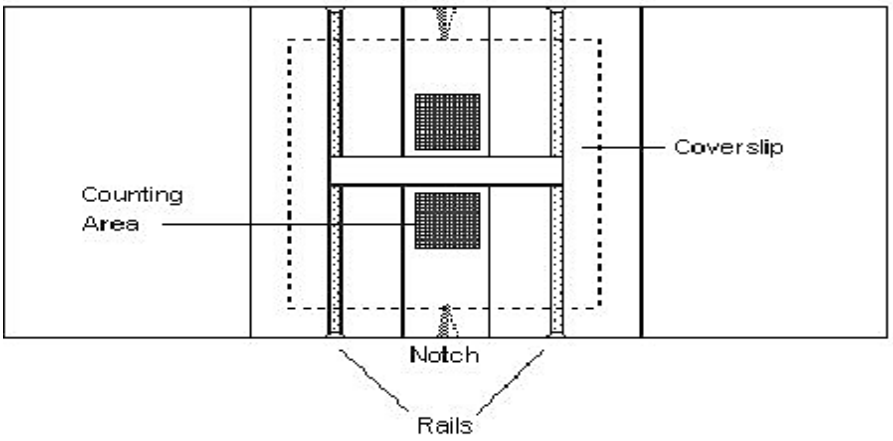



**Figure 2.2. Counting.** Figure was taken from (Mishell and Shigii, 1980).

The Haemocytometer was placed under a microscope and the 40X objective lense of the microscope was focused on the grid area of the counting chamber. Under the microscope, the central area of the grid was observed to be made up of triple ruled lines that in the centre form a 5x5 grid of 25 squares with a total volume of 0.1 $mm^3$ (Fig.2.3.). (Each of these 25 squares has an area of 0.04 $mm^2$ and a volume of 0.004 $mm^3$ with the depth of the chamber being 0.1mm). Five areas inside the grid were counted. Cells falling on the bordering triple lines were only counted if they were on either the top or on the left lines only.

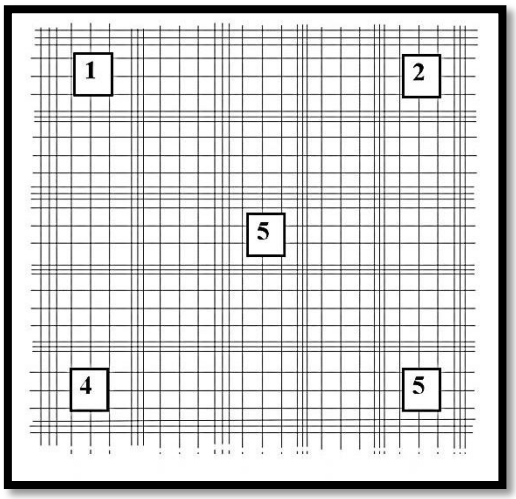



**Figure 2.3. The Haemocytometer grid**. Figure was taken from (Mishell and Shigii, 1980).

### 2.1.13 Calculating the Concentration of Cells

The centre triple ruled grid (consisting of 25 squares) has a volume of 0.1 $mm^3$. Cells were counted in only 5 out of the 25 squares of this grid representing a grid volume of 0.02 $mm^3$. To convert to the number of cells in 0.1 $mm^3$, the total number of cells in 5 squares were first divided by 5 (to give the total number of cells in 0.004 $mm^3$) and then were multiplied by 25 (to give the total number of cells in 0.1 $mm^3$). The haemocytometer is designed so that the number of cells in one set of 25 corner squares is equivalent to the number of cells x $10^4$/ml. Thus to convert this answer from cell/0.1 $mm^3$ to cells/ml, cells were multiplied by $10^4$ (0.1 $mm^3$ * $10^4$ =1000 $mm^3$= 1 $mm^3$).

For example:

Let the number (no.) of cells counted in 5 squares = **X.**

no. of cells/ ml suspension = $\mathbf{X \times 5 \times 10^4}$

Finally the total cell number/ml was multiplied by 2 to adjust for the 1:2 dilution in trypan blue.

## 2.2 In Vitro Flow Assay

### 2.2.1 Microslides

Important components of the flow assay are the microslides. These are glass capillaries with rectangular cross-section (0.3mm x 0.3mm; length 50 mm) (Vitro Dynamics Inc., USA; available through Camlab Ltd., UK) and in this study were coated with purified adhesion molecules. Prior to being used for coating, microslides were pre-treated with Aminopropyltriethoxysilane (APES) 4% in acetone (Sigma-Aldrich, UK) with molecular sieve (BDH Laboratory Supplies, UK) to ensure anhydrous. Recombinant human vascular cell adhesion molecule (VCAM-1) (R&D systems, UK) was used in all experiments.

### 2.2.2 Microslides Pre-treatment with APES

Microslides were immersed in nitric acid (50% v/v) (Fisher, UK) in distilled water for 24 hours to ensure they are chemically clean. The next day, they were washed for 3-4 hours thoroughly in beaker using running tap water and finally with deionised distilled water. They were then dried as thoroughly as possible at 37ºC or by sucking air through using a vacuum pump. Subsequently, microslides were placed in 50 ml polystyrene tubes and rinsed twice with anhydrous acetone (acetone with molecular sieve added to absorb any water) by gently inverting the tubes over 30 seconds. Using the same procedure as above, microslides were immersed in a freshly prepared solution of APES (4% v/v in anhydrous acetone) ensuring all capillaries were filled. They were subsequently removed from the APES and blotted out onto

tissue, ensuring that all capillaries were emptied. The procedure was repeated and the microslides were left in second change of APES overnight in the dark. The following day, the microslides were washed with two changes of anhydrous acetone followed by three washes with deionised distilled water by sucking a few ml through using a vacuum pump. Between each change care was taken to remove all the liquid from the microslides. Finally, the microslides were autoclaved at 121 ºC for 11 minutes and were stored aseptically indefinitely.

### 2.2.3 Coating of Microslides with VCAM-1

Recombinant human VCAM-1 was diluted to 10ug/ml in Dulbecco's PBS (Phosphate Buffered Saline with $MgCl_2$ and $CaCl_2$; from Sigma-Aldrich). In some initial experiments, desired serial dilutions were made to 5 µg/ml and 20 µg/ml. A short length of 2mm ID silicon rubber tubing (Fisher Scientific, UK) was attached to APES coated microslide and 50 µl of recombinant human VCAM-1 at final desired concentration was aspirated into a microslide using a pipettor inserted in the silicon tubing adapter. The recombinant human VCAM-1 coated microslide was incubated for two hours at 37ºC. Subsequently 500 µl of PBS with 1% (w/v) BSA (dilute from 7.5% culture-tested solution; Sigma-Aldrich) was aspirated as above and incubated at 37ºC for 2 hours to block non-specific binding sites.

### 2.2.4 Isolation of Peripheral Blood Lymphocytes (PBLs)

Whole blood from healthy individuals was collected in tubes coated with EDTA (anti-coagulant) (1.6 mg/ml) (Sarstedt, UK). Whole blood was gently layered onto a Histopaque gradient (2.5 ml Histopaque 1077 layered onto 2.5 ml of Histopaque 1119 (Sigma-Aldrich, UK) and centrifuged for 40 minutes at 1200 x g at room temperature. The PBMCs sedimented above the blood at the Histopaque interface. The PBMCs layer was collected and transferred into 15 ml Falcon tubes (Scientific Laboratory Supplies, UK). PBMCs were washed twice in

PBS + BSA (0.15%) 5 minutes by centrifugation at 500 x g then 320 x g at room temperature. PBLs were prepared by re-suspending PBMCs pellet in PBS + BSA (1%) and by panning PBMCs on culture plastic T25 flask (Falcon$^{TM}$, BD, Bioscience, UK) pre-coated with heat inactivated human serum. PBMCs were incubated at 37 ºC for 45 minutes to remove monocytes. Following incubation of PBMCs, the non-adherent cells were collected from the flask and transferred into 15 ml Falcon tubes. The volume of PBLs was made to10 ml with PBS + BSA (1%) and PBLs were centrifuged for 5 minutes at 320 x g at room temperature. The purified PBLs pellet was re-suspended in PBS + BSA (0.15%) at a final concentration of 1x10 $^{6}$cells/ml. For the static assay (section 2.3.2), the purified PBLs pellet was re-suspended in RPMI only at a final concentration of 1x10 $^{6}$cells/ml.

**2.2.5 Assay Design**

The microslide coated with VCAM-1 (10 µg/ml) was placed on a 75 x 25-mm coverslip (Fisher Scientific, UK).The coverslip and the microslide were incorporated into a parallel plate flow chamber and attached to a perfusion system mounted on the stage of phase contrast video microscope (Olympus IX70 with JVC TK8350 video camera attached) enclosed in a Perspex chamber at 37 ºC (Fig.2.4.), (previously described by Nash et al., 1992; Cooke et al., 1993; Rainger et al., 2001). At one end they were connected by flexible silicon to a Harvard withdrawal syringe pump, which was set to deliver flow at a rate equivalent to a wall shear stress of 0.05 Pa. At the other end they were connected to an electronic switching valve (Lee Products, Gerards Cross, UK). This valve selected flow from three reservoirs containing PBLs (PBMCs-minus plastic adherence cells) in PBS + BSA (0.15%), or cell-free wash buffer consisting of PBS + BSA (0.15%), or cell-free PBS + salmeterol/epinephrine at various concentrations. A suspension of either PBLs (1x10$^{6}$ cells/ml) or cell free wash buffer, or cell-free PBS + salmeterol/epinephrine (Sigma-Aldrich, UK) at various concentrations of 0.05 µM

-50 μM, was perfused through the chamber at a constant wall shear stress (τw) set at 0.05 Pa based on the formula: $Tw = (6\eta Q)/wh^2$

whereby: η = viscosity of perfusing medium (=7 x $10^{-3}$ Poise for aqueous buffer at 37 ºC);

Q = flow rate (yielding a flow rate of 0.05 Pa).

w= the width (4mm for the chamber); h= height (133μm for the chamber).

The microslide was perfused for 2 minutes with PBS + BSA (0.1%) to acclimatise cells. Subsequently a 4-minute bolus of PBLs was perfused over the VCAM-1 coated microslide followed by 2 minutes of cell-free wash buffer.

Video recordings were made of a series of microscope fields along the centreline of the flow channel after 2 minutes of wash-out. Following on from the video recordings of five different microscope fields, the microslide was perfused for 2 minutes with PBS + salmeterol at concentrations of either 0.5 μM, or 5 μM, or 50 μM, or epinephrine at a concentration of 0.05 μM. Cell-free wash buffer was perfused again over VCAM-1 microslide and video recordings of 5 different microscopic fields were made again at 2 minutes and 5 minutes following the PBS + salmeterol/epinephrine perfusion in order to observe the effect of salmeterol/epinephrine on the PBLs.

After video recordings, the flow was stopped and the manometer tubing was cut from the microslide. PBLs were detached from the microslide by carefully pipetting up and down the microslide volumes of EDTA (stock concentration 0.02%) (BD Vacutainer, UK)**.** Cells were collected in 2 ml eppendorf tubes (BD Biosciences, UK).When all cells had detached from the microslide (as determined from visual observations using phase contrast microscopy) PBLs were centrifuged at 500 x g for 3 minutes. Following on from centrifugation, the supernatant

was removed and the adherent lymphocytes were labelled with a cocktail of fluorescent-labelled monoclonal antibodies: CD3-PerCP, CD4-APC, CD8-APC-Cy7, CD28-PE-CY7 (Becton-Dickinson, UK) for 30 minutes at 4°C in the dark. At this stage, cells were washed with PBS + BSA (1%) and centrifuged for 5 minutes at 500 x g to remove unspecific binding of the antibodies to lymphocytes. The remaining cell pellet was re-suspended in100 µl PBS containing 2% paraformaldehyde, and stored in the dark at 4°C until analyses. Preparations were read within 18 hours. Gated lymphocytes ($10x10^3$-15 x $10^4$) were acquired from each preparation using a flow cytometer (FACS-Canto II, Becton Dickinson, UK). Data were analysed using Flowjo 7.6.1 (Treestar Inc, Ashland, OR, USA). In this way, we determined the percentages of each lymphocyte phenotype adhered to VCAM-1 under different conditions.

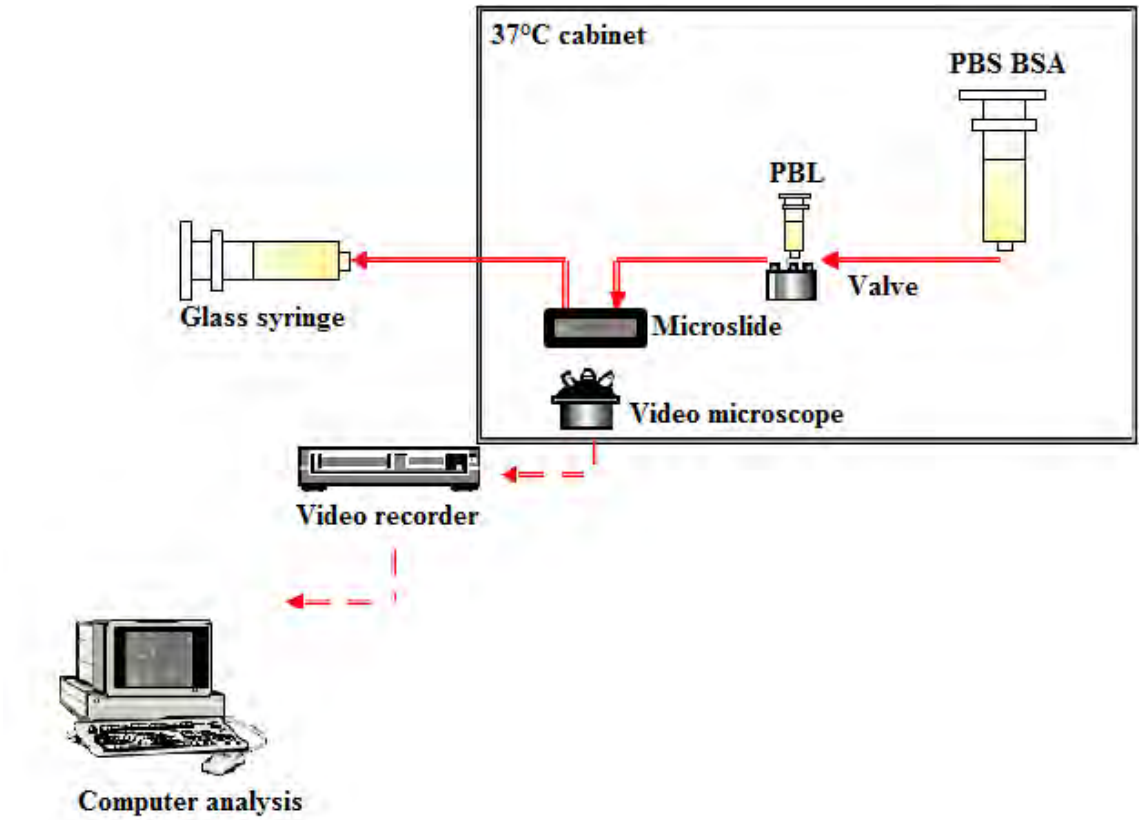



**Figure 2.4. The flow assay design.** The microslide coated with VCAM-1 was placed on a 75 x 25-mm coverslip and incorporated into a parallel plate flow chamber attached to a perfusion system mounted on the stage of phase contrast video microscope (enclosed in a Perspex chamber at 37 ºC). At one end, the microslide was connected to a Harvard withdrawal syringe pump and at the other end it was connected to an electronic switching valve. This valve selected flow from three reservoirs containing PBLs in PBS + BSA (0.15%), or cell-free wash buffer consisting of PBS + BSA (0.15%), or cell-free PBS + salmeterol at various concentrations. A suspension of either PBLs ($1x10^6$ cells/ml), or cell free wash buffer, or cell-free PBS + salmeterol / epinephrine at various concentrations of 0.05 µM -50 µM, was perfused through the chamber at a constant wall shear stress (τw) set at 0.05 Pa.

### 2.2.6 Analysis of Video Recordings under Flow Conditions

The adhesion and rolling behaviour of leukocytes were recorded and analysed offline using Image-Pro Plus software package. The total number of adherent leukocytes were counted and converted to cells/$mm^2/10^6$ perfused. In addition, leukocyte behaviour (rolling, firmly adherent) was analysed and expressed as percentage of total adherent cells. Rolling adherent cells were spherical and moved slowly over the VCAM-1 coated microslide. Stably adherent, otherwise referred to as the "activated "cells were stationary over the VCAM-1 coated microslide as previously described (Cooke et al., 1993). The rolling velocities of ten random cells were measured over ten seconds and were expressed as average rolling velocity per second (μm/sec).

## 2.3 Static Assay

### 2.3.1 Cell Culture Media

Endothelial cells were cultured in human microvascular endothelial cells (HMEC-1)/Medium 199 containing Earle's salts and L-Glutamine (M199-Gibco Invitrogen Compounds, UK) supplemented with 20% heat inactivated fetal calf serum (FCS), 10 ng/ml epidermal growth factor, 35 μg/ml gentamycin,1 μg/ml hydrocortisone, 2 mM L-glutamine (all from Sigma-Aldrich), and 2.5 μg/ml amphotericin B (Gibco Invitrogen Compounds).

### 2.3.2 Treatment of PBLs with Salmeterol or Epinephrine

PBLs were treated with salmeterol or epinephrine in the following way: 4 ml of PBLs ($4x10^6$) resuspended in serum free medium (RPMI-1640) was treated with either salmeterol (to make a final concentration of 5 and 50 μM respectively), or epinephrine (to make a final concentration of 0.05 μM epinephrine). This dose of epinephrine proved maximal efficacy (Dimitrov et al., 2010). Following treatment with salmeterol or epinephrine, PBLs were

incubated for 30 minutes at 37°C, 5% $CO_2$. Subsequently, cells were washed twice with RPMI and counted. Following counting, PBLs were diluted to a cell density of 2 x $10^6$ cells/ml in M199 + BSA (0.15%).To maintain consistency the same procedure was carried out with the untreated PBLs which served as a negative control as compared to the salmeterol/epinephrine treated PBLs samples.

### 2.3.3 Culture of Human Microvascular Endothelial Cell Line-1 (HMEC-1)

Endothelial cells (obtained from Prof. Gerard Nash, University of Birmingham) were retrieved from liquid nitrogen. The pellet was defrosted and re-suspended in 5 ml of cold HMEC-1/culture medium. Subsequently, HMEC-1 were plated onto 1% gelatin-coated T75 culture flasks (Falcon™, BD, Bioscience, UK) and cultured until confluent (approx. one to two days). To maintain a constant pH, the medium was replaced every day until the cells were fully confluent.

Upon confluency, the medium was removed and 2 mls EDTA (0.02%) (Sigma-Aldrich, UK) was added to HMEC-1 and left for 60 seconds. The supernatant was aspirated off and HMEC-1 were subsequently trypsinised (2.5 mg/ml) (Sigma-Aldrich, UK) in a 2:1 ratio with EDTA. When all the cultured HMEC-1 had detached, (as determined from visual observations using phase contrast microscopy) 5 ml of culture medium was added to stop the reaction. The contents of the T75 flask was transferred to a 15 ml Falcon tube and centrifuged at 500 x g for 5 minutes. The HMEC-1 were re-suspended in culture medium, plated out into 9 cm petri dishes (Appleton woods, UK) and incubated at 37 ºC. Confluent monolayers were yielded within 24 hours. Depending on the experimental protocol, TNF-α (100 U/ml, Sigma-Aldrich, UK) and/or IFN-γ (10 ng/ml, Peprotech Inc., UK) was added to confluent monolayers for 4 hours or 24 hours before the assay with lymphocytes or NK cells, respectively.

### 2.3.4 Natural Killer Cell (NK) Isolation

PBMCs from peripheral blood were isolated by density gradient centrifugation, as described above. The PBMCs layer was collected and transferred into 50 ml Falcon tubes. Cell suspension was centrifuged at 500 x g for 8 minutes. The supernatant was completely aspirated and the cell number was determined. PBMCs were then washed twice in MACS Buffer and centrifuged for 10 minutes at 4°C.The supernatant was again completely aspirated and the cell pellet was resuspended in 40 µl of MACS Buffer per $10^7$ total cells. Subsequently, 10 µl of NK cell biotin-antibody cocktail (Miltenyi Biotec, UK) was added per $10^7$ total cells. The cells were mixed well with the antibody and the sample was refrigerated for 15 minutes (4−8 °C). Following incubation, an additional 30 µl of MACS Buffer was added to $10^7$ total cells. This was followed by the addition of 20 µl of NK cell microbead cocktail (Miltenyi Biotec, UK). The sample was mixed well and refrigerated for an additional 15 minutes (4−8 °C), with occasional mixing. Following incubation, cells were washed by adding 2 ml of MACS Buffer per $10^7$ total cells and were centrifuged at 300 x g for 10 minutes. The supernatant was aspirated completely and up to $10^8$ cells were resuspended in 500 µl of MACS Buffer. The sample was ready to proceed for magnetic separation.

### 2.3.5 Magnetic Separation of NK cells

Magnetic separation was carried using LS MACS Column (Miltenyi Biotec, UK). The LS column was placed in the magnetic field of the MACS Separator (type-Miltenyi Biotec) and was prepared by rinsing with 3 ml of MACS Buffer. The eluent was collected and removed. The cell suspension (500 µl) was applied onto the column. The unlabelled NK cells that passed through were collected, and the column was washed three times with 3 ml of MACS Buffer to remove remaining NK cells. The new buffer was added only when the column

reservoir was empty. For the isolation of magnetically labelled non-NK cells, the column was removed from the separator and placed on a suitable collection tube. 5ml of MACS Buffer was pipetted onto the column and the magnetically labelled cells were immediately flushed out by firmly pushing the plunger into the column. Following collection, the volume of NK cells was made to 10 ml with MACS Buffer and NK cells were centrifuged at 300 x g for 10 minutes at 4 °C. The purified NK cell pellet was re-suspended in RPMI only at a final concentration of $1x10^6$ cells/ml.

Depending on the static assay experiment, NK cells were treated with salmeterol (to make a final concentration of 0.05 – 50 μM) or epinephrine (to make a final concentration of 0.05 μM) or not (which served as a negative control) and were incubated for 30 minutes at 37°C, 5% $CO_2$. Following incubation, cells were washed twice with RPMI Medium and were subsequently diluted to a cell density of $2x10^6$ cells/ml in M199 + BSA (0.15 %).

**2.3.6 Static Assay Design-Migration Through Endothelial Cells (EC)**

Whole blood was collected into EDTA tubes (BD Vacutainer, UK), maintained at room temperature and processed within 30 minutes after collection. PBLs/NK cells were isolated and treated with epinephrine or salmeterol or not (as described in section 2.3.2 and 2.3.5 respectively) and re-suspended in M199 + BSA (0.15%) at a final concentration of $2x10^6$ cells/ml.

HMEC-1 in individual 9 cm petri dishes were washed twice with M199 + BSA (0.15%) to remove residual cytokines, and 1ml of purified PBLs was added onto HMECs monolayer. It is important to note that in these experiments, salmeterol or epinephrine was added directly to PBLs (for 30 minutes at 37 ºC, 5% $CO_2$), and only following two washes of PBLs with RPMI (that would allow removal of salmeterol), were PBLs added to HMEC-1**.** Following adhesion of PBLs, to HMEC-1 monolayer, the lymphocytes were allowed to settle and adhere for 30

minutes at 37 ºC, 5% $CO_2$. Non adherent cells were carefully removed from the HMEC-1 by gentle washing with M199 + BSA (0.15%) and were subsequently transferred into labelled 15 ml Falcon tubes. Immediately after washing, video recordings of the endothelial surface were made using phase-contrast video microscopy. Five fields were briefly recorded to analyse the number of adherent cells above the monolayer; a single field was recorded for 5 minutes to follow lymphocyte behaviour. Manipulations and microscopy were carried out inside a Perspex box held at 37 ºC.

Following on from the video recordings, the medium containing non-adherent lymphocytes was aspirated from the HMEC-1 monolayer and collected into 15 ml Falcon tube. Subsequently, HMEC-1 were bathed in 2 ml of Accutase (Sigma-Aldrich, UK) for 5 minutes to detach HMEC-1 and lymphocytes. When all cells had detached, as determined from visual observations using phase contrast microscopy, 2 ml of culture medium was added to stop the reaction.

The contents of the petri-dish were transferred to a correctly labelled 15 ml Falcon tube and centrifuged at 500 x g for 5 minutes. At the same time, the non-adherent cells were also centrifuged at 500 x g for 5 minutes. Subsequently the non-adherent lymphocytes were transferred to their respectively labelled FACS tubes and the HMEC-1 with the adherent cells were transferred into their specifically labelled FACS tubes. FACS tubes containing cells were centrifuged again at 500 x g for 5 minutes. Following on from centrifugation, non-adherent and adherent lymphocytes were labelled with a cocktail of fluorescent-labelled monoclonal antibodies: CD3-PerCP, CD4-APC, CD8-APC-Cy7, CD56-PE for 30 minutes at 4°C in the dark. At this stage, cells were washed with PBS + BSA (1%) and centrifuged to remove unspecific binding of the antibodies to lymphocytes. The remaining cell pellet was re-suspended in100 µl PBS containing 2% paraformaldehyde, and stored in the dark at 4°C until

analyses. Preparations were read within 18 hours. Gated lymphocytes were acquired from each preparation using a flow cytometer (FACS-Canto II, Becton Dickinson, UK). Data were analysed using Flowjo 7.6.1 (Treestar Inc, Ashland, OR, USA). In this way, we calculated the percentage of lymphocytes adhered to HMEC-1 and the percentage of lymphocytes not adhered to HMEC-1 under different conditions.

### 2.3.7 Analysis of the Video Recordings under Static Conditions

The video recordings were analysed off-line using Image-Pro plus softaware (DataCell Ltd., Finchampstead, UK). In each video field, the total number of adherent cells were counted, averaged and then converted to cells per $mm^2$ using the calibrated microscope field dimensions. Each lymphocyte was classified as phase bright with distorted shape and adherent to the surface of HMEC-1 or phase dark if it had spread and migrated below the HMEC-1. Therefore the total number of phase bright lymphocytes and the total number of phase dark lymphocytes was also counted, averaged, and converted to cells per $mm^2$ using the calibrated microscope field dimensions. To determine the percentage of phase dark lymphocytes the total number of phase dark lymphocytes was divided by the total number of the adherent lymphocytes. Additional data are presented as total adhesion/$mm^2$/1x$10^6$ lymphocytes. The migration velocities of at least ten phase dark lymphocytes underneath the HMEC-1 were measured by digitizing a sequence of images 1 minute apart for 6 minutes. In each digitized image, at least ten cells were outlined using a pointer, and the position of their centroid was determined. Migration velocity (μm/min) was the averaged distance moved by the centroid per minute.

## 2.4 NK Degranulation Assay

### 2.4.1 K562 (Human Erythromyeloblastoid Leukemia Cell Line)

The K562 cell line is a highly undifferentiated human erythroleukemic cell line which does not express MHC Class 1 molecules on its surface. The K562 cell line was provided by John Hazeldine (Centre for Translational Inflammation Research, University of Birmingham) and maintained in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin (all from Sigma-Aldrich, UK) at $2x10^6$ cells/ml.

### 2.4.2 Stable MICA*009 Transfected Chinese Hamster Ovary Cells (T-CHO)

MICA-transfected CHO (MHC class I related antigen) cells were a gift from (Seema Shafi Department of Immunology, King's College, London). Transfected CHO-cells were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin and 1% streptomycin, 400 µg/ml hygromycin (all from Sigma-Aldrich**)** at a final concentration of $2x10^6$ cells/ml.

### 2.4.3 Chinese Hamster Ovary (CHO) Cells Culture Media

CHO cells, were a gift from **(**Seema Shafi Department of Immunology, King's College, London)**.** CHO cells were grown in RPMI 1640 supplemented with 10% FBS , 2 mM L-glutamine , 1% penicillin and 1% streptomycin at a final concentration of $2x10^6$ cells/ml.

### 2.4.4 Culture of CHO Cells

CHO cells were plated onto 1% gelatin-coated T75 culture flasks, and cultured until confluent (approx. one to two days). To maintain a constant pH, the medium was replaced every day until the cells were fully confluent. Upon confluency, the medium was removed and 6 ml PBS was added to CHO cells and left for 60 seconds. The supernatant was aspirated off and 2 ml of trypsin (x2) was added to CHO cells. Cells were subsequently incubated for 5-10 minutes at 37 ºC, 5% $CO_2$. When all the cultured CHO cells had detached, (as determined from visual observations using phase contrast microscopy) 5 ml of the respective culture medium was added to stop the reaction. The contents of the T75 flask was transferred to a 15 ml Falcon

tube and centrifuged at 500 x g, at 21 ºC for 6 minutes. The CHO / or CHO-transfected cells were re-suspended in their respective culture medium, plated onto 1% gelatin-coated T75 culture flasks and incubated at 37 ºC, 5% $CO_2$. Confluent monolayers were yielded within 24 hours.

### 2.4.5 Treatment of PBMCs with Salmeterol or Epinephrine

PBMCs freshly isolated from blood of healthy controls were treated with salmeterol or epinephrine in the following way: PBMCs were first pelleted by centrifuging at 500 x g for 6 minutes and resuspended in 3 ml serum free medium (RPMI 1640) with either 0.05 µM epinephrine, or 5 µM salmeterol or 50 µM salmeterol and then incubated for 30 minutes at 37°C, 5% $CO_2$. Following incubation, cells were washed twice with RPMI and counted. Subsequently PBMCs were diluted to a cell density of $2x10^6$ cells/ml in complete medium consisting of RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 50 IU/ml penicillin ( all from Sigma-Aldrich). To maintain consistency the same procedure was carried out with the untreated PBMCs which served as a negative control as compared to the salmeterol/epinephrine treated PBMCs samples**.**

### 2.4.6 NK Cell Killing Assay

Untreated or salmeterol/epinephrine treated PBMCs were re-suspended at $2x10^6$ cells/ml in RPMI 1640 (Sigma-Aldrich) containing 10 % FBS 2 mM L-glutamine, 50 IU/ml penicillin (all from Sigma-Aldrich). Like PBMCs, the K562 cell line was also re-suspended at $2x10^6$ cells/ml in RPMI 1640 containing 10 % FBS 2 mM L-glutamine, 50 IU/ml penicillin (all from Sigma-Aldrich). Following re-suspension of both of the types of cells, PBMCs were stimulated with MHC devoid, K562 cells at an effector to target ratio of 1:1.Medium alone was used as a negative control. CD107-a-PeCy5 antibody (eBioscience, UK) was added

directly to the cells (5 µl of antibody/0.1 ml of cell suspension). FACS tubes were covered with paraffin and cells were incubated for 1 hour at 37°C in 5 % $CO_2$. Following incubation, Monensin (Sigma-Aldrich) was added at a final concentration of 6 ug/ml and incubated for an additional 3 hours or 5 hours (depending on the experiment) at 37°C in 5 % $CO_2$. Subsequently, samples were spun for 8 minutes at 500 x g at 4°C. PBMCs were then stained for surface NK markers CD56-PE-CY7 (BD Biosciences) and CD3-PERCP for 30 minutes in ice. Following incubation, cells were washed and were re-suspended in 1% paraformaldehyde (Sigma-Aldrich). Multi-parameter cytokine staining was used to quantify the frequency of degranulating NK cells. The flow-cytometry was performed on a FACS-Canto II. A total of $3x10^4$-$2x10^5$ events were acquired and analysed using FlowJo software. Cells that fell within the lymphocyte population were gated and analysed. The CD3-/CD56+ population was also gated (within the lymphocyte population). An independent expression of CD107a was noted within the CD3-/CD56+ population. If the frequency of CD107a expressing CD3-/CD56+ cells was greater than in unstimulated control, the response was considered positive. Medium alone and CHO cells incubated with PBMCs were used as negative control

### 2.4.7 Gating Strategy for CD107a Positive NK and $CD8^+$ T Cells

As mentioned previously, cells that fell within the lymphocyte population were gated and analysed. Subsequently, the T cell population, the CD3+ CD8+ (CD8+ T cell) population and the CD3-/CD56+ (NK) population was also gated (within the lymphocyte population). As most of the remaining T cells (apart from CD8+ T cells) do not tend to have cytotoxic function, and thus would not express CD107a on their surface, the gated T cell-CD107a population (Fig.2.5.A) was used as a negative control for CD107a expression (Fig.2.5.B). The

same gate set for the negative control (Fig.2.5.B) was moved up and was also set for the samples in question (Fig.2.5.D,F).

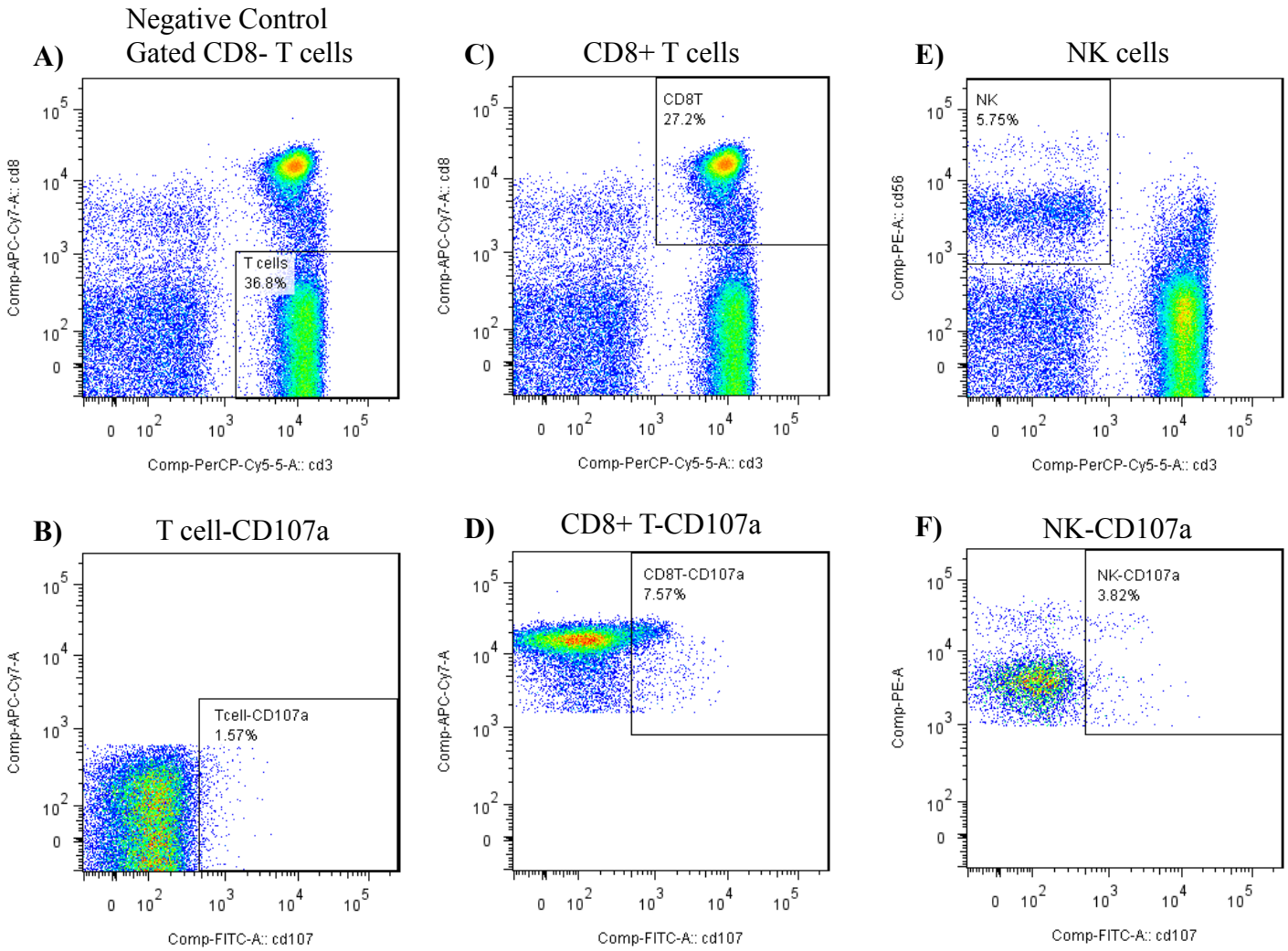



**Figure 2.5. Gating strategy for CD107a positive CD8+ T and NK cells.**
PBMCs were incubated with T-CHO, and CD8+ T CD107a and NK CD107a expression were determined.

**Table 2.1 Clinical Details of All Individuals Studied**

| Patient | Sex | Age |
|---|---|---|
| 1 | M | 50 |
| 2 | M | 27 |
| 3 | M | 43 |
| 4 | F | 23 |
| 5 | M | 43 |
| 6 | F | 44 |
| 7 | M | 55 |
| 8 | M | 36 |
| 9 | F | 26 |
| 10 | M | 29 |
| 11 | M | 47 |
| 12 | M | 44 |
| 13 | M | 28 |
| 14 | M | 34 |
| 15 | F | 37 |
| 16 | M | 40 |
| 17 | M | 42 |
| 18 | M | 31 |
| 19 | M | 50 |
| 20 | M | 36 |
| 21 | F | 35 |
| 22 | M | 28 |
| 23 | M | 27 |
| 24 | F | 46 |
| 25 | M | 32 |
| 26 | M | 37 |
| 27 | F | 26 |
| 28 | M | 51 |
| 29 | M | 33 |
| 30 | M | 22 |
| 31 | M | 24 |
| 32 | F | 38 |
| 33 | M | 55 |
| 34 | M | 48 |
| 35 | F | 25 |

**All individuals had a Caucasian background and were not under any medical treatment.** Individuals 1-20 were all CMV positive and were used for cytokine stimulation assay. Individuals 21-30 were used for flow and static assays. Individuals 31-35 were used for degranulation assay.

### 2.4.8 Statistical Analysis

The data in Chapters 3, 4 and 5 of this thesis were presented graphically as the mean ± SEM of n independent experiments. Variation between different (multiple) treatments was evaluated using paired sample t-test and were re-visited through the non- parametric Wilcoxon signed-rank test by SPSS programme (UOB). p (single-tailed) values $<0.05$ were considered statistically significant and were presented by a * symbol in Figures. Positive significant values only, are highlighted throughout the Chapters. Both, paired sample t-test and Wilcoxon signed rank test produced very similar results.

# CHAPTER 3

# EFFECT OF SALMETEROL ON IFN-γ PRODUCING CD4+ TLs AND CD8+ TLs

## 3.1 Introduction

The catecholamines Epi and NE represent the major stress hormones and are the main end products of SNS activation (Elenkov et al., 2000b). Indeed, previous studies have reported catecholamines to influence various aspects of the immune system. For example they influence proliferation of B and T cells, production of antibodies, the production of cytokines, antigen presentation by (APCs), the cytotoxicity of NK cells (Elenkov et al., 2000b; Madden et al., 1995; Padgett and Glaser, 2003; Wiegers et al., 1995; Yang and Glaser, 2000). However, relatively little is known about the effect of catecholamines on the function of the cytotoxic CD4+ T and CD8+ T lymphocytes. The memory CD8+ TLs subsets are also characterised by a higher expression of β2AR suggesting they are more responsive to β2AR stimulation than the naïve or central memory $CD8^+$ TLs (Dimitrov et al., 2010).

CD4+ TLs regulate and assist in active immune responses by releasing cytokines. Cytokines play a crucial role in maintaining the homeostasis within the immune system by mediating inflammatory and immune responses. Indeed, two main categories of CD4+ TLs are known with distinct cytokine secretion phenotype. These are Th-1 and Th-2. Whereas Th-2 are involved in promotion of humoral immunity by secreting IL-4, IL-10, and IL-13, Th-1 promote cellular immunity and stimulate the functional activity of CD8+ TLs by secretion of IFN-γ, IL-2, and TNF-β (Elenkov, 2008).

The main aim of the study presented here was to investigate the effect of salmeterol on IFN-γ producing CD4+ TLs and CD8+ TLs. Subsequently, we wanted to investigate whether the effect of salmeterol on IFN-γ producing CD4+ TLs and CD8+ TLs was direct, i.e., affected the T cells, or indirect by affecting the APCs or both. For this reason, the present study

employed three different approaches. Initially, we examined the IFN-γ producing CD4+ T and CD8+ T cells in salmeterol treated PBMCs. Secondly we investigated IFN-γ producing CD4+ T and CD8+ T cells on incubation with salmeterol treated APCs. Thirdly, we investigated the effect of salmeterol on the proportion of IFN-γ producing CD4+ T and CD8+ T cells. Previous studies have reported that salmeterol inhibits the production of IFN-γ by human PBMCs (Mohede et al., 1996). Consistent with the literature, we hypothesised that β2AR stimulation (salmeterol) inhibits the percentage of IFN-γ producing CD4+ TLs and CD8+ TLs. Since the β2AR is expressed on CD4+ TLs, CD8+ TLs as well as on the surface of APCs (Borger et al., 1998; Goyarts et al., 2008; Elenkov et al., 1996), we hypothesised that salmeterol would inhibit IFN-γ producing CD4+ TLs and CD8+ TLs both directly (by affecting β2AR expressed on CD4+ TLs and CD8+ TLs) and indirectly (by affecting β2AR expressed on APCs). The clinical details of individuals used in this assay are shown in Table 3.1.

**Table 3.1 Clinical Details of Individuals Studied for Cytokine Stimulation Assay**

| Patient | Sex | Age |
|---|---|---|
| 1 | M | 50 |
| 2 | M | 27 |
| 3 | M | 43 |
| 4 | F | 23 |
| 5 | M | 43 |
| 6 | F | 44 |
| 7 | M | 55 |
| 8 | M | 36 |
| 9 | F | 26 |
| 10 | M | 29 |
| 11 | M | 47 |
| 12 | M | 44 |
| 13 | M | 28 |
| 14 | M | 34 |
| 15 | F | 37 |
| 16 | M | 40 |
| 17 | M | 42 |
| 18 | M | 31 |
| 19 | M | 50 |
| 20 | M | 36 |

**All individuals had a Caucasian background and were not under any medical treatment.**
Individuals 1-20 were all CMV positive and were used for cytokine stimulation assay.

## 3.2 Results

### 3.2.1 Optimising the Assay - Initial Studies

Initial experiments described in this paragraph served to optimise the assay. PBMCs were treated for 30 minutes with salmeterol (50 μM) after which they were activated by incubation with phorbol myristate acetate (PMA) (25 ng/ml) and ionomycin (IO) (1 μg/ml) (a stimuli independent on the effect of antigen presenting cells, which served as high positive control (Godoy-Ramirez et al., 2004) and incubated for 1 hour and 30 minutes at 37°C, 5 % $CO_2$. Following incubation, monensin was added to inhibit intracellular secretion of cytokines. Cells were then incubated for a further period of 5 hours and stained with respective

antibodies. The percentage of IFN-γ producing CD4+ and CD8+ T cells was determined using FACS analysis. Our initial data showed that PMA and IO were potent activators of PBMCs, and increased the number of IFN-γ producing CD4+ and CD8+ TLs by 24 and 28 fold respectively, as compared to the untreated control (Fig.3.1.A-B). On the other hand, salmeterol proved to have a inhibitory effect on the production of IFN-γ producing CD4+ and CD8+ TLs, inducing a 5.7 fold and 4.1 fold decrease respectively compared to the positive control (Fig.3.1 .A-B). The choice of using PMA and IO in the initial trials relates to the fact that PMA + IO is a very potent stimulator of IFN-γ and since the number of antigen specific cytokine responding T cells is usually low, and difficult to detect. However one of the main drawbacks of PMA and IO is that it causes a dramatic decrease in CD4 expression, thus it can only be an activator of choice when the examination of the precise lymphocyte subpopulation is not important. Therefore for the next set of experiments, PMA + IO were replaced by Staphylococcus enterotoxin type B (SEB) superantigen, which acts as a positive control for IFN-γ producing T cells. As with PMA+IO, SEB stimulates T cells directly, and thus does not require antigen presentation by APCs.

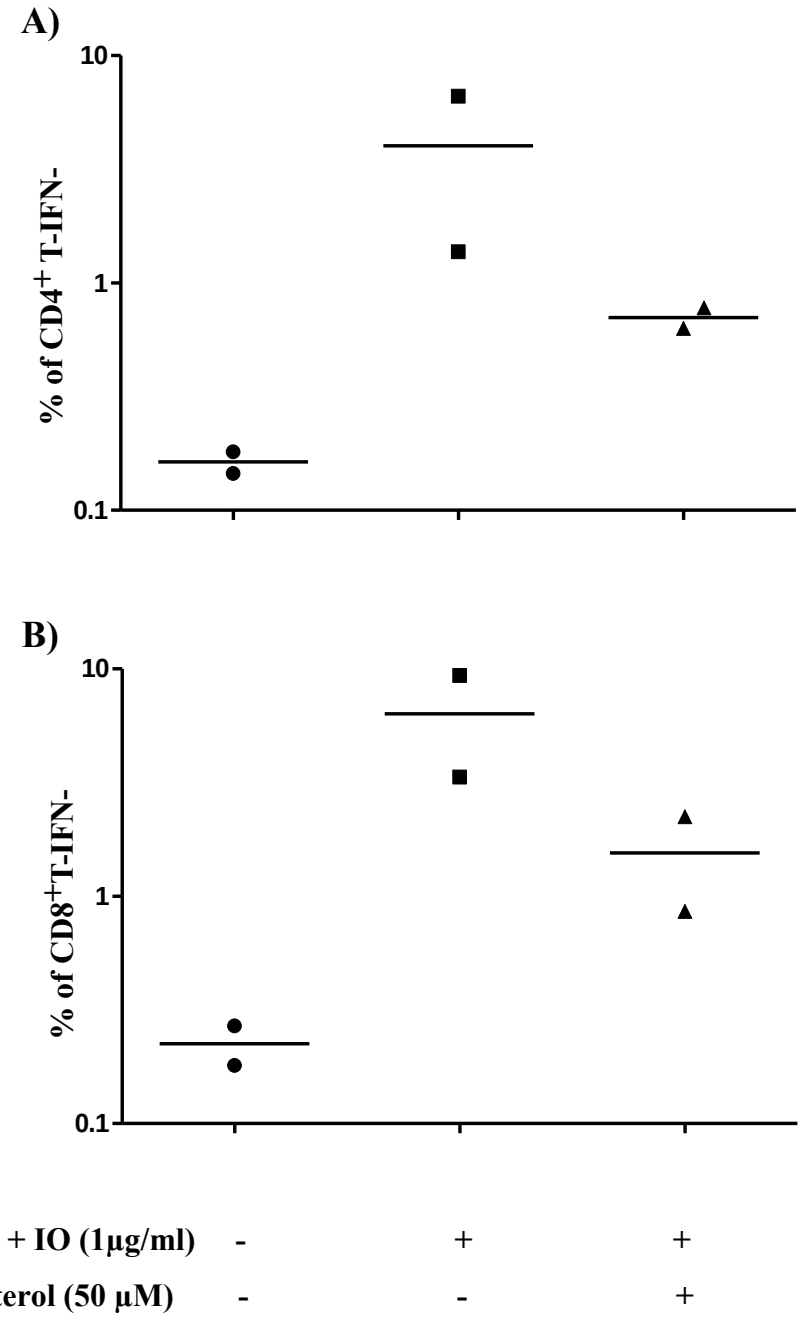



**Figure 3.1. (A-B). Effect of salmeterol on IFN-γ producing CD4+ T (A) and CD8 +T (B) cells in whole PBMCs stimulated with PMA + IO and/ or salmeterol.** PBMCs were isolated from blood and were incubated for 30 min at 37°C, 5% $CO_2$ in the presence or absence of 50 µM salmeterol. Following incubation of PBMCs with salmeterol (50 µM), PBMCs were washed twice after which they were subsequently stimulated with PMA + IO and incubated for 1 hr 30 min at 37°C, 5 % $CO_2$. After 1 hr 30 min of incubation of PBMCs with PMA + IO, monensin was added to inhibit intracellular secretion of cytokines. Cells were then incubated for a further period of 5 hours and stained with respective antibodies. The percentage of IFN-γ producing CD4+ and CD8+ T cells was determined using FACS Analysis. Horizontal lines represent mean values of 2 replicates (n=2). No statistics were carried out based on two values.

### 3.2.2 Effect of Salmeterol on the Production of CD4+ T-IFN-γ+ and CD8+ T-IFN-γ+ cells. Stimulation of PBMCs with SEB

In the experiment presented below, PBMCs were exposed to salmeterol (50 μM) before activation by SEB superantigen (Fig.3.2.A-B). This activation does not require specific antigen processing but evolves from direct binding to MHC class II molecules (Qasim et al., 1991). The results presented in Fig.3.2.A show that stimulation of PBMCs with SEB caused a significant 12.4 fold increase in the percentage of IFN-γ+ CD4+ TLs (p=0.001 using paired t-test; p=0.006 using Wilcoxon signed rank test) (Fig.3.2.A). In addition, the stimulation of PBMCs with SEB induced a significant 34.7 fold increase in the percentage of IFN-γ+ CD8+ TLs (p=0.001 using paired sample t-test; p=0.006 using Wilcoxon signed rank test) (Fig.3.2.B).

Exposure of PBMCs to the $\beta_2$AR agonist salmeterol before activation by SEB inhibited significantly the IFN-γ producing CD4+ TLs (p=0.009 using paired sample t-test; p=0.006 using Wilcoxon signed rank test), reducing the proportion of IFN-γ+ cells by 1.2 fold compared to the positive control (Fig.3.2.A). Similarly salmeterol significantly inhibited the IFN-γ producing CD8+ TLs (p=0.003 using paired sample t-test; p=0.009 using Wilcoxon signed rank test) resulting in a 1.6 fold reduction compared to the stimulated sample (Fig.3.2.B).

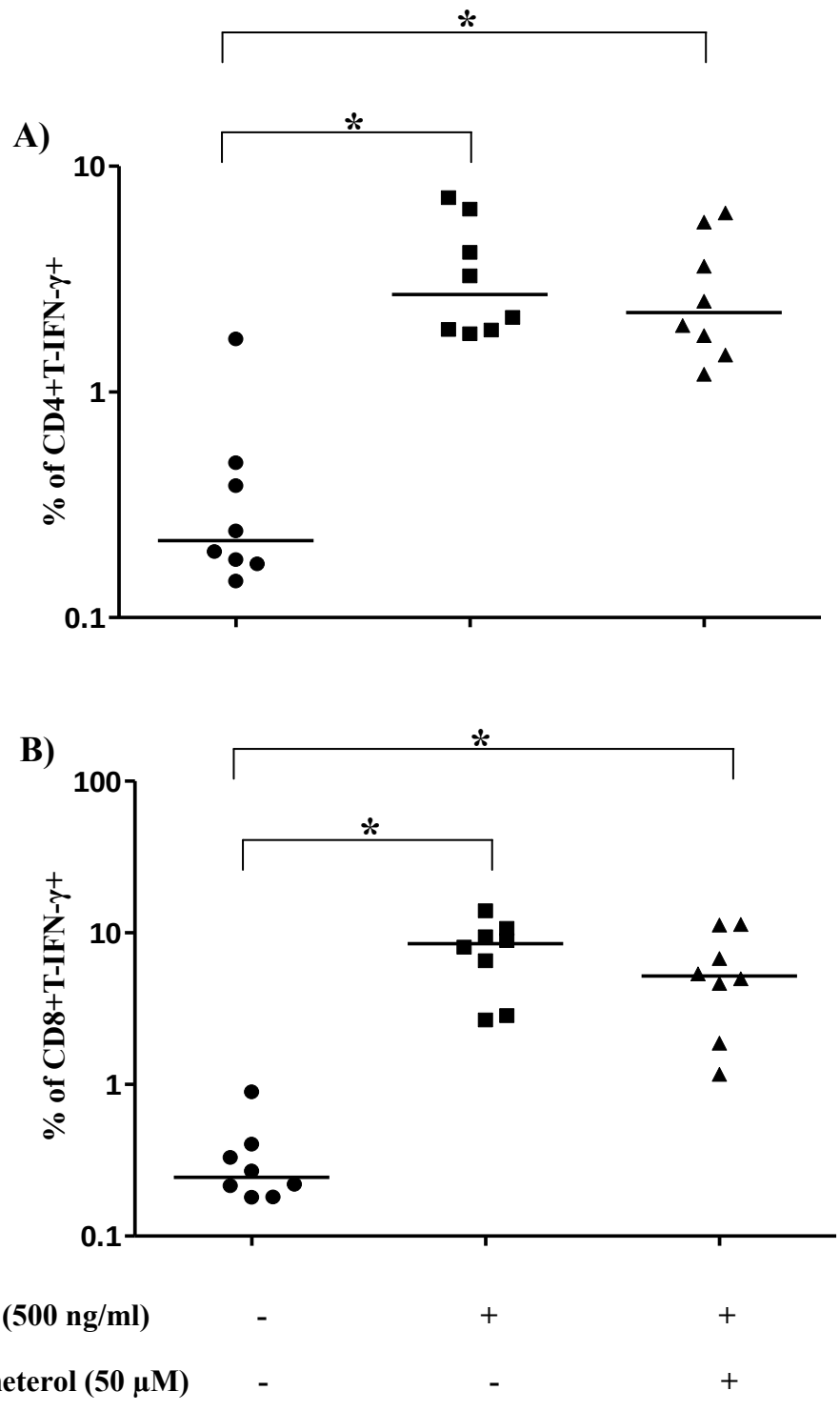



**Figure 3.2. (A-B). Effect of salmeterol on IFN-γ producing CD4+ T (A) and CD8+ T (B) cells in whole PBMCs stimulated with SEB and/or salmeterol.** PBMCs were isolated from blood and were incubated for 30 min at 37°C, 5% $CO_2$ in the presence or absence of 50 µM Salmeterol. For specific conditions refer to legend for (Fig. 3.1.). Results are shown as the mean values of 8 replicates (n=8). *$p<0.05$ by paired sample t-test, or Wilcoxon signed rank test for comparison to untreated control. The horizontal brackets indicate what is being compared. Flow cytometer data of a representative sample is shown in **Fig. 3.5-3.6. (A-D).**

### 3.2.3 The Effect of Salmeterol on the Proportion of CD4+ T-IFN-γ+ and CD8+ T-IFN-γ+ cells. Stimulation of PBMCs with CMV lysate (AD169) or CMV-pp65

To be able to detect and analyse activation of CMV-specific CD4+ and CD8+ T cells, PBMCs were stimulated with either CMV lysate (30 µl of CMV lysate/500 µl RPMI) or CMV pp65 (referred to as pp65 in the Figures; at a final concentration of 0.6 nM (~1 µg/ml) of peptide) following exposure to salmeterol (Fig 3.3.A-B). The choice of the CMV lysate was justified by the fact that CMV lysate induces a potent CD8+ T cell response, resulting in the secretion of effector cytokines such as IFN-γ. The CMV pp65 protein is a virion tegument protein and represents the main component of the enveloped subviral particle. Similarly to CMV lysate, CMV pp65 is an immunodominant target for CD4+ and CD8+ T cell responses to CMV, and induces secretion of IFN-γ, IL-2, and TNF-α. The production of these effector cytokines (IFN-γ, in our case) permits identification and analysis of CMV specific T cells. It is important to note that unlike the superantigen SEB, CMV lysate and CMV pp65 recombinant protein require antigen processing by APCs, to allow presentation to CD4+ T cells via MHC class II molecules on the surface of APCs and presentation to CD8+ T cells via MHC class I molecules on the surface of APCs (Arrode et al., 2002; Pita-Lopez et al., 2009; Reiser et al., 2011) .

The results, shown in Fig. 3.3-A, indicate that treatment of PBMCs with CMV lysate (30 µl/500 µl volume) caused a significant 5.5 fold increase in the percentage of CD4+ T IFN-γ+ cells (p=0.028 using paired sample t-test; p=0.043 using Wilcoxon signed rank test) and that pre-treatment of PBMCs with salmeterol reduced the percentage of IFN-γ producing CD4+ T cells by 1.3 fold following stimulation with CMV lysate (30 µl CMV lysate/500 µl buffer) (this increase was significant when p value was analysed using Wilcoxon signed rank test p=0.043) (Fig. 3.3-A). Similarly to CMV, treatment of PBMCs with CMV pp65 (0.6 nM~1 µg/ml) induced a significant 10.8 fold increase in the percentage of CD8+ T-IFN-γ+ cells

(p=0.017 using paired sample t-test; p=0.043 using Wilcoxon signed rank test), however a slightly more potent effect of salmeterol was observed on CD8+ TLs compared to the CD4+ TLs as salmeterol caused a significant 1.5 fold reduction in the percentage of CD8+ T-IFN-γ+ cells (p=0.004 using paired sample t-test; p=0.014 using Wilcoxon signed rank test) compared to the positive control (Fig. 3.3-B). Whilst our results suggest that salmeterol reduces the IFN-γ producing T cells, it was not clear whether the effect of salmeterol on T cells was direct or indirect. This is because in addition to T lymphocytes, β2AR are present on other cells within the lymphocyte population such as B cells, as well as on other antigen presenting cells such dendritic cells and monocytes (Borger et al., 1998; Goyarts et al., 2008). Thus this β2 agonist could influence the percentage of IFN-γ producing T cells, by binding to the β2AR on APCs (dendritic cells or B cells) and affecting the antigen processing or inhibiting the antigen presenting capability of these cells (APCs). In fact, several studies have reported catecholamines to inhibit the antigen presentation by epidermal Langerhans cells (Goyarts et al., 2008; Seiffert et al., 2002). To investigate whether salmeterol, affects the percentage of IFN-γ producing T cells directly or non-directly (i.e. as a consequence of an effect on APCs), or both, it was necessary to use a more direct approach of experiment.

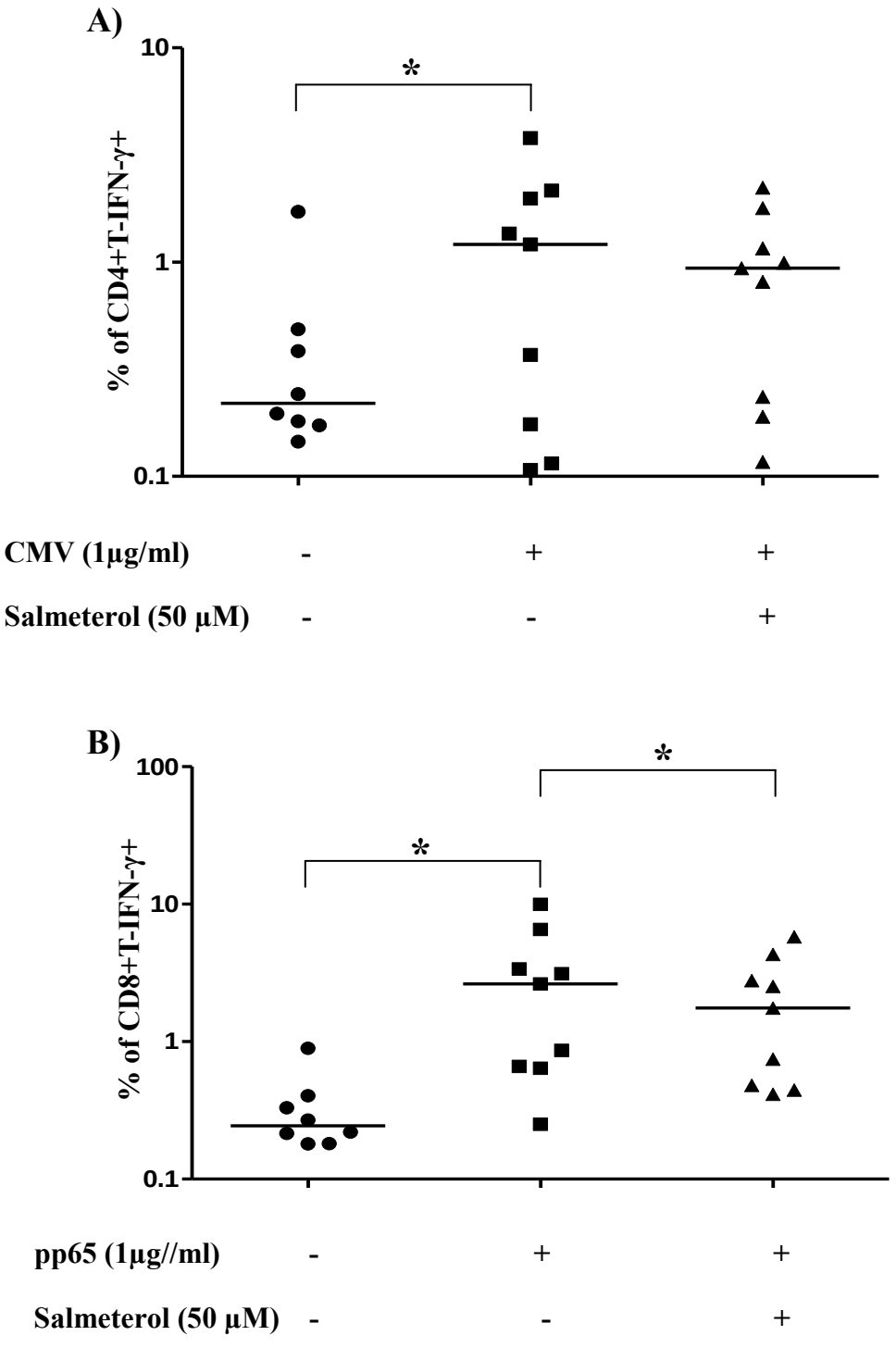



**Figure 3.3. (A-B). Effect of salmeterol on IFN-γ producing CD4+ T (A) and CD8+ T (B) cells in whole PBMCs stimulated with CMV lysate (A) or CMV pp65 (B) and/ or salmeterol.** For specific conditions refer to legend for (Fig. 3.1.). Results are shown as the mean values of 9 replicates (n=9). *$p<0.05$ using paired sample t-test and/ or Wilcoxon signed rank test. The horizontal brackets indicate what is being compared. Flow cytometer data of a representative sample is shown in **Fig. 3.7-3.8. (A-D)**.

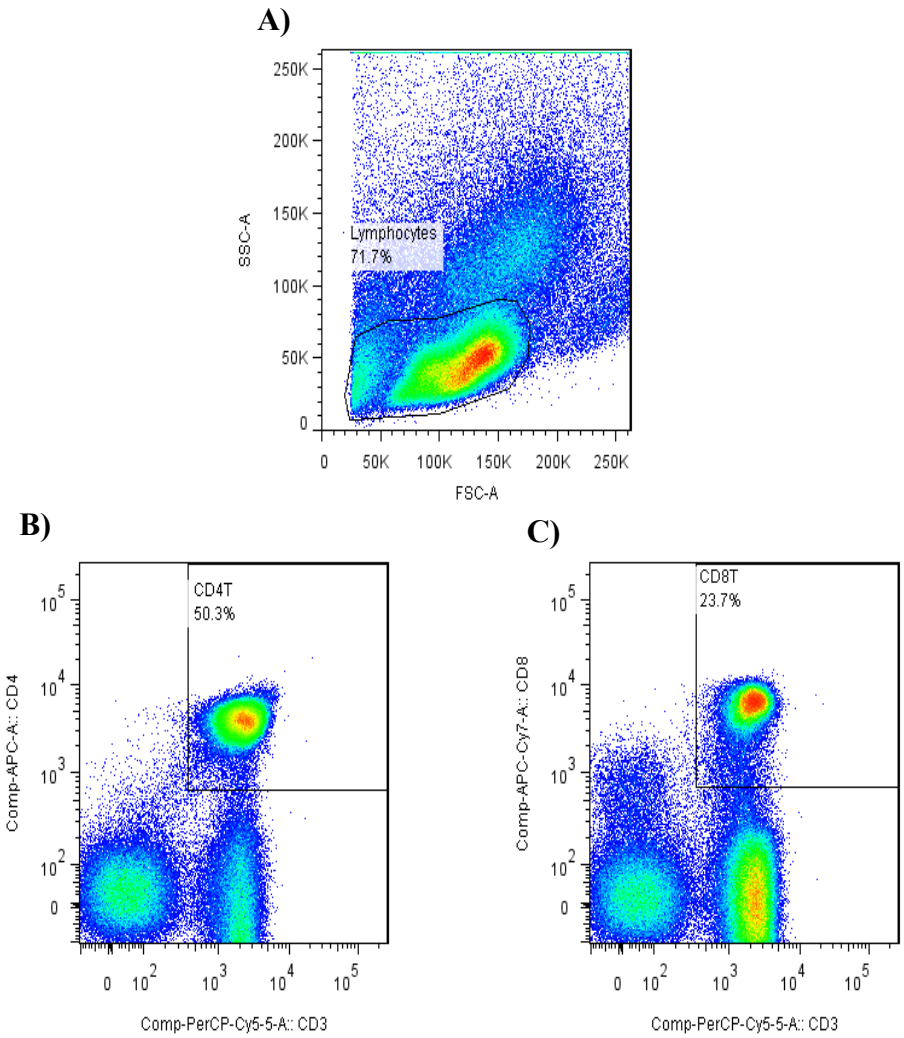



**Figure 3.4. (A-C). Representation of lymphocytes, CD4+ T and CD8+ T populations in Flow Jo graphs. Figures are representative scatter plots for data in Fig. 3.3. (A-B).** A total of 2 x $10^5$-7 x $10^5$ PBMC events were acquired using a dual-laser flow cytometer and analysed using FlowJo software. Cells that fell within the lymphocyte population were gated and analysed (A). Within the lymphocyte population, the CD3+/CD4+ T (B), and CD3+/CD8+ T (C) cell population was also gated.

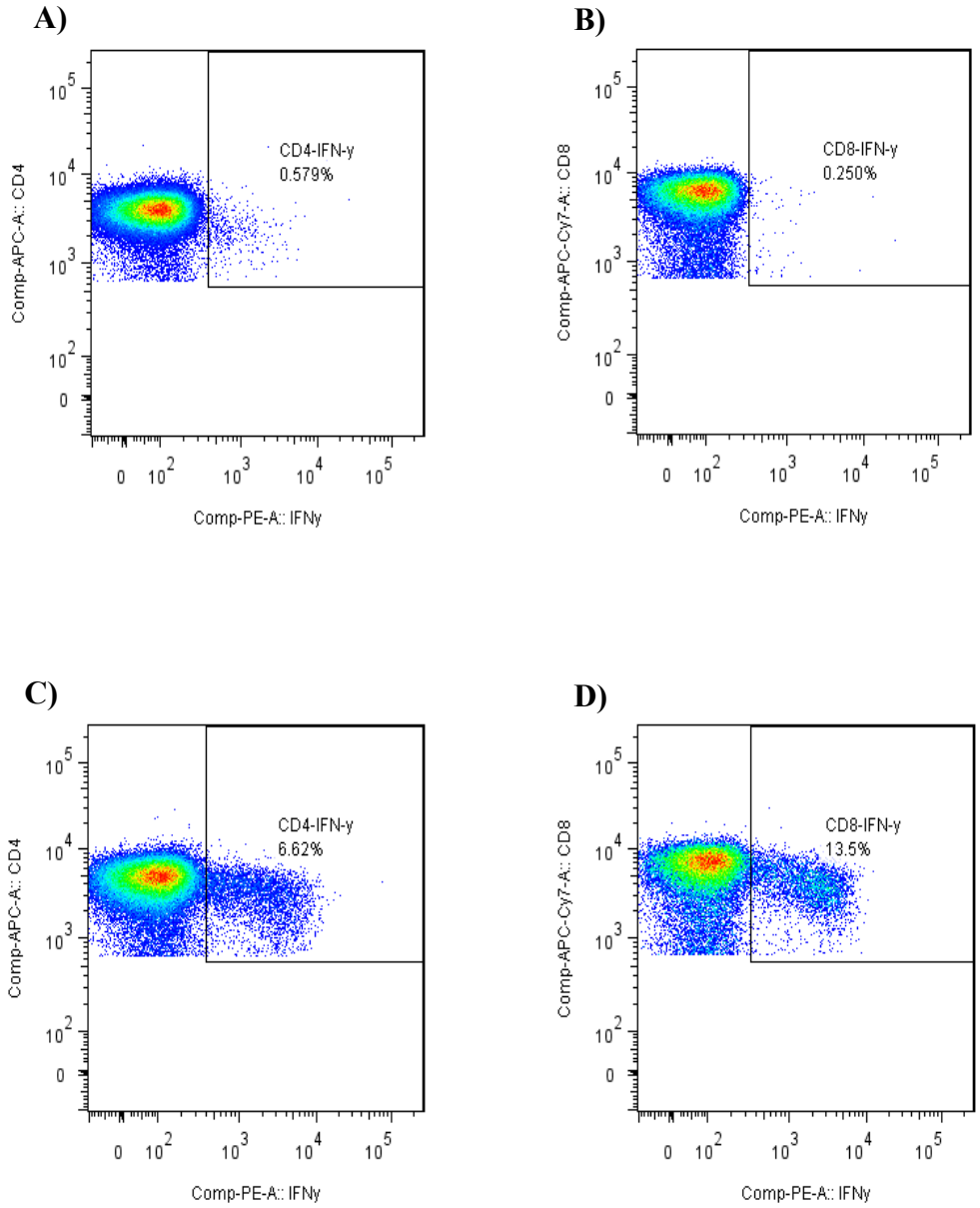



**Figure 3.5. (A-D). Effect of SEB on IFN-γ producing CD4+ T (A) and CD8+ T (B) cells. Figures are representative scatter plots for data in Fig. 3.3. (A-B).** The CD4+ and CD8+ T subpopulations were gated within the PBMCs population. The percentage of IFN-γ producing CD4+ T (C) and CD8+ T cells (D) in SEB treated PBMCs was gated and compared to the unstimulated CD4+ T (A) and CD8+ T cells (B) in control.

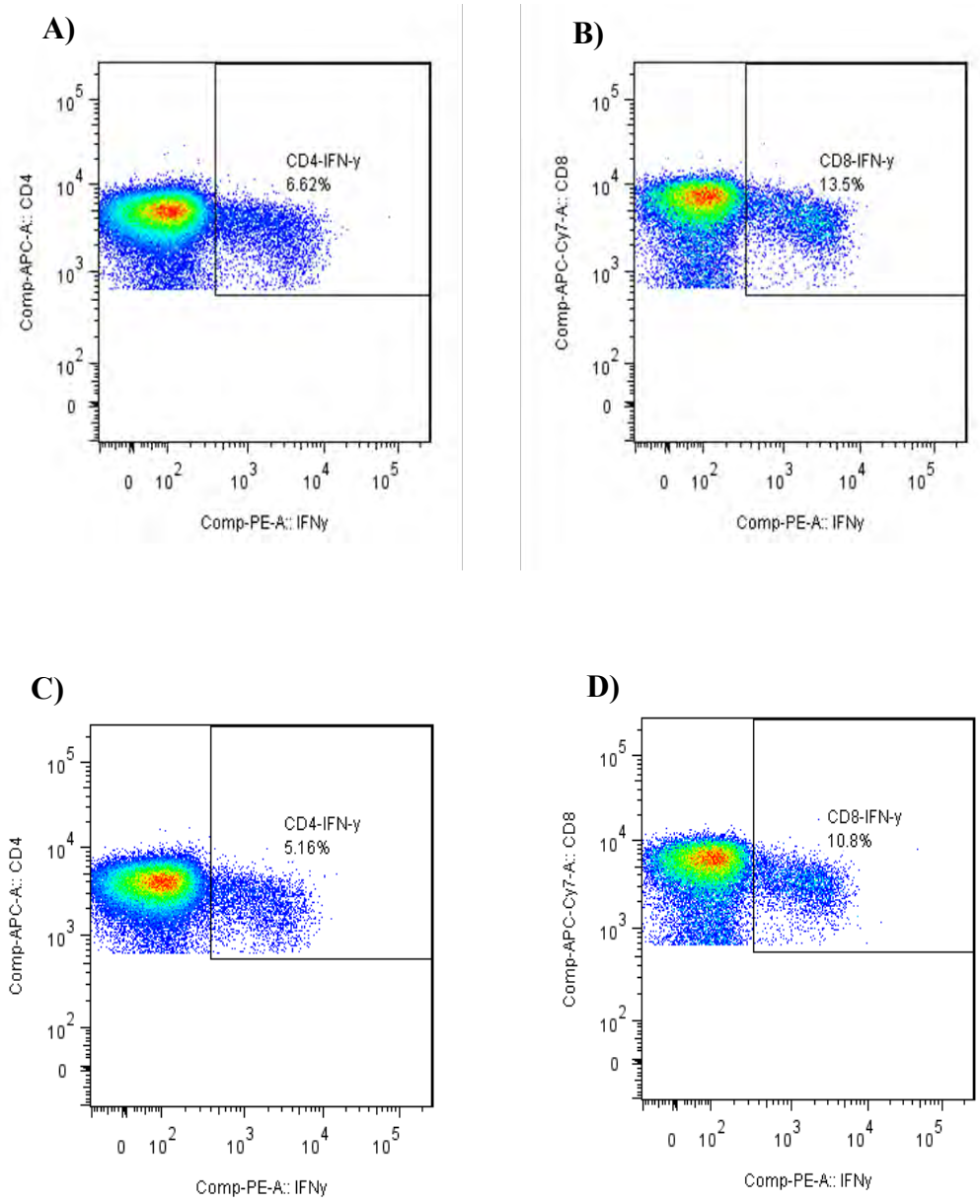



**Figure 3.6. (A-D). Effect of salmeterol on IFN-γ producing CD4+ and CD8+ T cells following treatment of PBMCs with SEB. Figures are representative scatter plots for data in Fig. 3.3. (A-B).** The CD4+ and CD8+ T subpopulations were gated within the PBMCs population. The percentage of IFN-γ producing CD4+ T (A) and CD8+ T cells (B) in SEB stimulated PBMCs was gated and compared to the percentage of IFN-γ producing CD4+ T (C) and CD8+ T cells (D) in PBMCs treated with salmeterol and stimulated with SEB.

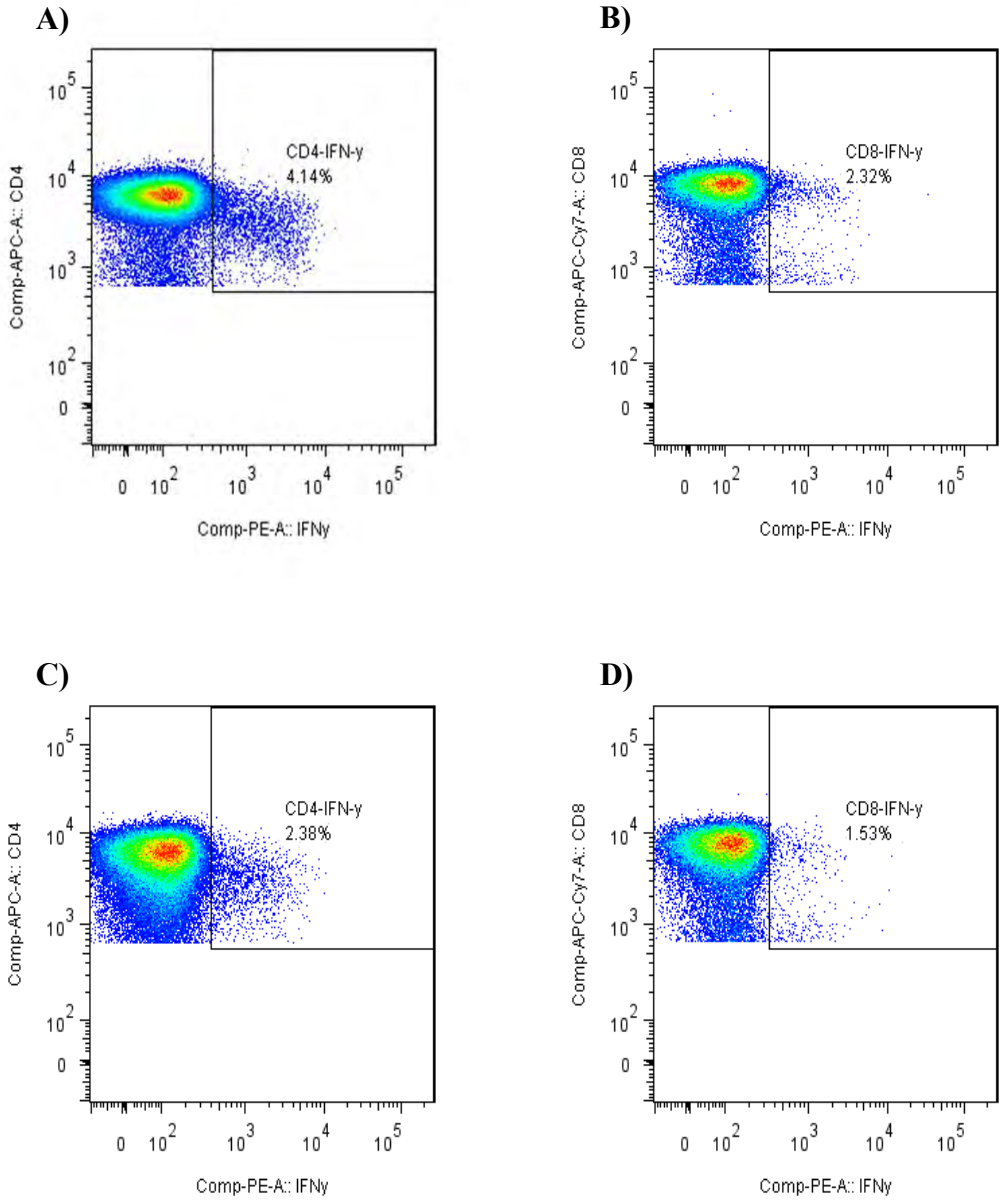



**Figure 3.7. (A-D). Effect of salmeterol on IFN-γ producing $CD4^+$ and $CD8^+$ T cells following treatment of PBMCs with CMV lysate. Figures are representative scatter plots for data in Fig. 3.3. (A-B).** The $CD4^+$ and $CD8^+$ T subpopulations were gated within the PBMCs population. The percentage of IFN-γ producing $CD4^+$ T (A) and $CD8^+$ T cells (B) in CMV lysate stimulated PBMCs was gated and compared to the percentage of IFN-γ producing $CD4^+$ T (C) and $CD8^+$ T cells (D) in PBMCs treated with salmeterol and stimulated with CMV lysate.

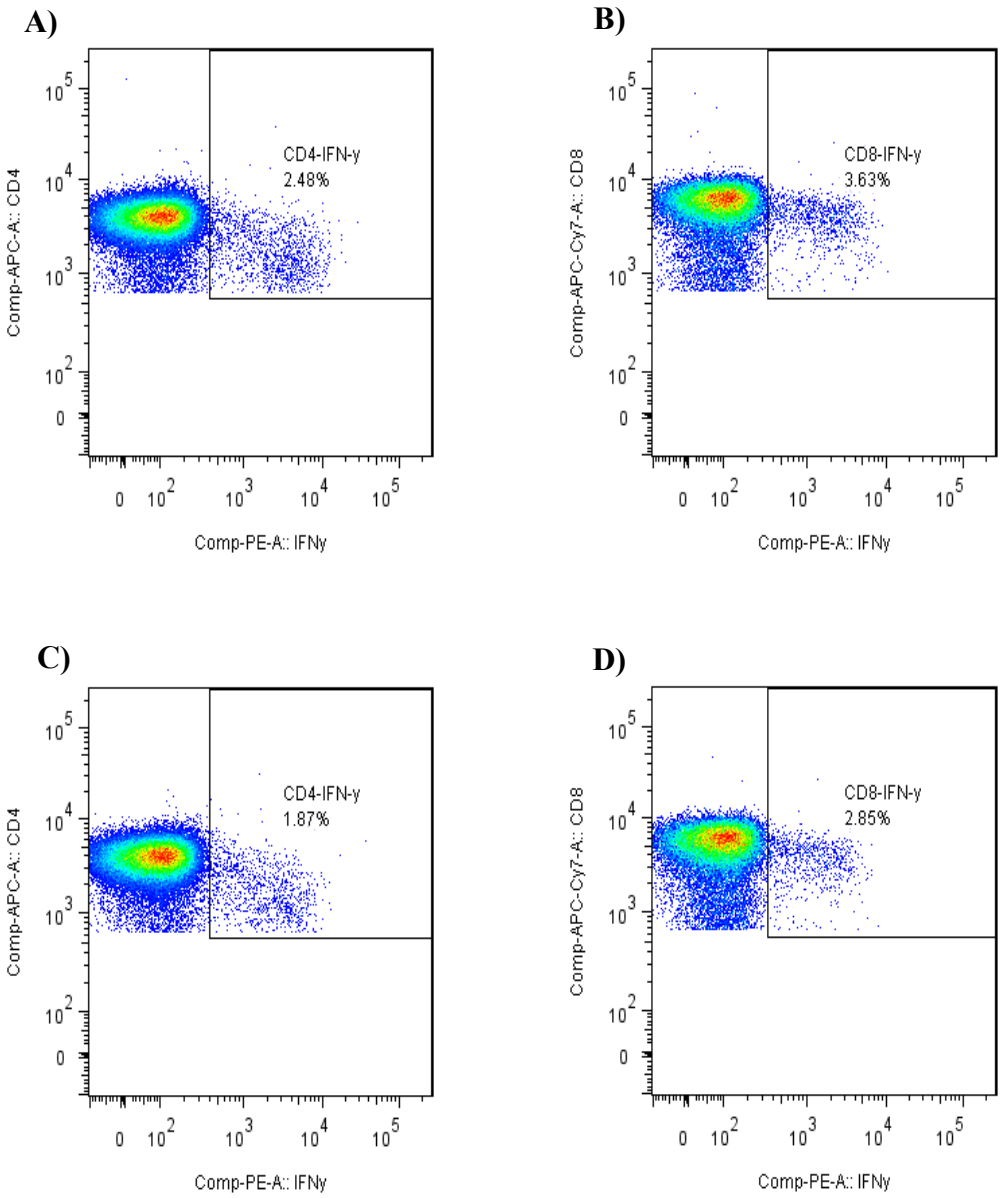



**Figure 3.8. (A-D). Effect of salmeterol on IFN-γ producing CD4$^+$ and CD8$^+$ T cells following treatment of PBMCs with CMV pp65. Figures are representative scatter plots for data in Fig. 3.3. (A-B).** The CD4$^+$ and CD8$^+$ T subpopulations were gated within the PBMCs population. The percentage of IFN-γ producing CD4+ T (A) and CD8+ T cells (B) in CMV pp65 stimulated PBMCs was gated and compared to the percentage of IFN-γ producing CD4+ T (C) and CD8+ T cells (D) in PBMCs treated with salmeterol and stimulated with CMV pp65.

### 3.2.4 The Indirect Effect of Salmeterol on the Percentage of IFN-γ Producing CD4+ and CD8+ T cells

In the next set of experiments, T cells were isolated from PBMCs and the remaining cells, which are to a large extent APCs like B cells and monocytes, (PBMCs – T cells), were treated with salmeterol (50 μM). Following this treatment, the APCs were added back to the isolated T cells and all cells were stimulated with SEB (Fig. 3.9. A-B). Our results showed that SEB significantly increased the percentage of CD4+ T-IFN-γ+ (3.2 fold; p=0.031 using paired sample t-test; although a borderline significance was not reached using Wilcoxon signed rank test, p=0.054) and CD8+T-IFN-γ+ cells (4.2 fold; p=0.032 using paired sample t-test, although p=0.054 using Wilcoxon signed rank test) (Fig. 3.9. A-B). In contrast, salmeterol caused a small but statistically significant reduction in the percentage of IFN-γ producing CD4+ T cells (1.19 fold inhibition; p=0.009 using paired sample t –test, although p=0.054 using Wilcoxon signed rank test) and CD8+ T cells (1.53 fold inhibition; p=0.003 using paired sample t-test, although p=0.054 using Wilcoxon signed rank test), as compared to the positive control (Fig. 3.9. A-B). Stimulation of PBMCs with CMV pp65, following exposure of APCs to salmeterol, reduced the percentage of IFN-γ producing CD8+ T cells by 1.8 fold (Fig. 3.10. B). In contrast, the effect of salmeterol on IFN-γ producing CD4+ T cells was difficult to determine as no increase on the % of CD4+ T-IFN-γ+ was observed following stimulation of PBMCs with CMV lysate (Fig.3.10-A). Altogether, these results suggest that salmeterol inhibits the IFN-γ producing CD4+ and CD8+ TLs indirectly, by affecting the APCs.

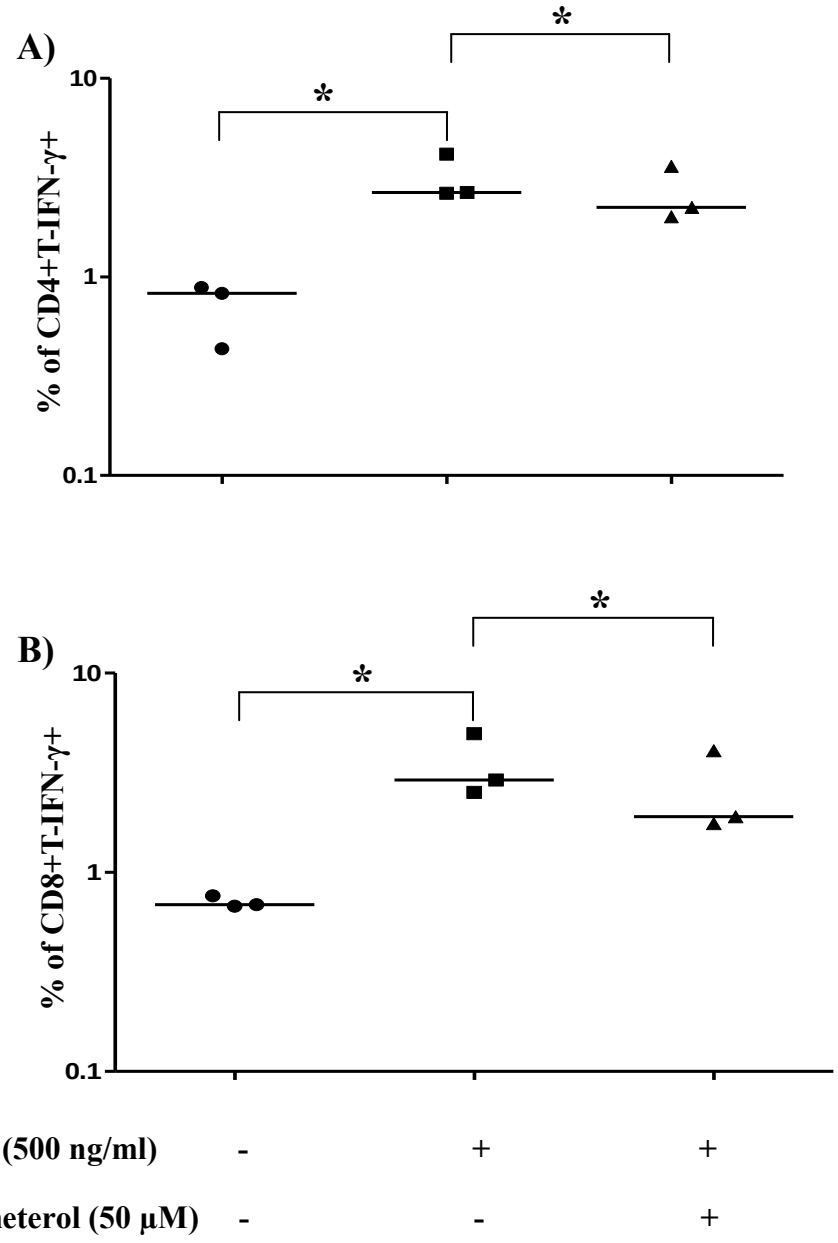



**Figure 3.9. (A-B). Effect of salmeterol on IFN-γ producing CD4+ T (A) and CD8+ T (B) cells following treatment of APCs and stimulation of PBMCs with SEB.** T cells were isolated from PBMCs and the remaining APCs were incubated for 30 min at 37°C, 5% $CO_2$ in the presence or absence of 50 µM salmeterol. Following incubation with salmeterol, APCs were washed and T cells were added back to the APCs. PBMCs were then stimulated with SEB and incubated for 1 hr 30 min at 37°C, 5% $CO_2$. Subsequently, monensin was added to inhibit the intracellular secretion of cytokines. Cells were incubated for a further period of 5 hours and stained with respective antibodies. The percentage of IFN-γ producing T cells was determined using FACS analysis. Results are shown as the mean values of 3 replicates (n=3). *$p<0.05$ using paired sample t-test. The horizontal brackets indicate what is being compared. Flow cytometry data of a representative sample is shown in **Fig. 3.11-3.12. (A-D)**.

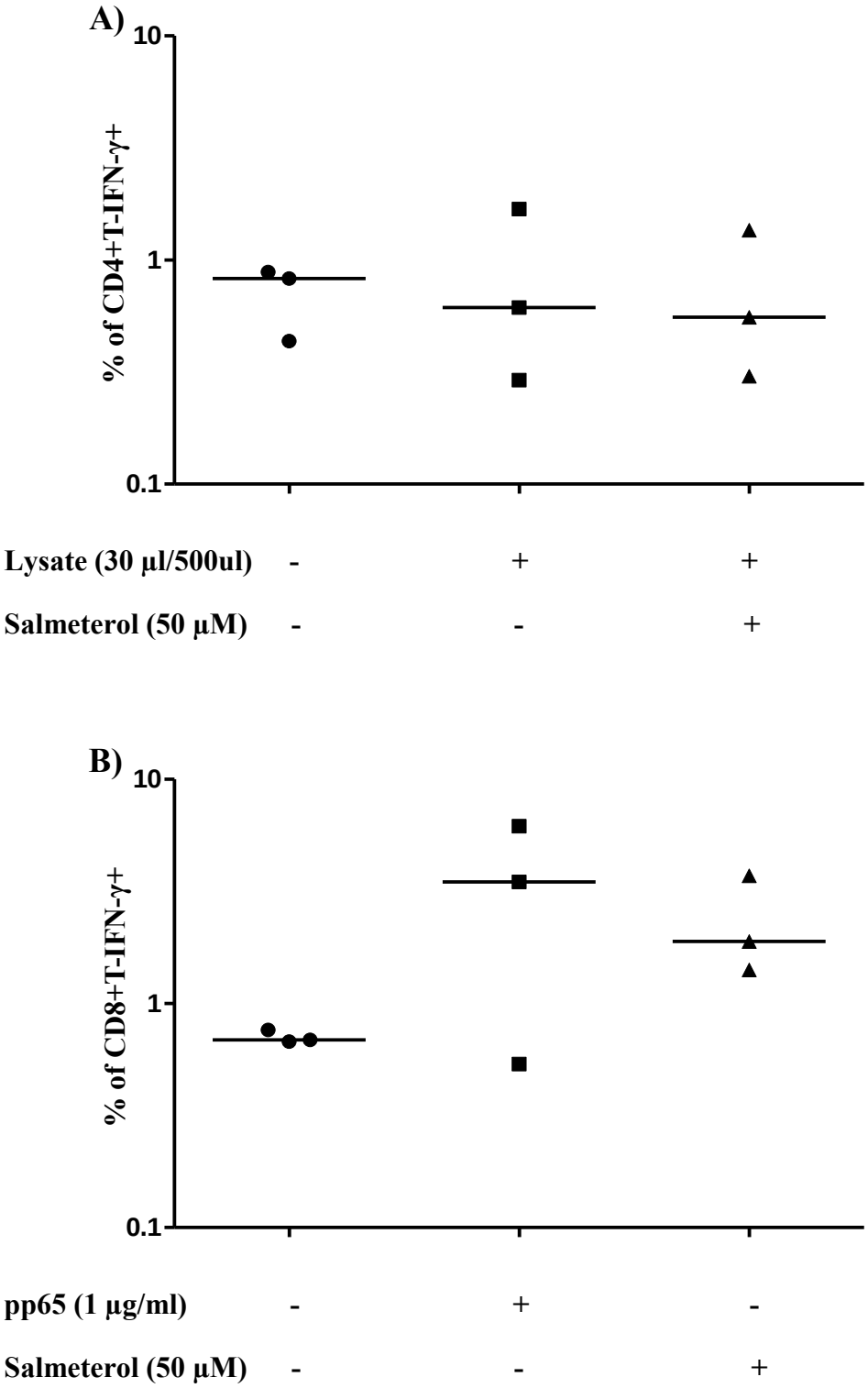



**Figure 3.10. (A-B). Effect of salmeterol on IFN-γ producing CD4+ T (A) and CD8+ T (B) cells following treatment of APCs and stimulation of PBMCs with CMV-lysate (A) or CMV pp65 (B).** Results are shown as the mean values of 3 replicates (n=3). For specific conditions refer to (Fig. 3.9.). Flow cytometer data of a representative sample is shown in **Fig. 3.13-3.14. (A-D).**

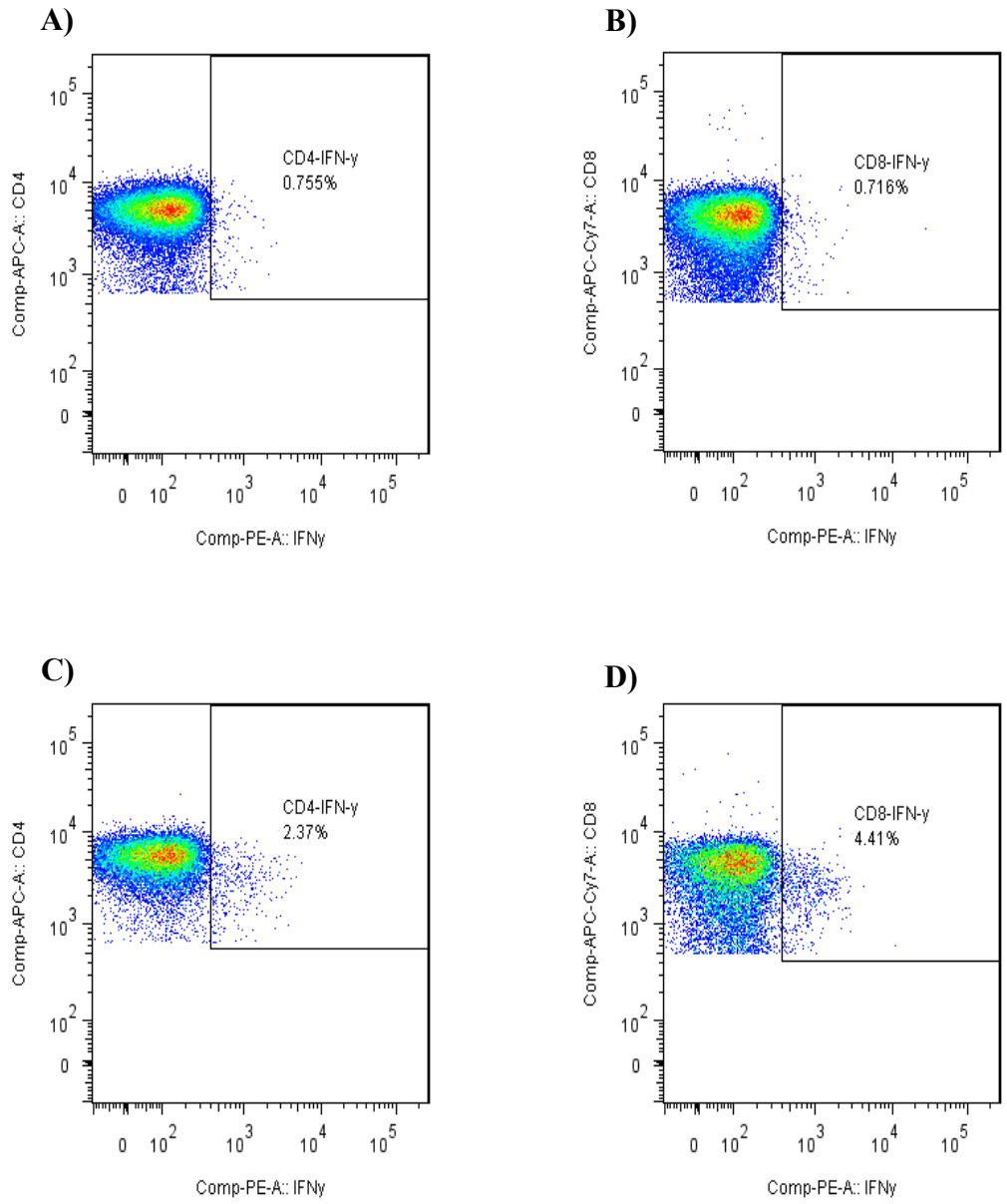



**Figure 3.11. (A-D). Effect of SEB on IFN-γ producing $CD4^+$ T (A) and $CD8^+$ T (B) cells in SEB stimulated PBMCs. Figures are representative scatter plots for data in Figures 3.9. (A-B).** The $CD4^+$ and $CD8^+$ T subpopulations were gated within the lymphocyte population. The percentage of IFN-γ producing CD4+ T (C) and CD8+ T (D) cells in SEB treated PBMCs was gated and compared to the percentage of IFN-γ producing CD4+ T (A) and CD8+ T cells (B) in unstimulated control.

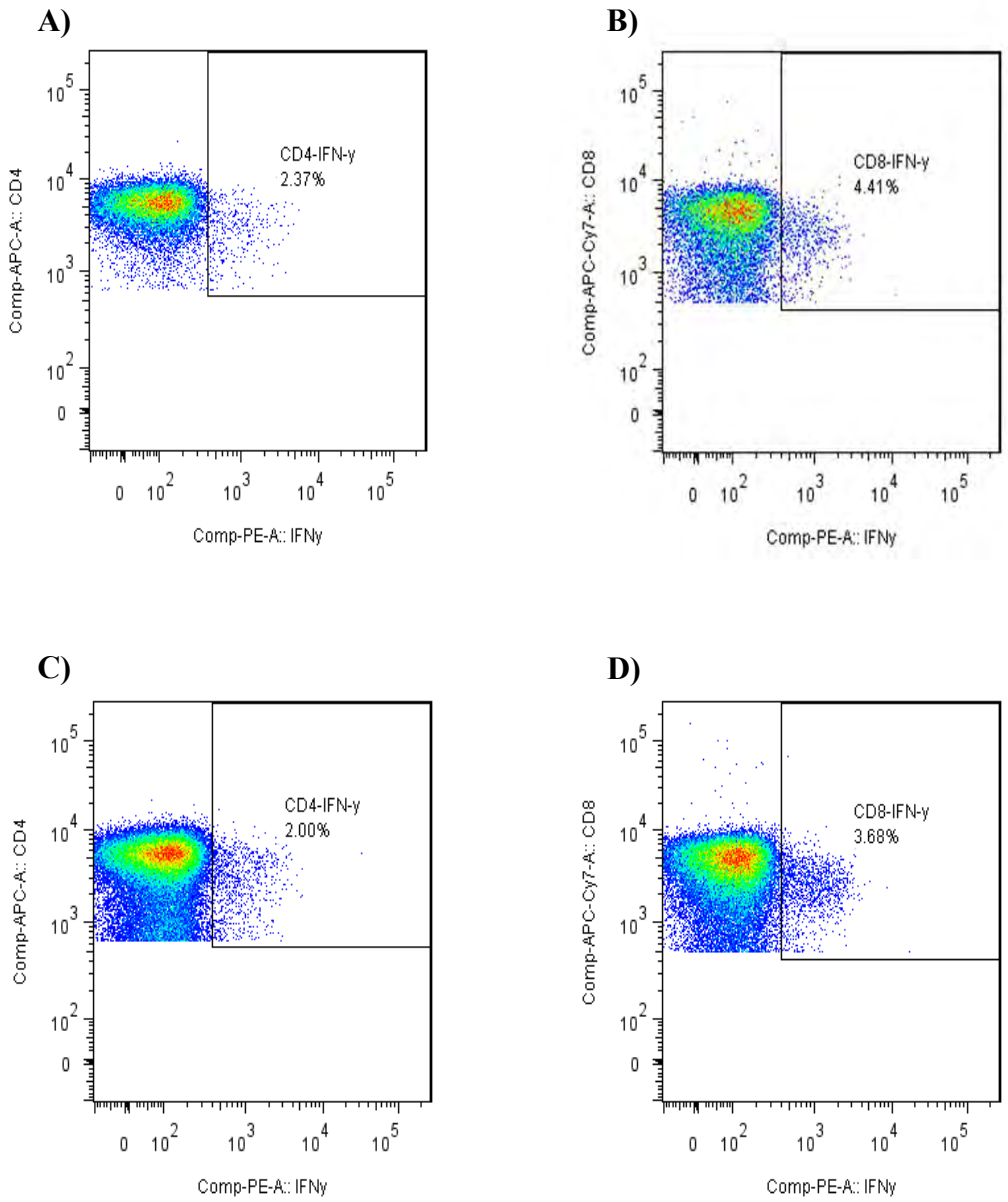



**Figure 3.12. (A-D). Effect of salmeterol on IFN-γ producing $CD4^+$ and $CD8^+$ T cells following treatment of APCs with or without salmeterol and stimulation of PBMCs with SEB. Figures are representative plots for data in Fig. 3.9. (A-B).** The $CD4^+$ and $CD8^+$ T subpopulations were gated within the lymphocyte population. The percentage of IFN-γ producing CD4+ T (A) and CD8+ T cells (B) in SEB stimulated PBMCs was gated and compared to the percentage of IFN-γ producing CD4+ T (C) and CD8+ T cells (D) in APCs treated with salmeterol and PBMCs stimulated with SEB.

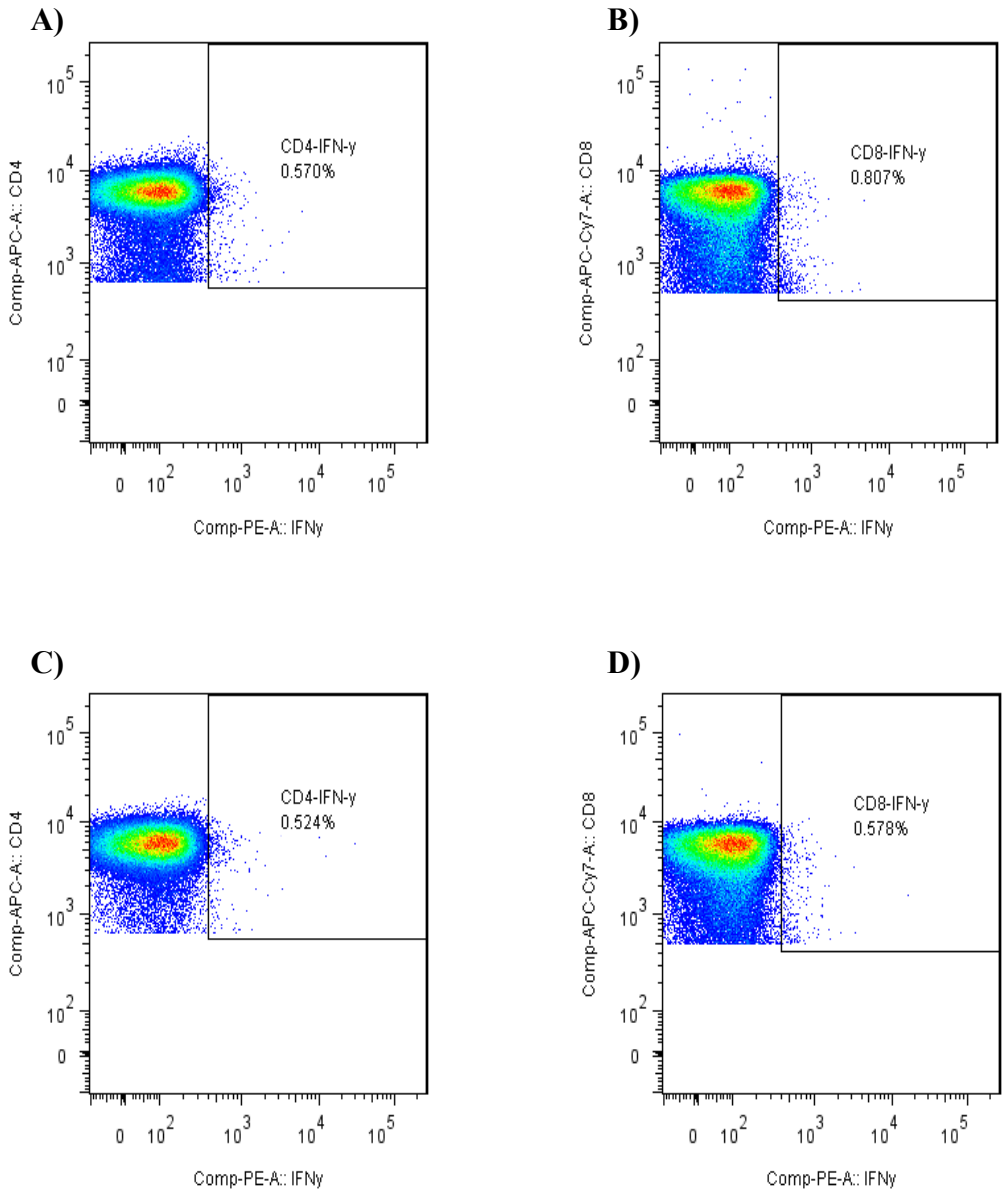



**Figure 3.13. (A-D). Effect of salmeterol on IFN-γ producing CD4$^+$ and CD8$^+$ T cells following treatment of APCs with or without salmeterol and stimulation of PBMCs with CMV lysate. Figures are representative plots for data in Fig. 3.10. (A-B).** The CD4$^+$ and CD8$^+$ T subpopulations were gated within the PBMCs population. The percentage of IFN-γ producing CD4+ T (A) and CD8+ T cells (B) in CMV lysate stimulated PBMCs was gated and compared to the percentage of IFN-γ producing CD4+ T (C) and CD8+ T cells (D) in APCs treated with salmeterol and PBMCs stimulated with CMV lysate.

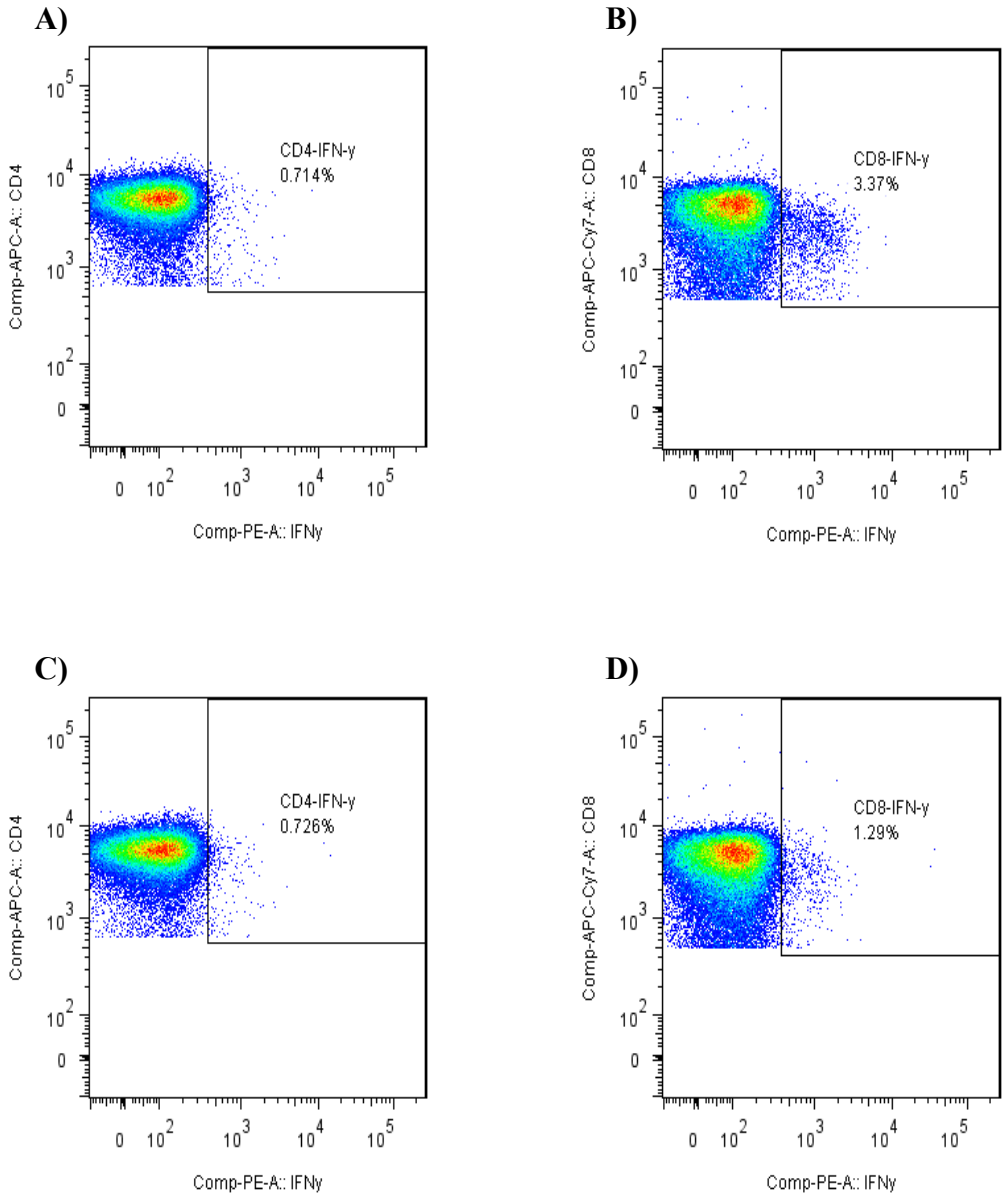



**Figure 3.14. (A-D). Effect of salmeterol on IFN-γ producing CD4$^+$ and CD8$^+$ T cells following treatment of APCs with or without salmeterol and stimulation of PBMCs with CMV pp65. Figures are representative scatter plots of data in Fig. 3.10. (A-B).** The CD4$^+$ and CD8$^+$ T subpopulations were gated within the PBMCs population. The percentage of IFN-γ producing CD4+ T (A) and CD8+ T cells (B) in CMV pp65 stimulated PBMCs was gated and compared to the percentage of IFN-γ producing CD4+ T (C) and CD8+ T cells (D) in APCs treated with salmeterol and PBMCs stimulated with CMV pp65.

### 3.2.5 The Direct Effect of Salmeterol on IFN-γ Producing CD4+ and CD8+ T cells

To investigate whether salmeterol also has a direct effect on the percentage of CD4+ T-IFN-$\gamma^+$ and CD8+ T- IFN-$\gamma^+$, we isolated CD4+ and CD8+ T cells from PBMCs and incubated these T cells with salmeterol (50 µM). Following treatment of T cells with salmeterol, were the T cells added back to the remaining PBMCs (containing APCs such as monocytes and B cells) and incubated with SEB. The results showed that SEB significantly increased the percentage of CD4+ T-IFN-$\gamma^+$ (5.2 fold; p=0.001 using paired sample t-test; p=0.001 using Wilcoxon singed rank test) and CD8+ T-IFN-$\gamma^+$ (4.5 fold; p=0.003 using paired sample t-test; p=0.001 using Wilcoxon singed rank test). Salmeterol (50 µM) induced a significant 1.8 fold inhibition on the percentage of IFN-γ producing CD4+ T cells (p=0.001 using paired-sample t-test; p=0.001 using Wilcoxon singed rank test) (Fig. 3.15. A) and a significant 2.1 fold inhibition on the percentage of IFN-γ producing CD8+ TLs (p=0.001 using paired sample t-test; p=0.001 using Wilcoxon singed rank test) compared to the positive control (Fig. 3.15. B). Another interesting observation is the fact that the percentage of IFN-γ producing CD4+ and CD8+ T cells following stimulation with SEB is ~5-9 fold lower than the positive control observed in the previous experiments (Fig. 3.2). These discrepancies probably reflect the large donor to donor variation on SEB induced IFN-γ producing T cells. One of the factors which appears to have an influence on the T cell response to SEB is age. As shown in Fig. 3.16. B, it is mostly the 40-49 and 50-59 age group that respond better to SEB, whilst the 30-39 and the 20-29 age group stand at a lower end of the spectrum characterised by lower stimulation of IFN-γ production from T cells in response to SEB. This could possibly be explained as older people are characterised by a higher proportion of effector memory T cells, which respond to infections, more vigorously and faster. On the other hand, gender does not seem to influence T cell IFN-γ production in response to SEB. Both, females and males had a similar response,

and this is observed throughout the chapters of this thesis. However, it is important to note that in all three studies of this thesis, the majority of participants were males, thus a more precise answer on whether gender influences T cell IFN-γ production in response to SEB, would have been achieved if equal number of male and female participants had been recruited.

We next asked whether salmeterol inhibits the IFN-γ producing T cells in a dose dependent manner. Two concentrations of salmeterol (5 and 50 μM) were analysed (Fig. 3.17. A-B). Our data show that stimulation of PBMCs with SEB significantly increased the percentage of IFN-γ producing CD4+ ($p=0.014$ using paired sample t-test; $p=0.003$ using Wilcoxon singed rank test) and CD8+ T cells ($p=0.049$ using paired sample t-test; $p=0.003$ using Wilcoxon singed rank test) compared to the unstimulated PBMCs. In addition, salmeterol at concentration of 50 μM, significantly suppressed the percentage of IFN-γ producing CD4+ TLs ($p=0.039$ using paired sample t-test; $p=0.030$ using Wilcoxon singed rank test) by 1.27 fold compared to the positive control (Fig. 3.17. A). In contrast, at a concentration of 5 μM, salmeterol had a smaller suppressive effect, inhibiting the percentage of IFN-γ production by 1.1 fold. Similarly, a 50 μM concentration of salmeterol caused a significant inhibition on the percentage of IFN-γ producing CD8+ T cells (1.8 fold decrease) compared to the positive control ($p=0.016$ using paired sample t-test; $p=0.003$ using Wilcoxon singed rank test) (Fig. 3.17. B). In contrast, a 5 μM concentration of salmeterol caused a 1.6 fold inhibition, relative to the positive control, which did not reach significance using paired sample t-test but reached significance using Wilcoxon singed rank test as $p=0.011$. These findings suggest that salmeterol inhibits the IFN-γ production in a dose dependent manner.

To examine, whether the inhibitory effect of salmeterol on the percentage of IFN-γ producing CD4+ and CD8+ TLs, was consistent when PBMCs were stimulated with different stimuli,

PBMCs were stimulated with either CMV lysate or CMV pp65 (Fig. 3.18. A-B). The results show salmeterol to cause a 1.2 fold inhibition on the percentage of IFN-γ producing CD4+ T cells, when PBMCs were stimulated with CMV lysate (significant value was reached only using Wilcoxon singed rank test p=0.022) (Fig. 3.18. A). More importantly, following stimulation of PBMCs with CMV pp65, the percentage of IFN-γ producing CD8+ T cells was reduced by 3.4 fold (significant value was reached only using Wilcoxon singed rank test p=0.022) (Fig. 3.18. B). Altogether, these results suggest that salmeterol has a suppressive effect on the percentage of IFN-γ producing CD4+ T and CD8+ T cells by a direct effect on these cells, as opposed to indirectly via APCs.

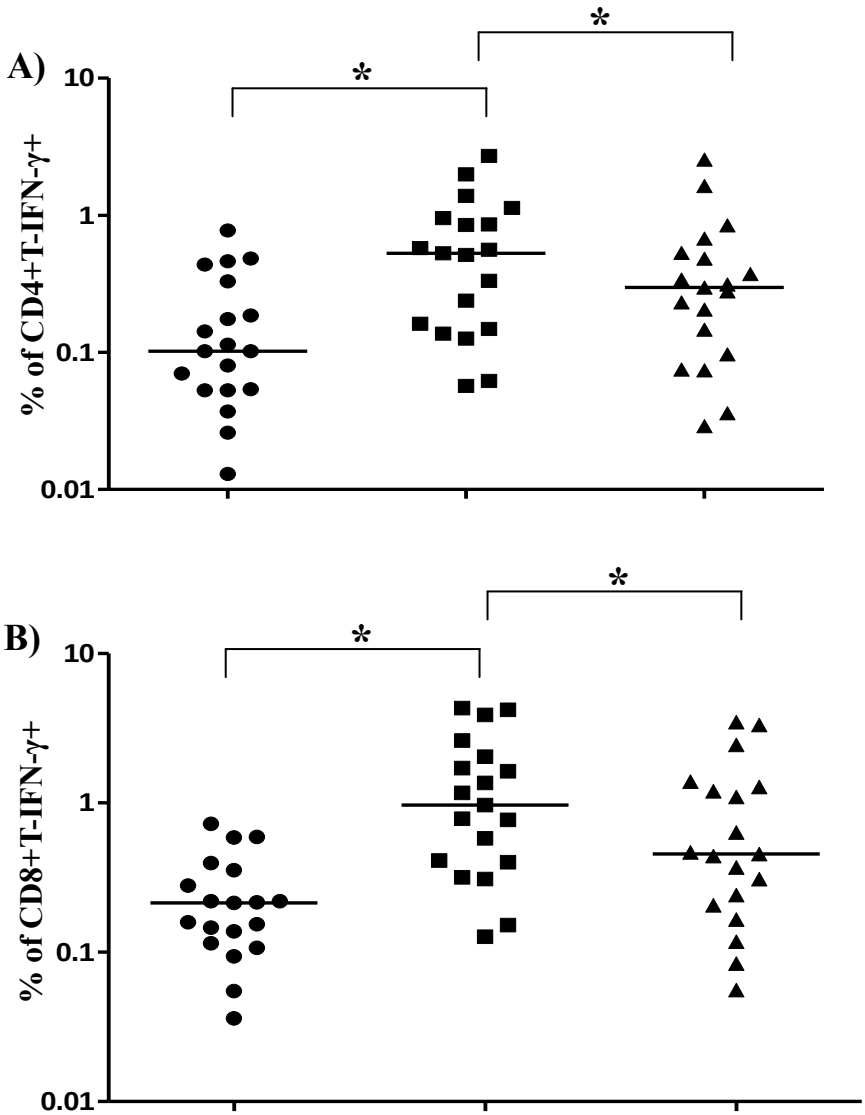



**Figure 3.15. (A-B). Salmeterol inhibits IFN-γ producing CD4+ T (A) and CD8+ T (B) cells stimulated with SEB.** T cells were isolated from PBMCs and incubated for 30 min at 37°C, 5% $CO_2$ in the presence or absence of 50 µM salmeterol. Following incubation with salmeterol, T cells were washed and added to the rest of the PBMCs. Subsequently, PBMCs were stimulated with SEB and incubated for 1 hr 30 min at 37°C, 5 % $CO_2$. Following incubation of PBMCs with SEB, monensin was added to inhibit intracellular secretion of cytokines. Cells were incubated for a further period of 5 hours and stained with respective antibodies. The percentage of IFN-y producing T cells was determined using FACS Analysis. Results are shown as the mean values of 20 replicates (n=20). *$p<0.05$ using paired sample t-test and/or Wilcoxon singed rank test.

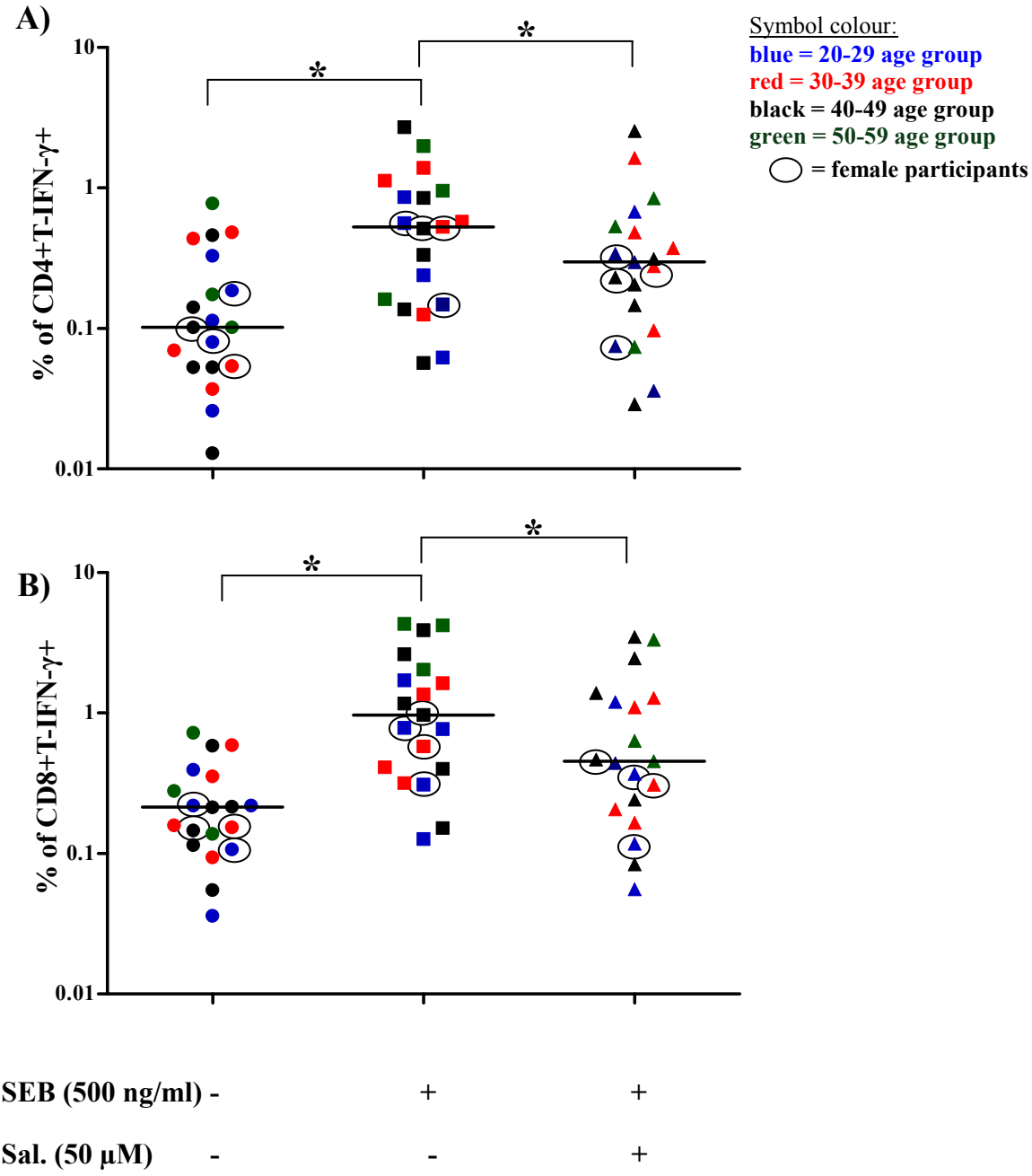



**Figure 3.16. (A-B). Salmeterol inhibits IFN-γ producing CD4+ T (A) and CD8+ T (B) cells stimulated with SEB in different age –groups.** For specific conditions refer to (Fig. 3.15). ). *p<0.05 using paired sample t-test and/ or Wilcoxon singed rank test.

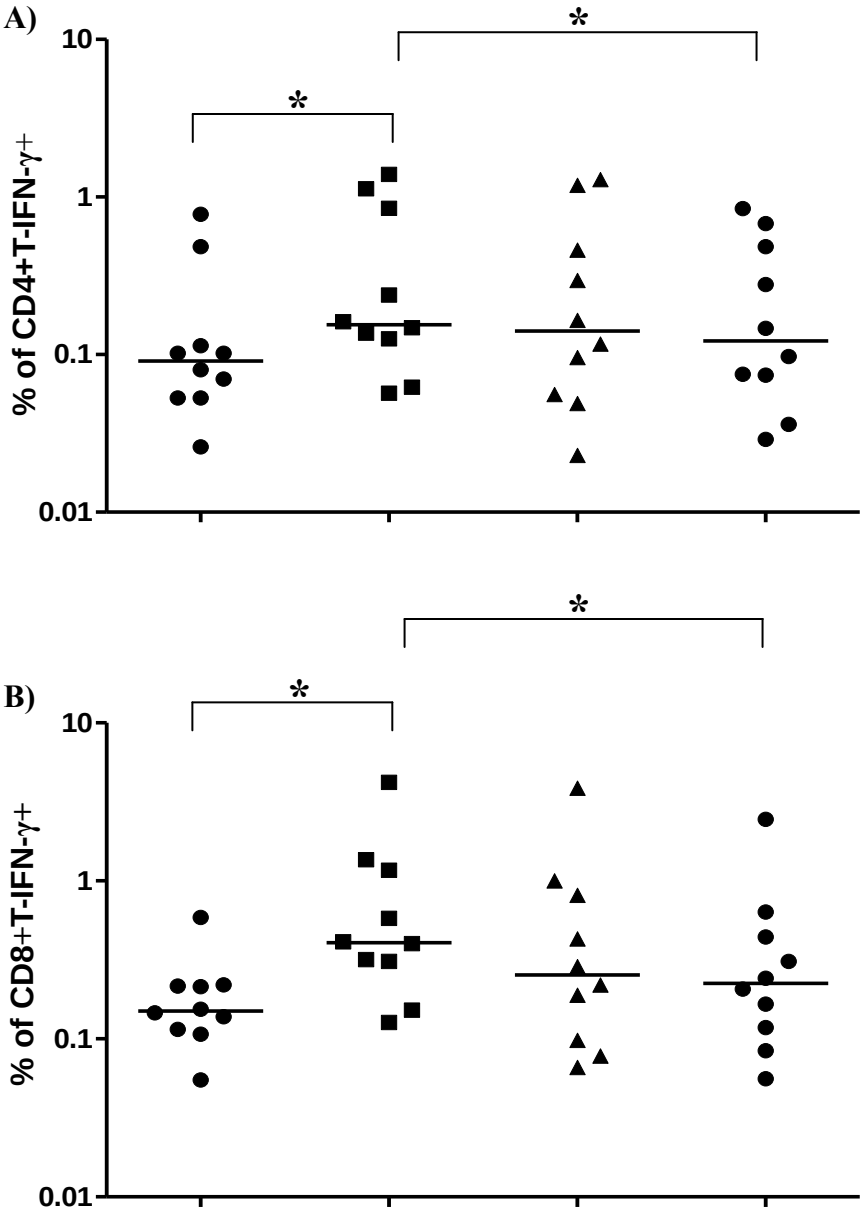
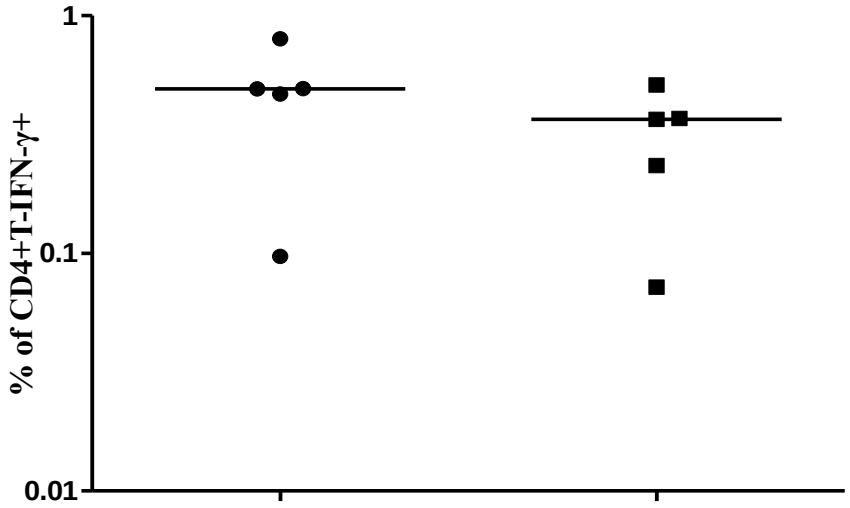
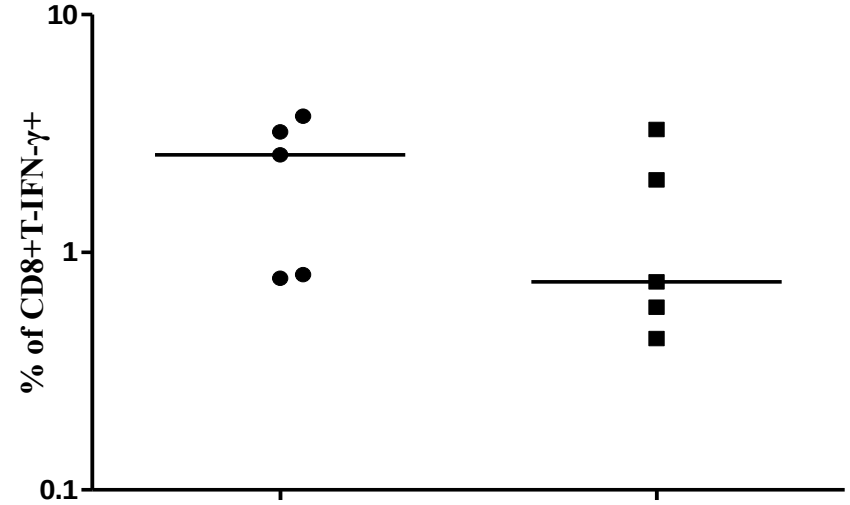



**Figure 3.17. (A-B). Effect of Salmeterol on IFN-γ producing CD4+ T (A) and CD8+ T (B) in PBMCs stimulated with SEB.** Results are shown as the mean values of 10 replicates (n=10). For specific conditions refer to (Fig. 3.15). *p<0.05 using paired sample t-test and/ or Wilcoxon singed rank test.




**CMV (30 µl/500 ul)** + +

**Salmeterol (50 µM)** - +




**pp65 (1 µg/ml)** + +

**Salmeterol (50 µM)** - +

**Figure 3.18. (A-B). Effect of salmeterol on IFN-γ producing CD4+ T (A) and CD8+ T cells (B) in PBMCs stimulated with CMV lysate (A) or CMV pp65 (B) respectively.** Results are shown as the mean values of 5 replicates (n=5). For specific conditions refer to (Fig.3.15).

### 3.2.6 Salmeterol Inhibits IFN-γ Producing CD4+ and CD8+ T cells via a $\beta_2$ Adrenergic-Dependent Pathway

To confirm that the effects of salmeterol were mediated by stimulation of $\beta_2$AR, T cells were treated with salmeterol (50 μM) in the presence or absence of different concentrations (10, 25 and 50 μM) of the selective $\beta_2$ antagonist, ICI 118, 551 (Fig. 3.19. A-B). In the absence of ICI 118,551, salmeterol induced a 1.5 fold suppression on the percentage of IFN-γ producing CD4+ TLs (Fig. 3.19. A) and 1.1 fold suppression on the IFN-γ producing CD8+ TLs (Fig. 3.19. B). The inhibition of IFN-γ producing T cells by salmeterol was completely reversible by ICI118,551 when used at concentration of 25 μM in both CD4+ and CD8+ T lymphocytes. These findings confirm that salmeterol-induced IFN-γ inhibition is mediated by $\beta_2$AR. Our data (Fig. 3.19. A) also show that ICI 115, 881 at a concentration of 10 μM did not completely reverse the inhibition of salmeterol in IFN-γ producing CD4+ TLs as compared to the negative control (i.e., SEB + salmeterol), whilst it did not have any effect on the percentage of IFN-γ producing CD8+ TLs (Fig. 3.19. B). In contrast, treatment of T cells with salmeterol (50 μM) and ICI118, 551 (50 μM) completely reversed the suppressive effect on CD8+ TLs (Fig. 3.19. B), whilst it reversed the inhibition of IFN-y producing CD4+ TLs by 1.18 fold (Fig. 3.19. A).

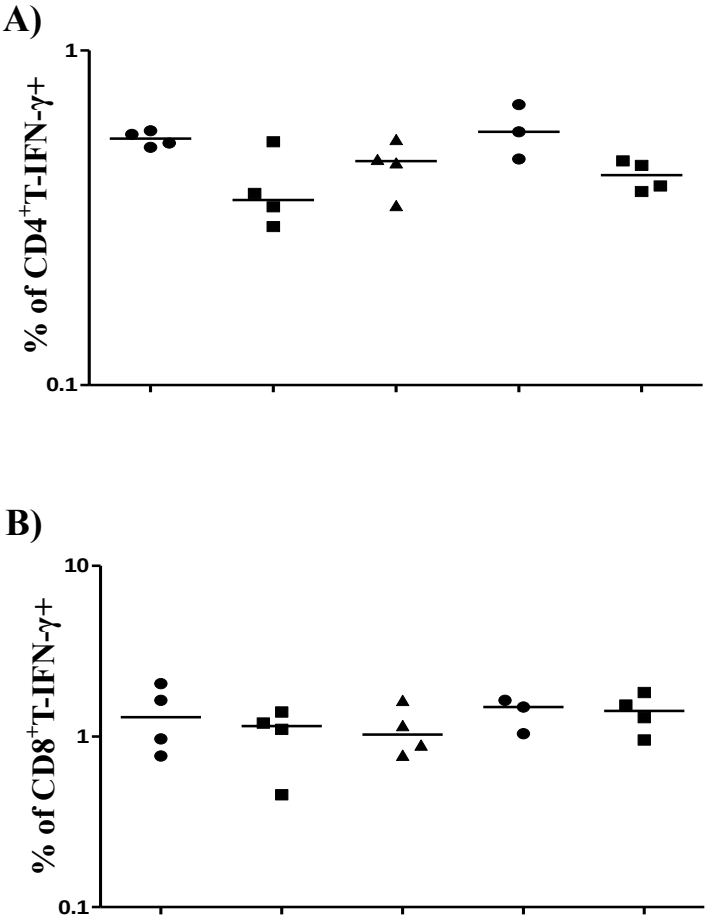



**Figure 3.19. (A-B). Effect of salmeterol and ICI 115, 881 (10-50 µM) on IFN-γ producing CD4+ T (A) and CD8+ T (B) in PBMCs stimulated with SEB.** T cells were isolated from PBMCs and incubated for 30 min at 37°C, 5% $CO_2$ in the presence or absence of 50 µM salmeterol and/or ICI (10-50 µM). Following incubation of T cells with salmeterol (50 µM) and/or ICI (10-50 µM), T cells were washed and added to the rest of the PBMCs. Subsequently, PBMCs were stimulated with SEB and incubated for 1 hr 30 min at 37°C, 5 % $CO_2$. Following incubation, monensin was added to PBMCs to inhibit intracellular secretion of cytokines. Cells were incubated for a further period of 5 hours and stained with respective antibodies. The percentage of IFN-γ producing T cells was determined using FACS Analysis. Results are shown as the mean values of at least 3 replicates (n=3). Flow cytometer data of a representative sample (n=1) is shown in **Fig. 3.20-3.22. (A-D).**

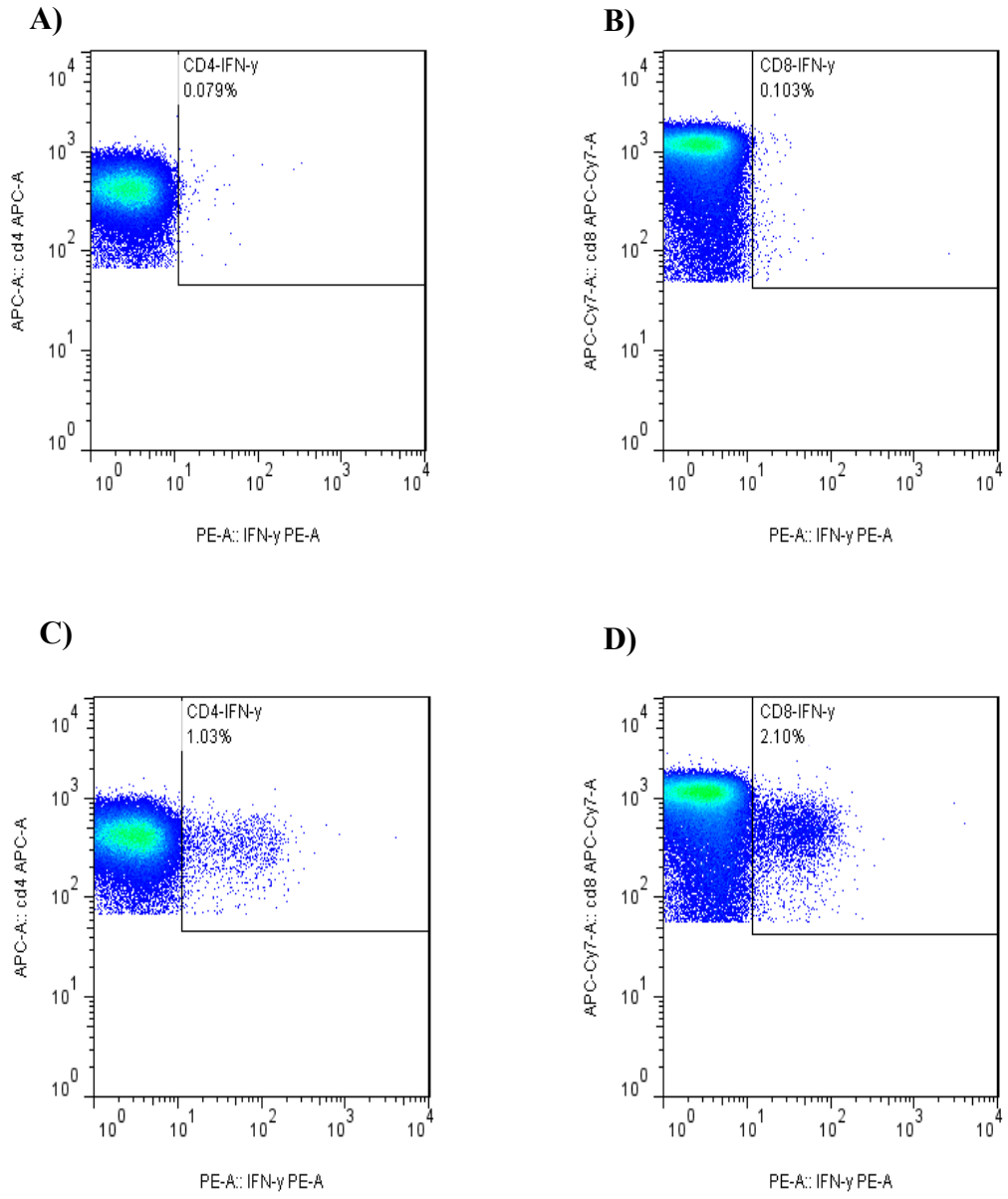



**Figure 3.20. (A-D). Effect of SEB on IFN-γ producing $CD4^+$ T (A) and $CD8^+$ T (B) cells in PBMCs treated with SEB. Figures are representative plots for data in Fig. 3.18. (A-B).** The $CD4^+$ and $CD8^+$ T subpopulations were gated within the PBMCs population. The percentage of IFN-γ producing CD4+ T (C) and CD8+ T cells (D) in SEB stimulated PBMC was gated and compared to the percentage of IFN-γ producing CD4+ T (A) and CD8+ T cells (B) in unstimulated control.

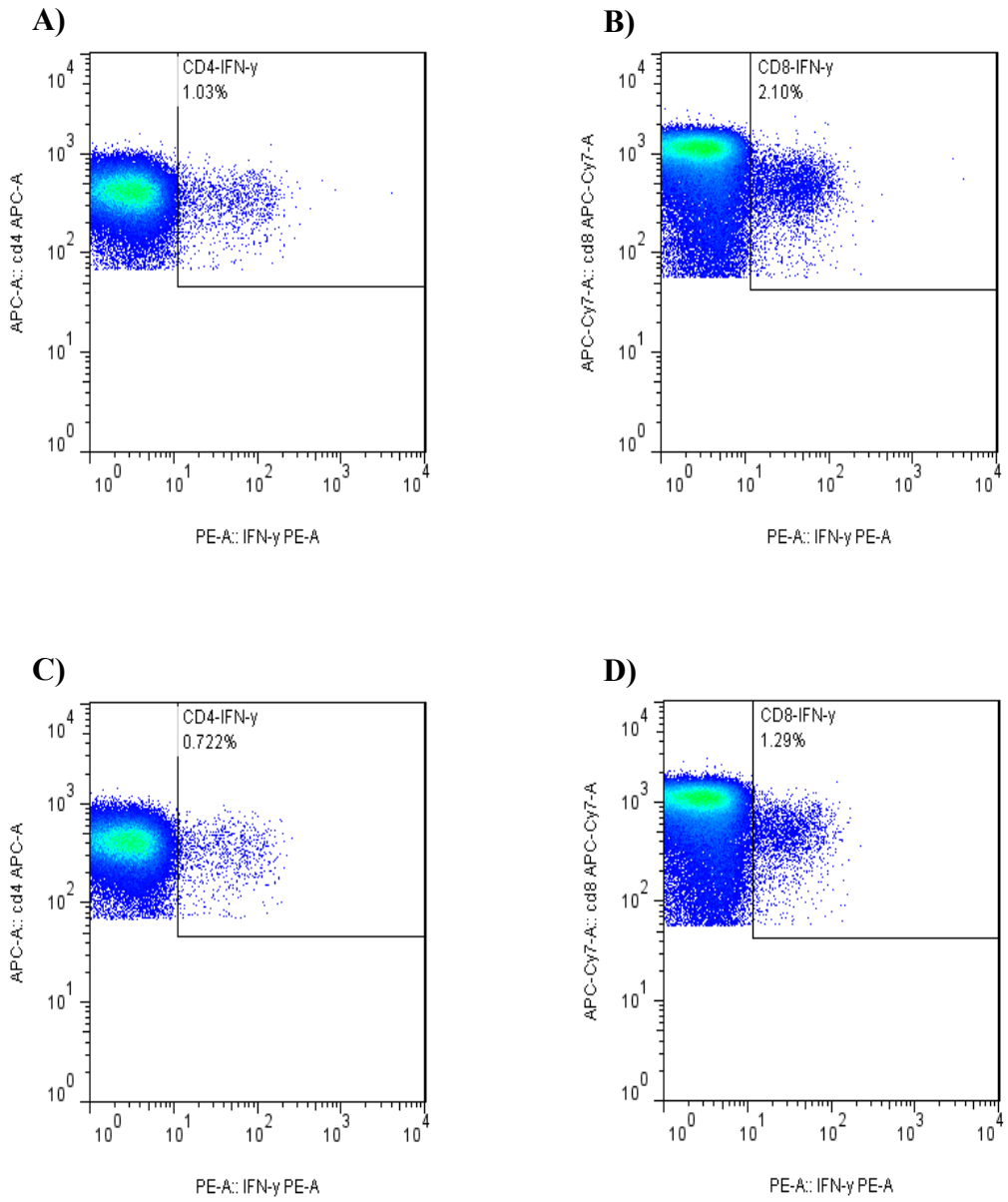



**Figure 3.21. (A-D). Effect of salmeterol (50 uM) on IFN-γ producing $CD4^+$ and $CD8^+$ T cells following treatment of T cells with or without salmeterol and stimulation of PBMCs with SEB. Figures are representative plots for data in Fig. 3.18. (A-B).** The $CD4^+$ and $CD8^+$ T subpopulations were gated within the PBMCs population. The percentage of IFN-γ producing CD4+ T (A) and CD8+ T cells (B) in SEB stimulated PBMCs was gated and compared to the percentage of IFN-γ producing CD4+ T (C) and CD8+ T cells (D) in T cells treated with salmeterol and PBMCs stimulated with SEB.

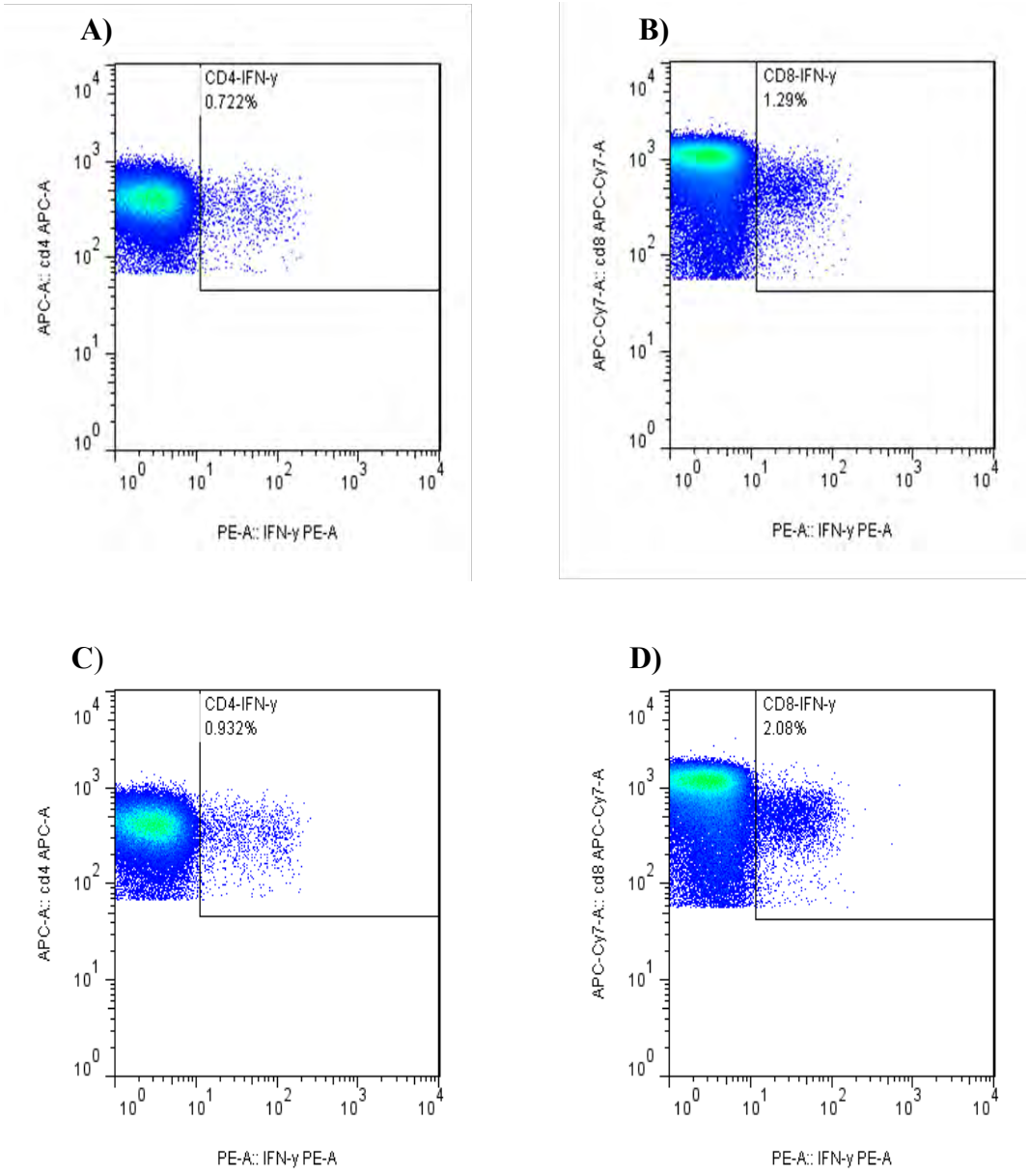



**Figure 3.22. (A-D). Effect of salmeterol (50 uM)and ICI 115, 551 (25 uM) on IFN-γ producing $CD4^+$ and $CD8^+$ T cells after treatment of T cells with salmeterol and with or without ICI 115, 551; followed by stimulation of PBMCs with SEB. Figures are representative plots for data in Fig. 3.18. (A-B).** The $CD4^+$ and $CD8^+$ T subpopulations were gated within the PBMCs population. In A) and B), the CD4+ and CD8+T cells respectively were treated with salmeterol only. In C) and D), CD4+ and CD8+T cells respectively were treated with salmeterol and ICI 115, 551.

### 3.3 Discussion

It has been reported that catecholamines and β2AR agonists have inhibitory effects on lymphocyte function. For example salmeterol, a potent long-acting β2AR agonist (Bail et al., 1991; Butchers et al., 1991; Nials et al., 1993) inhibits IFN-γ production by PBMCs (Mohede et al., 1996; Salicru et al., 2007). However, there is a lack of information regarding the effect of salmeterol on CD4+ T and CD8+ T lymphocyte subsets. In this study the effect of salmeterol on the cytotoxic CD4+ T and CD8+ T cells, was measured in vitro by means of measuring the % of IFN-γ producing CD4+ and CD8+ T cells in PBMCs from CMV positive individuals. By *in vitro* testing we were able to exert substantial experimental control and hereby determine direct effects of salmeterol on T cells at the single cell level, and differentiate these from indirect effects on T cells as result of modulation via other cell types like APCs that may also be responsive to salmeterol. For this purpose the present study employed three different approaches in order to take into account all of these aspects. Initially, we examined the effect of salmeterol on the IFN-γ producing CD4+ T and CD8+ T cells in treated PBMCs stimulated with SEB and demonstrated that salmeterol (50 μM) diminished significantly the proportion of IFN-γ producing T cells (Fig. 3.2. A-B), as compared to the SEB stimulated PBMCs. Secondly we investigated the effect of salmeterol on IFN-γ producing CD4+ T and CD8+ T cells when not the T cells, but the APCs were incubated with salmeterol. Our findings suggest that salmeterol significantly inhibits IFN-γ producing CD4+ T and CD8+ T lymphocytes by inhibiting the function of APCs (Fig. 3.9. A-B). The exact mechanism responsible for mediating this immune inhibitory effect remains to be elucidated, but it is possible that catecholamines affect several pathways including the expression of class II and costimulatory molecules on APCs, the efficiency of antigen processing, as well as changes in cytokine production and response to cytokines. For example,

Seiffert et al. (2002) demonstrated that catecholamines inhibit the antigen presenting capability of epidermal Langerhans cells from BALB/c mice. Furthermore, other studies have reported that the stress affected population of Langerhans cells (LC) possess a significant decrease in γ-interferon induced major histocompatibility class II (Ia) expression and an obvious alteration in LC morphology (characterised by decrease number of dendrites and cell size) (Hosoi et al., 1998; Kawaguchi et al., 1998). Since cytotoxic CD4+ T and CD8+ T cells would respond to presentation of CMV pp65 antigen via MHC II or MHC I respectively on APCs (Arrode et al., 2002; Pita-Lopez et al., 2009; Reiser et al., 2011) by secretion of IFN-γ, one of the ways by which salmeterol could inhibit IFN-γ producing T cells could be by affecting MHC II or MHC I expression and inhibiting antigen processing by APCs.

Finally, we investigated whether salmeterol could also have a direct effect on the percentage of IFN-γ producing CD4+ and CD8+ TLs, by treating only T cells with salmeterol. Our results indicate that salmeterol at higher concentration (50 μM) significantly inhibits both IFN-γ producing CD4+ and CD8+ TLs (Fig. 3.16-3.17. A-B). One mechanism responsible for reducing the secretion of some cytokines (i.e., IFN-γ, TNF-α, IL-1, IL-2) by monocyte/macrophages or T cells is elevation of intracellular cAMP levels (Loza et al., 2006; Sanders et al., 1997; Sekut et al., 1995).

Supporting our findings, Betz and Fox. (1991) demonstrated that prostaglandin $E_2$ ($PGE_2$), which similarly increases cAMP levels through activation of adenylate cyclase, inhibited the pigeon cytochrome c-induced IFN-γ production levels by a pigeon cytochrome c specific Th-1 cell clone. Similarly, Snijdewint et al. (1993) and Van der Pouw-Kraan et al. (1992) have also shown that increased cAMP levels correspond to a decreased level of IFN-γ production from T cells. Importantly, β2AR selective agonist terbutaline ($10^{-8}$-$10^{-5}$ M) inhibited IFN-γ production from murine (BALB/c mice) Th-1 cells (Sanders et al., 1997), whereas the β2AR

selective agonist salbutamol ($10^{-5}$ M) suppressed the level of IFN-γ production by T cells activated with PHA and PMA (Kohm and Sanders, 2001; Paul-Eugene et al., 1993).

In line with these, both isoproterenol (a βAR non selective agonist) and fenoterol (a β2AR selective agonist) inhibited the Con A-induced IFN-γ production by human T cells in a dose dependent manner (Borger et al., 1998). These findings are consistent with our data which show that at concentration of 50 μM, salmeterol induced a higher inhibition of IFN-γ producing T cells as compared to a concentration of 5 μM. However, in contradiction to previous reports that have demonstrated complete inhibition of Th-1 cytokine production by cAMP elevating agents (Betz and Fox, 1991; Gajewski et al., 1990; Snijdewint et al., 1993), salmeterol never induced a complete inhibition of IFN-γ producing CD4+ T or CD8+ TLs in the model system presented here. One reason that might explain this discrepancy is that different cAMP-increasing agents have distinct cellular dynamics resulting in different T cell responses. For example, whereas surface receptor cAMP ligands increase cAMP mainly at the site of receptor stimulation, for a transient period of time, other cAMP analogues may increase cAMP levels for a long period of time, allowing cAMP levels to diffuse throughout the cell (Bauman et al., 1994). Thus, whereas the cAMP levels resulting from a non-receptor-mediated change may be extensive and long lasting, the increased cAMP levels resulting from a receptor mediated change may be localised and transient inducing thus a limited change in cell function.

Finally, the present study, examined the selectivity of β2AR stimulation, using ICI118, 551; a β2AR selective antagonist. Our results demonstrated that salmeterol inhibits IFN-γ producing T cells. Moreover this effects seemed to act through $\beta_2$AR, because incubation of T cells with salmeterol (50 μM) and ICI 118,551 (25 μM), completely reversed the inhibitory effect of salmeterol. These data are consistent with findings by Borger et al. (1998) which show that

only stimulation of β2AR expressed on the surface of T cells is sufficient to influence the production of IFN-γ. Overall our data suggest that salmeterol affects T cell subsets directly by stimulating β2AR expressed on the surface of T cells, and indirectly by stimulating β2AR expressed on antigen presenting cells. The use of specific antigens (i.e. CMV lysate and pp65) in these studies as opposed to SEB was important to dissect these responses (see section 3.2.3).

Another important observation from our data is that the inhibitory effect of salmeterol on IFN-γ is stronger for CD8+ T as compared to CD4+ T cells. One reason that could explain these findings is the fact that the number of β2AR on CD8+ T cells is higher (2434-5967 binding sites/cell) than the number of β2AR on the surface of CD4+ T lymphocytes (619-1237 binding sites per cell) (Wahle et al., 2001). It has been reported that lymphocytes exhibiting reduced β2AR densities, also exhibited a reduced catecholamine response (Baerwald et al., 1999). Throughout our experiments the stimulatory effect of SEB, CMV lysate and CMV pp65 on T cells showed large variation. These differences are probably the result of the PBMCs populations in different individuals, which may vary, and thus may reflect a diverse starting population of T cells and the measurement of function at the population versus single cell level (Loza et al., 2006). This would be particularly relevant for the CMV studies as different individuals would have different numbers of memory cells, due to age for example (Ben-Smith et al., 2008) (as was confirmed in Fig. 3.16. B, whereby the 41-49 and 50-59 age group responded better to SEB) and for CMV pp65 studies where specific human leukocyte antigen (HLA) haplotypes may influence the response. Gender however, did not make any difference in T cell response to SEB, as both females and males responded in a very similar way. One of the issues presented in these studies is the dilution of salmeterol, carried out with ethanol, as salmeterol is only sparingly soluble in water. In order

to eliminate the possibility that the small effect of salmeterol is not due to the solvent, we could have included controls. However, the fact that β2-antagonist ICI118, 551 was diluted with water and reversed the effect of β2 agonist salmeterol, suggest that ethanol did not add to the affect of salmeterol. Yet, future studies on the effect of ethanol on cells should be assessed to better understand how ethanol could affect the cells in short term studies. This is because, it is important to note that the vast majority of studies on the effects of ethanol have been done in vivo on long term alcoholics and not in short term studies.

In conclusion, our data presented evidence that β2AR stimulation (salmeterol) exerts an inhibitory effect on IFN-γ producing CD4+ T and CD8+ T lymphocytes. The salmeterol induced inhibition of the IFN-γ producing T cells appears explained by direct effects on T cells as well as indirectly, possibly via affecting processing and/or presentation by APCs. While the exact mechanisms underlying these effects remain to be elucidated, studies by Loza et al. (2006) have reported that the inhibition of PMA-stimulated IFN-γ production by PGE2 and β-agonist is a result of inhibition of p38 phosphorylation. P38 is a member of the mitogen activated protein (MAP) kinase subfamily that is involved in regulating cellular responses to inflammation. Significantly, IFN-γ production is dependent on p38 activation. Continuing studies will provide a better understanding on the role of β2AR agonist in controlling the development of protective cytotoxic T cell responses and thus contributing towards the treatment of serious diseases in which cytokines play a pathogenic role.

# CHAPTER 4

# EFFECTS OF β2-ADRENERGIC AGONISTS ON LYMPHOCYTE ADHESION AND MIGRATION

## 4.1 Introduction

Migration of leukocytes from the blood, through various organs and back into the blood is essential to the maintenance of an effective immune defense network. In effect, the recruitment of white blood cells at sites of injury and infection is crucial to allowing sufficient surveillance of tissues for infectious pathogens. This recruitment is a process that can be split into five successive steps. Initially, flowing leukocytes marginate to the vessel wall and are (i) captured to the vascular endothelium that lines the vessel and (ii) roll along it. Subsequently leukocytes (iii) become rapidly activated (seconds) and arrested on the endothelium monolayer in a stable secondary adhesion that can be potentially reversible over minutes. The final step involves leukocytes (iv) spreading and migrating along and then across the endothelial lining of the blood vessel through (v) both the apical and basolateral basement membranes and finally through stroma in the underlying tissues (Ley et al., 2007; Ludwig et al., 2009). This migration of lymphocytes to inflamed tissues occurs as a result of the interactions of specific leukocytes' cell surface receptors (e.g., selectins, integrins, chemokine receptors) with their ligands found on endothelial cells (Akiyama, 1996; Johnston et al., 1996; Ley et al., 2007). Tissue migration of lymphocytes is critically controlled by modulation of integrin affinity on the surface of leukocytes as well as by molecular signals (e.g. chemokines) which are displayed on the endothelium (Alon et al., 1995; Middleton et al., 2002; Springer, 1995). These molecular signals cause the transcriptional upregulation and expression of adhesion molecules such as VCAM-1, ICAM-1, MAdCAM-1 (Fabbri et al., 1999; Francischetti et al., 2010; Springer, 1995). Thus a number of adhesion molecules and chemokines are involved to support the process of tissue infiltration in a controlled manner.

Previous studies have documented that the process of leukocytes trafficking and subsequent cellular immune responses in the local tissues are modulated by stress, as best documented in lymphocytosis (Anane et al., 2009a; Anane et al., 2010a; Atanackovic et al., 2006; Campbell et al., 2009). One factor mediating this response is βAR activation. It has been demonstrated that β2AR stimulation modifies the adhesive (and possibly migratory) behaviour of leukocytes in a subset-specific manner (Dimitrov et al., 2010). For example stress lymphocytosis is largely driven by a selective increase in cytotoxic lymphocytes such as NK cells and γδ and memory cytotoxic CD8+ T cells, with less contribution observed from other non-cytotoxic lymphocytes subsets such as T helper CD4+ and B (CD19+) lymphocytes although expressing functional β2AR (Anane et al., 2010a; Benschop et al., 1996c; Campbell et al., 2009).

In this chapter we examined the effects of β2AR stimulation, induced by different concentrations of salmeterol (a β2-agonist) and epinephrine, on adhesive and migratory behaviours of leukocytes. Specifically, we tested CD8+ T cells and NK cells to VCAM-1 coated glass capillaries (microslides) (see Fig. 4.1) in a flow based assay in vitro. The results of this chapter are presented in means ± SEM and all significant data are indicated by a p-value, derived from paired sample t-tests. The clinical details of individuals used in this assay are shown in Table 4.1.

**Table 4.1 Clinical Details of Individuals Studied for Flow and Static Assays**

| Patient | Sex | Age |
|---|---|---|
| 21 | F | 35 |
| 22 | M | 28 |
| 23 | M | 27 |
| 24 | M | 46 |
| 25 | M | 32 |
| 26 | M | 37 |
| 27 | F | 26 |
| 28 | M | 31 |
| 29 | M | 33 |
| 30 | M | 22 |

**All individuals had a Caucasian background and were not under any medical treatment.**
Individuals 21-30 were used for flow and static assays.

## 4.2 Methods

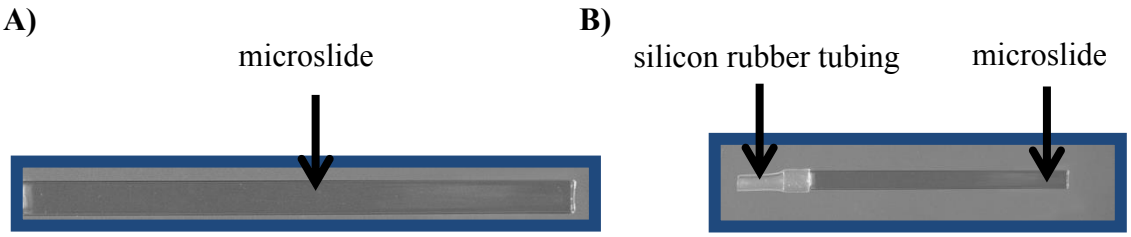



**Fig 4.1. Represents: A)** The microslide**.** This is a glass capillary with rectangular cross-section (0.3mm x 0.3mm; length 50 mm) and in this study was used to coat with purified adhesion molecules. **B)** A short length of silicon rubber tubing attached to aminopropyltriethoxysilane (APES) coated microslide. This was essential before the addition of recombinant human VCAM-1 at final desired concentration.

The decision to study VCAM-1 was based on previous findings which showed that VCAM-1 is a receptor that supports lymphocyte capture and stable adhesion under physiological flow conditions (Abbitt and Nash, 2001; Alon et al., 1995; Berlin et al., 1995; Johnston et al., 2000; Johnston et al., 1996; Miles et al., 2008). This is in contrast to ICAM-1 which is unable to support lymphocyte capture *in vitro* under physiological shear, but is functional in static assays (Berlin et al., 1995; Johnston et al., 2000). All flow experiments were carried in a Perspex chamber at 37 ºC (previously described by Nash et al.,1992; Cooke et al.,1993; Rainger et al., 2001) in the presence of flow at a rate equivalent to a wall shear stress of 0.05 Pa, in order to mimic *in vivo* conditions (in post-capillary venules). In all experiments, the microslide was perfused for 2 minutes with PBS + BSA (0.15%) to acclimatise the cells. Subsequently a 4-minutes bolus of PBLs (peripheral blood lymphocytes without monocytes) was perfused over the VCAM-1 coated microslide followed by 2 minutes of cell-free wash buffer. Video recordings were made of a series of microscope fields along the centreline of the flow channel after 2 minutes of wash-out or otherwise dependent on the specific experiment.

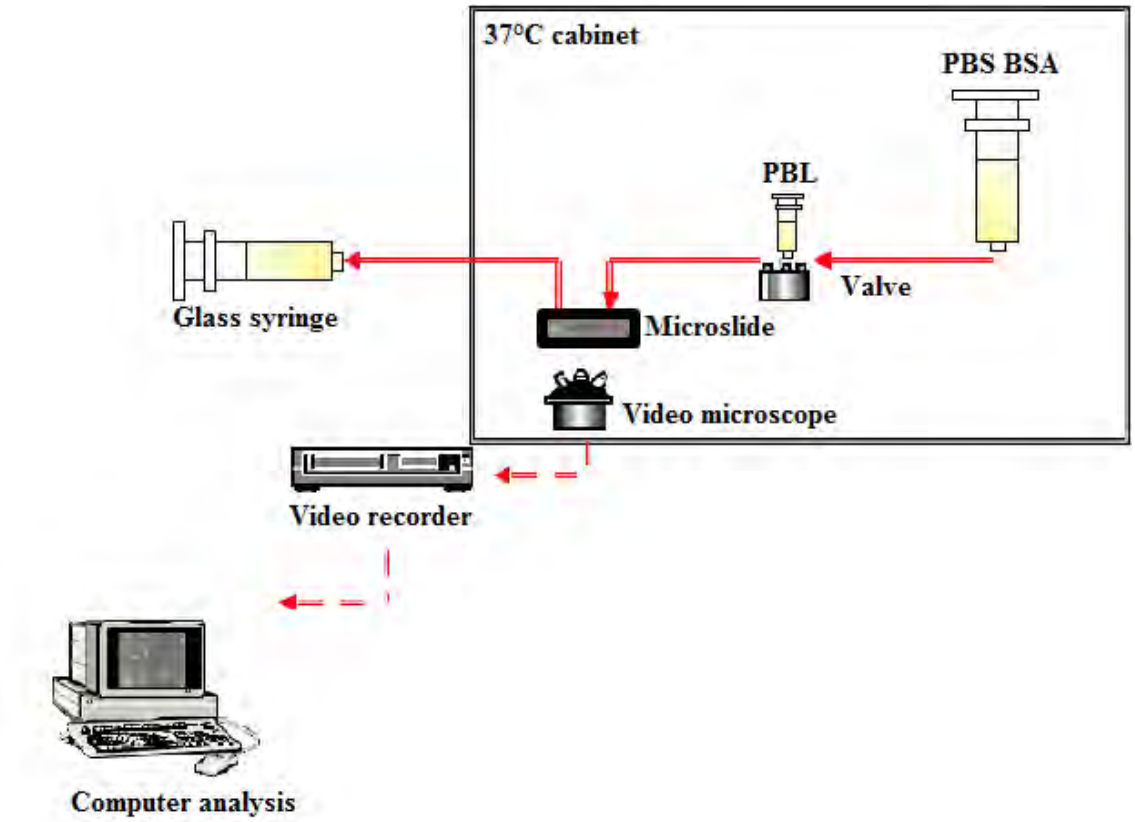



**Figure 4.2. The flow assay design.** The microslide coated with VCAM-1 was placed on a 75 x 25-mm coverslip and incorporated into a parallel plate flow chamber attached to a perfusion system mounted on the stage of phase contrast video microscope (enclosed in a Perspex chamber at 37 ºC). At one end, the microslide was connected to a Harvard withdrawal syringe pump and at the other end it was connected to an electronic switching valve. This valve selected flow from three reservoirs containing PBLs in PBS + BSA (0.15%), or cell-free wash buffer consisting of PBS + BSA (0.15%), or cell-free PBS + salmeterol / epinephrine at various concentrations. A suspension of either PBLs ($1x10^6$ cells/ml), or cell free wash buffer, or cell-free PBS + salmeterol / epinephrine at various concentrations of 0.05 µM -50 µM, was perfused through the chamber at a constant wall shear stress (τw) set at 0.05 Pa.

## 4.3 Results

### 4.3.1 Optimising the Flow Assay

The overall aim of this flow assay was to determine the effects of the β2AR (salmeterol) stimulation on adhesive and migratory behaviour of leukocytes. Previous data have shown that stress/ epinephrine induces a rapid release of effector memory T and NK cells into the peripheral blood and that this effect occurs within 1-2 minutes (Anane et al., 2009; Anane et al., 2010; Atanackovic et al., 2006; Benschop et al., 1996b; Campbell et al., 2009; Dhabhar et al., 1995; Dimitrov et al., 2010; Silberman et al., 2003; Simpson et al., 2007; Viswanathan and Dhabhar, 2005).

Whilst taking into account previous findings and addressing the aim of this flow assay, it was important to design a protocol that could answer the question of how these cells were induced to move. A series of assay variables to investigate the effect of salmeterol on PBLs binding to adhesion molecules needed to be considered. These included:

(a) The concentration of the adhesion molecule used to coat the microslides.
(b) The duration of salmeterol perfusion and
(c) the timing of the recordings following the salmeterol perfusion.

### 4.3.2 Determining Which VCAM-1 Concentration Best Captures PBLs *In Vitro*

In the initial experiments a 4 minutes bolus of PBLs ($1x10^6$ cells/ml) was perfused over different concentrations of purified VCAM-1 coated microslides (5-20 μg/ml) through the chamber at a constant wall shear stress (τw) set at 0.05 Pa under flow. Initially, behaviour and adhesion of PBLs was evaluated at 2 and 9 minutes after wash-out (Fig. 4.3. A-B). The data show that when VCAM-1 was used at a concentration of 5 μg/ml, ~80% of cells could be clearly seen to roll, whereas ~ 20% of cells were stationary, adhered onto the VCAM-1 coated

microslide (Fig. 4.3. A-B). In contrast, at 10 µg/ml VCAM-1, ~42% of cells rolled and 58% of cells were firmly adherent, while at 20 µg/ml VCAM-1 ~21% of PBLs were rolling and ~79% firmly adherent (Fig .4.3. A-B). Moreover, increasing VCAM-1 concentration from 5 µg/ml – 10 µg/ml significantly increased the number of rolling PBL at 2 minutes ($p=0.046$ using paired sample t-test; $p=0.034$ using Wilcoxon signed rank test) and 9 minutes following wash-out ($p=0.028$ using paired sample t-test; $p=0.034$ using Wilcoxon signed rank test). In addition increasing VCAM-1 concentration from 5 µg/ml to 20 µg/ml significantly increased the number of stationary adherent PBLs at both 2 minutes ($p=0.013$ using paired sample t-test; $p=0.034$ using Wilcoxon signed rank test) and 9 minutes after wash-out perfusion ($p=0.036$ using paired sample t-test; $p=0.034$ using Wilcoxon signed rank test). In contrast no significant change was observed in the total number of rolling and stationary adherent PBLs (i.e. total adhesion) at any VCAM-1 concentration used (i.e. between 5 and 10 µg/ml, or 5 and 20 µg/ml, or 10 and 20 µg/ml). Similarly, no change was observed in the number of rolling and stationary adherent PBLs between 2 and 9 minutes after wash out, indicating that the number of lymphocytes adherent to VCAM-1 remained stable (Fig. 4.3. A-B). This was an important concept in the next set of experiments, whereby we aimed to investigate the effect of epinephrine or salmeterol at 0, 2 and 9 minutes following lymphocytes perfusion, allowing confirmation that any change in the percentage or the number of adherent PBLs to VCAM-1 coated microslide is due to the effect of treatment. As we wanted to investigate whether salmeterol had any effect on lymphocyte recruitment from flow, we initially decided to coat the mircroslides with 10 µg/ml VCAM-1 assuming that the effect of salmeterol would be strong enough to detach the rolling PBLs. (These data showed that 10 µg/ml VCAM-1 caused 42% of cells to roll).

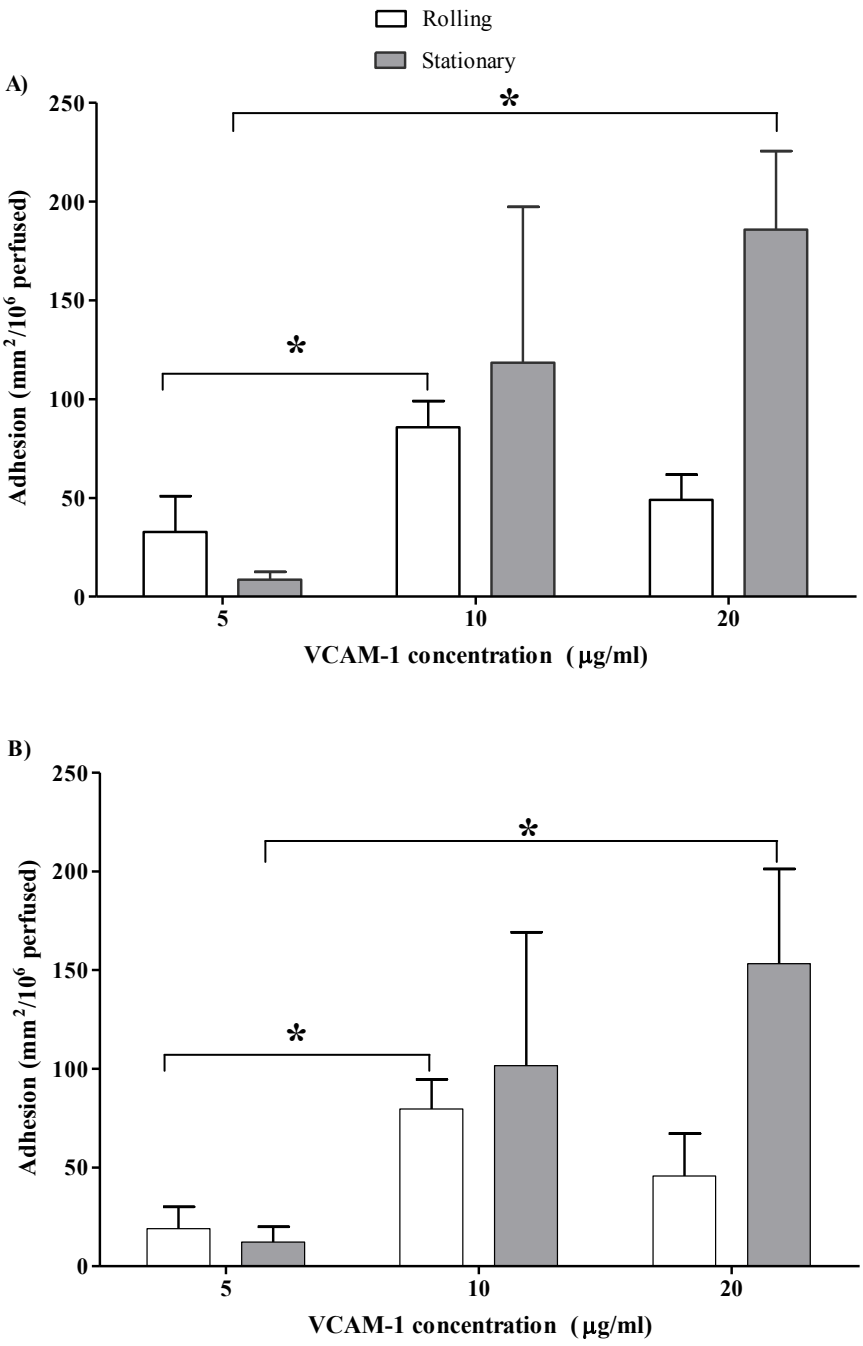



**Fig.4.3. Effect of increasing VCAM-1 concentration on PBLs captured from flow.** Microslides were coated with increasing concentrations of purified human VCAM-1. A 4-min bolus of lymphocytes was perfused over the VCAM-1 and adhesion was assessed 2 min (A) and 9 min (B) after wash out. Data are mean ± SEM from four independent experiments. *$p<0.05$ indicates that the effect of increasing VCAM-1 concentration on PBLs capture from flow was significant (using paired sample t-test and/or Wilcoxon singed rank test) compared to 5 µg/ml VCAM-1.

### 4.3.3 Determining Epinephrine/Salmeterol Perfusion and Timing of Recordings

A second potentially important factor was the duration of epinephrine / salmeterol perfusion and the timing of the analysis following the epinephrine / salmeterol perfusion. Since the release of effector memory T and NK cells into the peripheral blood occurs within 2 minutes following stress or epinephrine (Benschop et al., 1996c), we hypothesised that epinephrine or salmeterol would cause a rapid (within 2 to 5 minutes) detachment of lymphocytes under conditions of flow. For this reason we decided to perfuse epinephrine or salmeterol over adhered lymphocytes for a period of 2 minutes, and measure its effect at 0, 2, and 8 minutes following perfusion (Fig. 4.4).

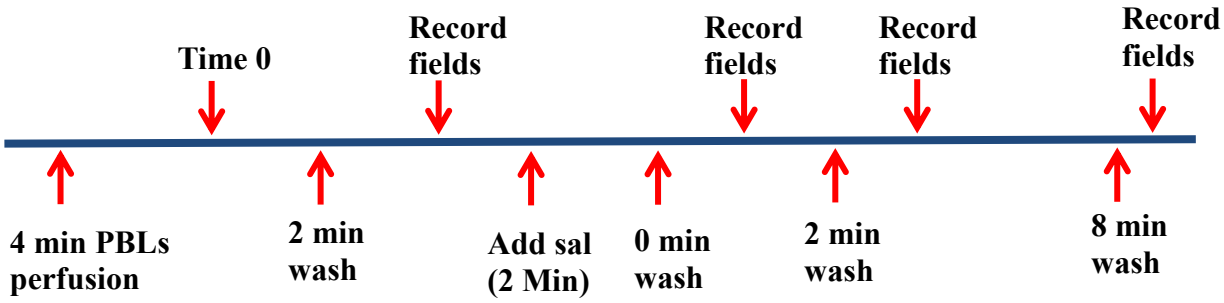



**Figure 4.4. Epinephrine/Salmeterol Perfusion and Timing of Recordings.**

In addition to video recordings, which allowed analyses of the adhesion and rolling behaviour of leukocytes, we were also interested to determine the phenotype of the PBLs adhered to VCAM-1, and had to design a method that would allow us to collect enough PBLs from the microslides, to perform flow cytometry and represent an overall picture of the results.

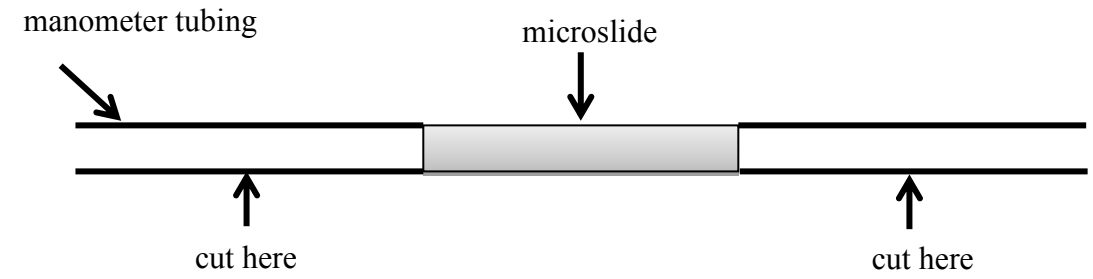
### 4.3.4 Different Options of Retrieving PBLs from Microslide

Four different options were tested to retrieve PBLs from microslides (Fig. 4.5. A-D). In all retrieval options manometer tubing was connected at both sides of the microslides and the

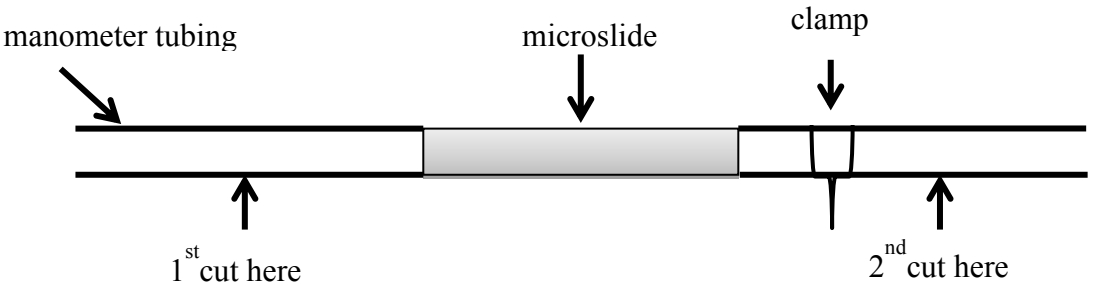
options differed only on the number of clamps used and the position of where the manometer tubing was first cut following the stoppage of the flow. In option 1, no clamps were used. To collect PBLs, left side tubing was cut and the right side tubing was carefully removed from the microslide. Volumes of 200 µl of EDTA were pipetted up and down in the microslide to collect as many adherent cells as possible in 2 ml eppendorf tubes. This was the same for all options. In option 2, the right tubing was clamped and the left tubing was cut after the stoppage of the flow, followed by the right side of tubing. In option 3, the right tubing was clamped and cut, followed by the left side of tubing. In option 4, both the left and the right side tubing was clamped and cut when the flow was stopped.

The results showed that retrieval options 2, 3, 4 resulted in higher percentages of lymphocytes (CD4+ T, CD8+ T, and NK cells) collected from the microslide. In contrast, retrieval option 1 was excluded, as it only collected ~45% of adherent lymphocytes. Although almost no difference in results between retrieval options 2, 3, and 4 were observed (Fig. 4.6. A-B), we decided to choose option 4 and clamp both, the left and the right side tubing as a precaution to minimise PBLs loss.

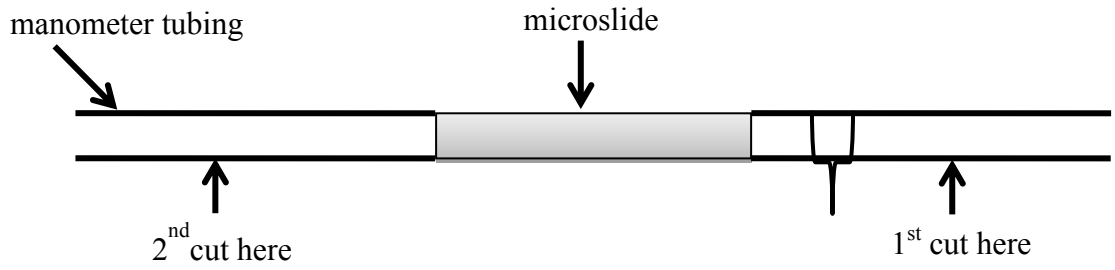
**A) Option 1**




**B) Option 2**




**C) Option 3**




**D) Option 4**

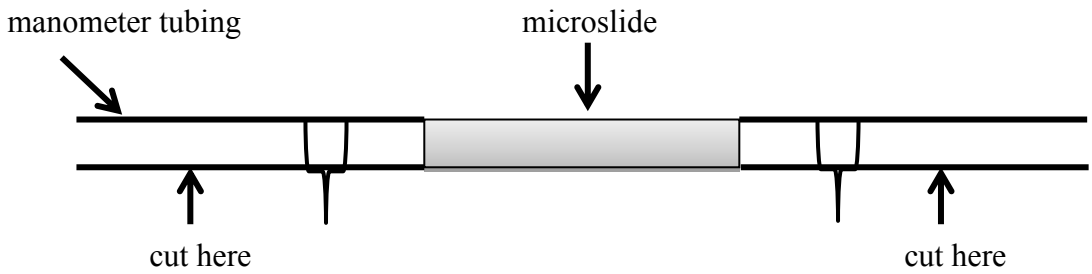



**Figure 4.5. Different Options of Retrieving PBLs from Microslide.**

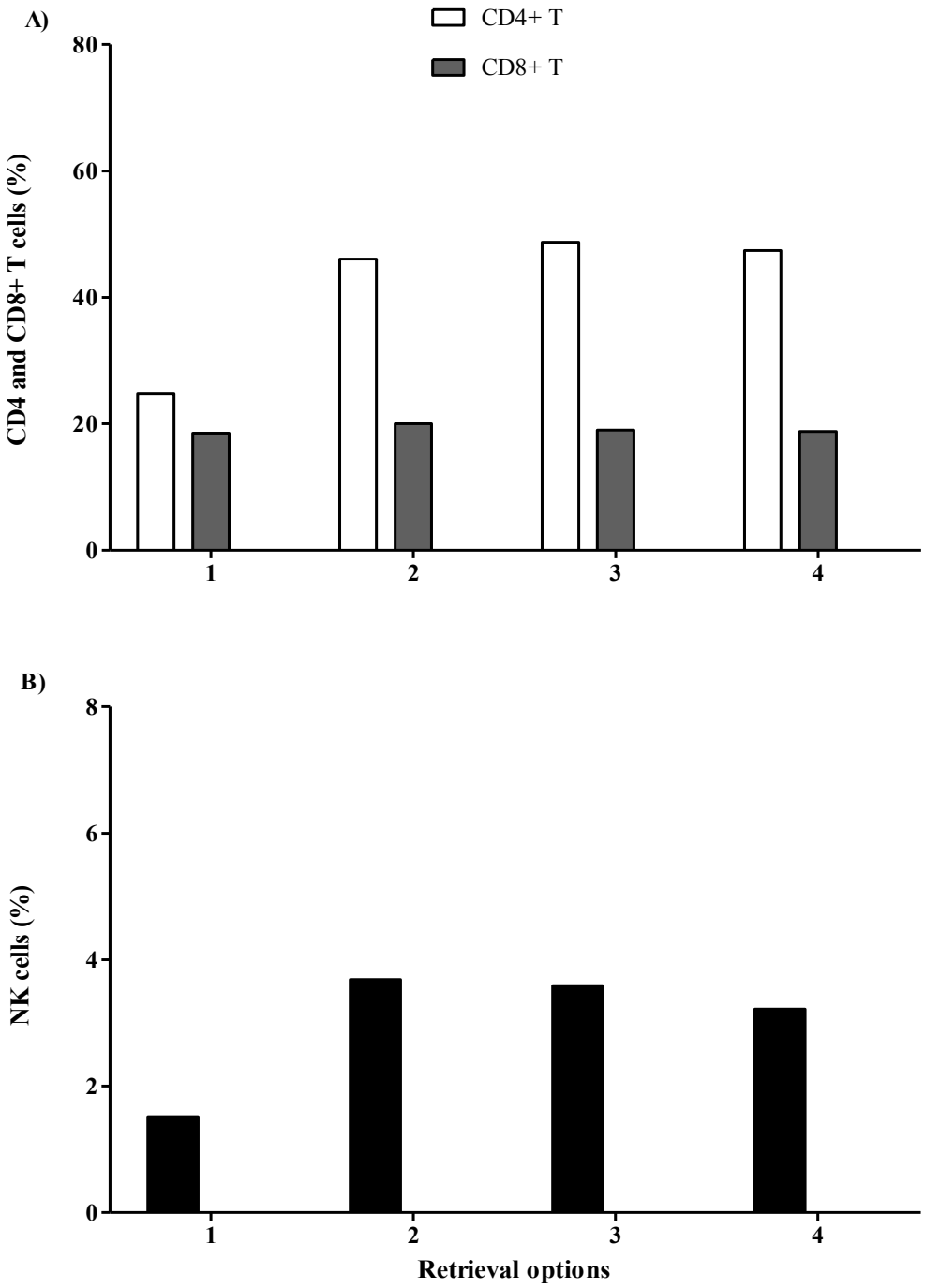



**Figure 4.6. Comparison of lymphocyte retrieval options for collecting CD4+ T, CD8+ T and NK cells.** Cells were collected, labelled and analysed by flow cytometry to assess the percentage of CD4+ T, CD8+ T (A) and NK cells (B) attached to the VCAM-1. The data are the mean from at least two independent experiments. No statistics were carried out based on two independent experiments.

### 4.3.5 Effect of Epinephrine and Salmeterol on PBLs Rolling and Adhesion

In the next set of experiments, the effect of epinephrine or salmeterol treatment on lymphocytes behaviour and rolling velocity was investigated. A 4-minutes bolus of lymphocytes was perfused over 10 μg/ml VCAM-1 coated microslide at a wall shear stress of 0.05 Pa. Salmeterol or epinephrine was added at time 0 following wash-out and their effect on PBLs behaviour was measured following 2 and 8-minutes of wash out respectively. The results show that the percentage of rolling PBLs was slightly increased on treatment with salmeterol or epinephrine as compared to the untreated control (from ~9% in the control condition to ~12 and 16 % rolling PBLs upon treatment with salmeterol and epinephrine respectively), whereas the percentage of the stationary adherent PBLs was decreased at a low degree (from ~91% to ~88% upon treatment with salmeterol and to ~84% following treatment with epinephrine) (Fig. 4.7. A-B). Perfusion of salmeterol (5 μM) resulted in a ~2% increase in the rolling of PBLs at 0 minute following washout compared to the control condition (as compensated by a ~2% decrease in the percentage of adherent PBLs). This change remained persistent at 2 minutes but was increased ~1% following 8 minutes after wash out (Fig. 4.7. A). By comparison, epinephrine (0.05 μM) resulted in a ~3% increase at 0 minute compared to untreated control and rose to a ~7% increase in the rolling of PBLs following 8 minutes of washout (as compensated by a ~7% decrease in stationary adherent PBLs). The number of adherent PBLs was reduced non-significantly when 5 uM salmeterol (by an average of ~15%) or 0.05 μM epinephrine (by an average of~19%) was perfused when compared to the untreated control following 0 to 8 minutes of wash-out. (These discrepancies might reflect different donor PBLs) (Fig. 4.8.). In addition, neither epinephrine nor salmeterol affected total lymphocyte recruitment to a 10 μg/ml VCAM-1 coated microslide, over time (0-8 minutes). Next the effect of salmeterol or epinephrine on the average rolling velocity of PBLs was

investigated (Fig. 4.9.). A salmeterol concentration of 5 μM increased the rolling velocity by ~ 0.5 μm/sec compared to the control following 0 minute of wash-out (i.e. from ~2.8 μm/sec to ~3.3 μm/sec) or by~0.7 μm/sec following 8 minutes of washout (i.e. from 2.8 μm/sec to 3.5 μm/sec). Similarly to salmeterol, epinephrine (0.05 μM) increased the rolling velocity by ~ 0.4 μm/sec compared to the control (i.e. from ~2.8 μm/sec to ~3.1 μm/sec) following 2 minutes of washout, and by a ~2.6 μm/sec increase (i.e. from ~2.8 μm/sec-~5.4 μm/sec) following 8 minutes of washout, although this difference was not significant possibly due to PBLs donor variation. The results were analysed using paired sample t-test and Wilcoxon signed rank test and no significant difference was found in both cases as described above.

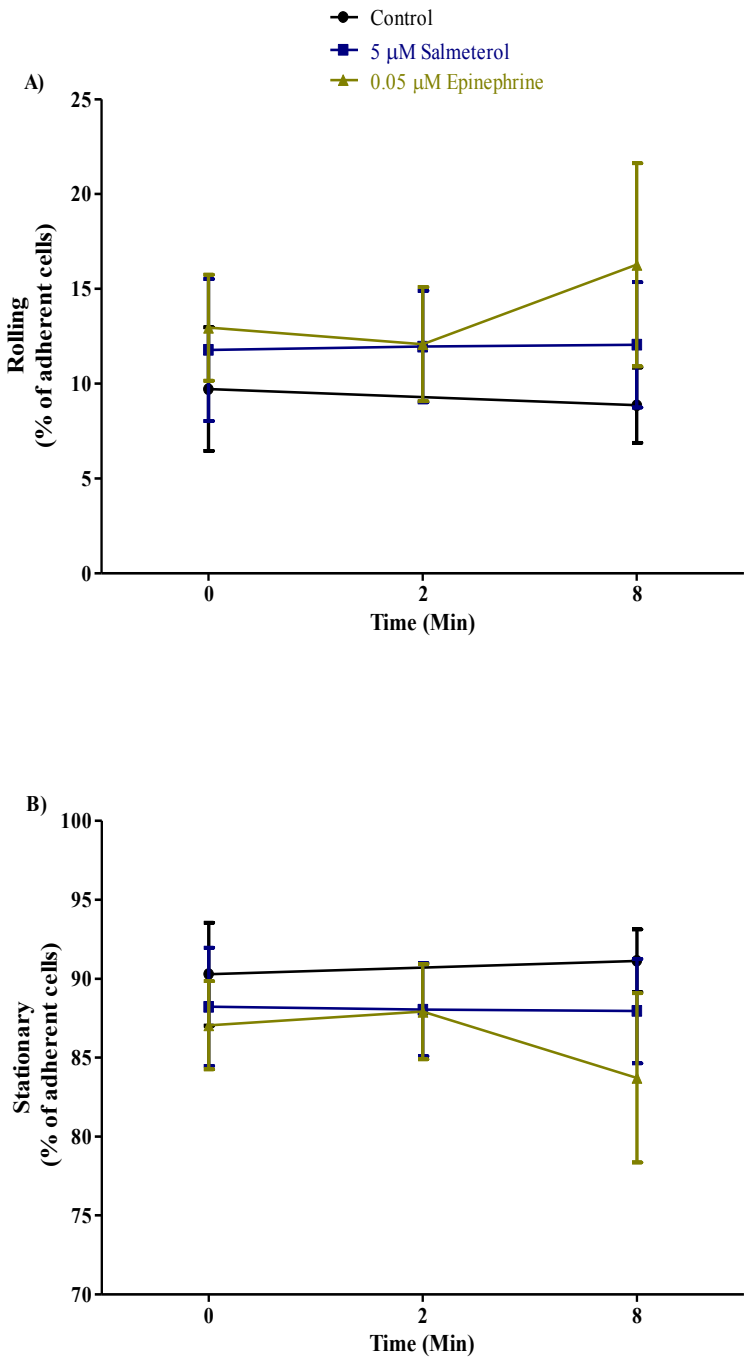



**Figure 4.7. Effect of salmeterol and epinephrine on lymphocytes recruitment to VCAM-1 coated microslide in a flow based assay.** A 4-min bolus of lymphocytes was perfused over 10 µg/ml VCAM-1 coated microslide at a wall shear stress of 0.05 Pa. Salmeterol or epinephrine was added at time 0 and their effect on PBLs behaviour ((A) rolling-(B) stationary) was measured following 2 and 8-min of wash out respectively. The data are the mean ± SEM from five independent experiments.

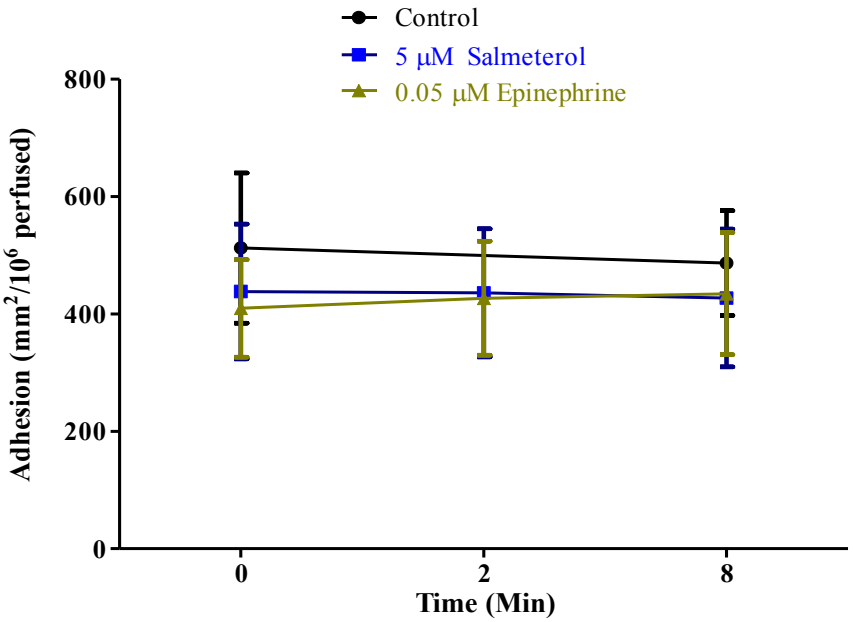



**Figure 4.8. Effect of salmeterol and epinephrine on lymphocytes adhesion to VCAM-1 coated microslides from flow.** The data are the mean ± SEM from five independent experiments. For conditions see Figure legend 4.7.

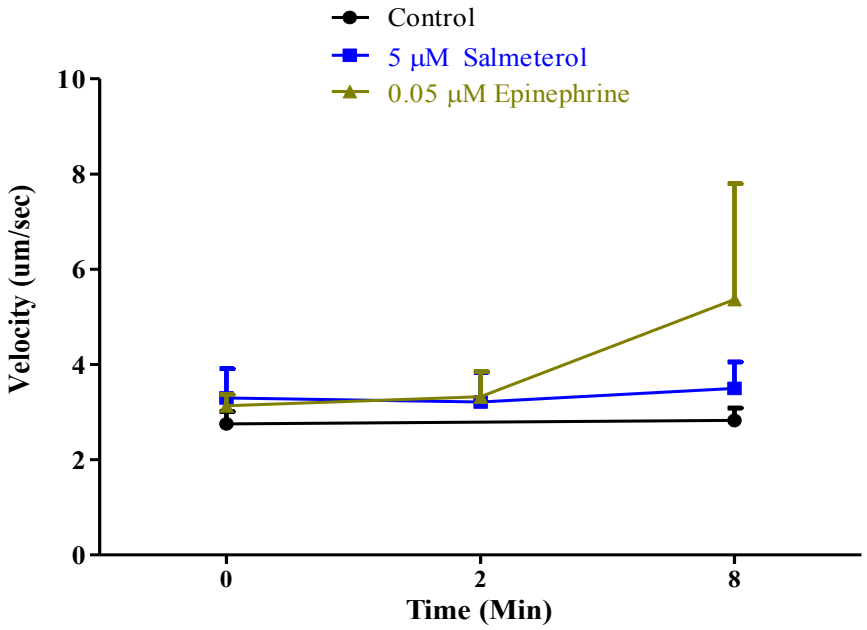



**Figure 4.9. Effect of salmeterol or epinephrine on the rolling velocity of lymphocytes recruited to VCAM-1 coated microslides from flow.** The data are the mean ± SEM from five independent experiments. For conditions see Figure legend 4.7.

### 4.3.6 Effect of Different Concentrations of Salmeterol on PBLs Adhesion and Rolling

As epinephrine at concentration of 0.05 µM and salmeterol at concentration of 5 µM did not significantly affect the recruitment, behaviour, or the average rolling velocities of PBLs to 10 µg/ml of VCAM-1 coated microslide, it was considered whether a wider range of salmeterol concentrations would have any affect. Therefore, in the next set of experiments, we measured the effects of various salmeterol concentrations (0; 0.5; 5; 50 µM) on the lymphocytes recruitment, behaviour and rolling velocities (Fig. 4.10-4.12). As above, a 4-minutes bolus of lymphocytes was perfused over 10 µg/ml VCAM-1 coated microslide at a wall shear stress of 0.05 Pa. A specific concentration of salmeterol was added at time 0 and its effect on PBLs behaviour was measured following 2 and 8-minutes of wash out respectively (Fig. 4.10. A-B). The data indicates that over time 0.5 µM salmeterol moderately increased the percentage of rolling cells and decreased adherent PBLs (Fig. 4.10. A-B). In contrast salmeterol at a concentration of 50 µM, caused a significant increase in the percentage of rolling PBLs ($p=0.007$ using paired sample t-test; $p=0.014$ using Wilcoxon signed rank test) and a significant decrease in the percentage of adherent PBLs ($p=0.009$ using paired sample t-test; $p=0.014$ using Wilcoxon signed rank test) over time from 0 to 8 minutes after wash-out (Fig. 4.10. A-B). Similarly salmeterol 50 µM, significantly decreased the percentage of rolling PBLs at 8 minutes following wash-out as compared to the untreated control ($p=0.032$ using paired sample t-test; $p=0.033$ using Wilcoxon signed rank test). Surprisingly at salmeterol concentration of 5 µM there was no effect on PBLs behaviour.

The overall data indicate that salmeterol at concentrations of 0.5 and 50 µM, increased the rolling and decreased adhesion of PBLs (in consistency with what is observed in an individual during exercise or when stressed). In contrast a salmeterol concentration of 5 µM had almost no effect on lymphocytes behaviour as demonstrated by the percentage of rolling and adherent

PBLs to 10 μg/ml VCAM-1 coated microslides.

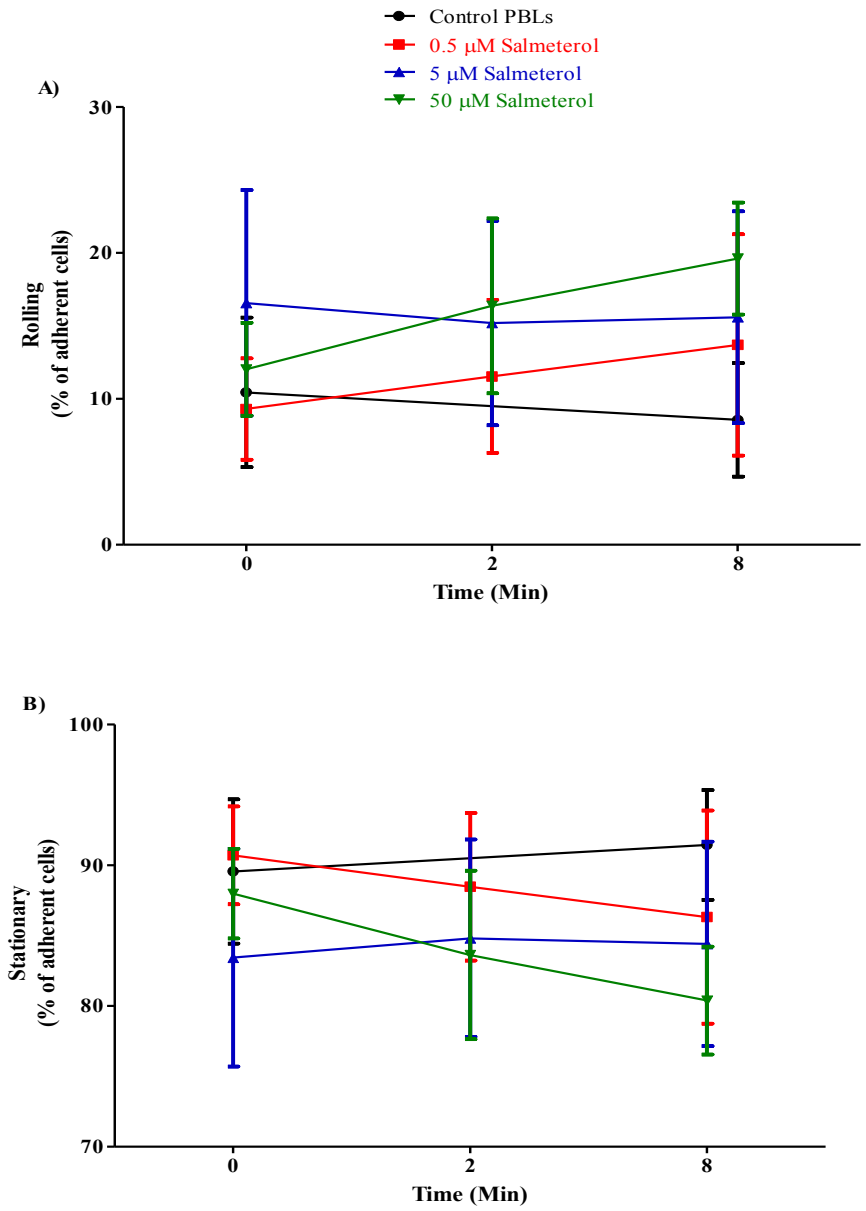



**Figure 4.10. Effect of different concentrations of salmeterol on lymphocytes recruitment to VCAM-1 coated microslide in a flow based assay.** A 4-min bolus of lymphocytes was perfused over 10 µg/ml VCAM-1 coated microslide at a wall shear stress of 0.05 Pa. Salmeterol was added at time 0 and its effect on PBL behaviour was measured following 2 and 8-min of wash out. The data are the mean ± SEM from five-six independent experiments.

We next examined the effect of various concentrations of salmeterol on the total number of PBLs recruited to 10 µg/ml of VCAM-1 coated microslide. As observed previously the number of recruited PBLs only slightly differed in between different salmeterol concentrations (0 to 5 µM) (Fig. 4.11.). At baseline (0 minute) following washout, 0.5 and 5 µM salmeterol induced ~20% and a ~14% decrease respectively. In contrast, at a concentration of 50 µM, a ~45 % decrease in the number of PBLs recruited to 10 µg/ml VCAM-1 coated microslide was observed compared to the untreated control. Interestingly salmeterol at 50 µM significantly decreased the percentage of total PBLs as compared to 0.5 µM ($p=0.050$ using paired sample t-test; $p=0.058$ using Wilcoxon signed rank test) and 5 µM ($p=0.019$ using paired sample t-test; $p=0.023$ using Wilcoxon signed rank test) respectively at baseline (0 minute) following washout. This significant decrease persisted at 5 µM following 8 minutes of washout ($p=0.010$ using paired sample t-test; $p=0.014$ using Wilcoxon signed rank test). Over time (from 0 to 8 minutes) adhesion moderately decreased in untreated PBLs, slightly increased in 0.5 µM salmeterol treated PBLs, and significantly increased in 5 µM salmeterol treated PBLs ($p=0.0043$ using paired sample t-test; $p=0.014$ using Wilcoxon signed rank test). In contrast total adhesion remained almost unchanged between 0-8 minutes after wash-out following treatment of cells with 50 µM salmeterol.

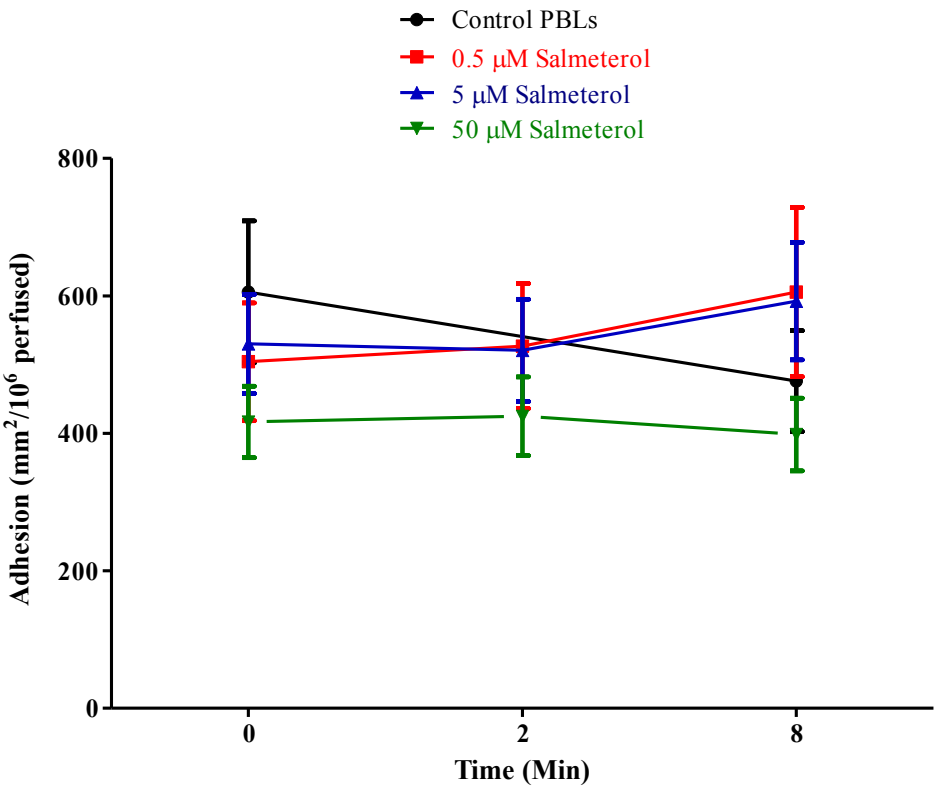



**Figure 4.11. Effect of different concentrations of salmeterol on lymphocytes adhesion to VCAM-1 coated microslide in a flow based assay.** The data are the mean ± from five-six independent experiments. For specific conditions see Figure legend 4.10.

We next investigated the effect of different concentrations of salmeterol on the average rolling velocity of PBLs (Fig. 4.12). Our data show that 50 µM salmeterol, significantly increased rolling velocity with time from 0 to 8 minutes following wash-out (from an average of 2.5 µm/sec to an average of 3.6 µm/sec respectively; p=0.023 using paired sample t-test; p=0.023 using Wilcoxon signed rank test). At 0 minute following 50 µM salmeterol perfusion, the PBLs average rolling velocity was increased by ~0.1 µm/sec as compared to the untreated control. This increase was slightly more pronounced at 2 minutes of washout (~0.2 µm/sec) and significantly more pronounced following 8 minutes of washout (~1.3 µm/sec increase; p=0.024 using paired sample t-test; p=0.023 using Wilcoxon signed rank test) compared to the untreated control. In addition, perfusion of 50 µM salmeterol significantly increased the PBLs rolling velocity compared to 0.5 µM (p=0.009 using paired sample t-test; p=0.023 using Wilcoxon signed rank test) and 5 µM salmeterol (p=0.040 using paired sample t-test; p=0.038 using Wilcoxon signed rank test) after 8 minutes of wash-out. By comparison, there was almost no effect of 0.5 µM salmeterol on rolling velocity and a small increase (~0.6 µm/sec at 2 minutes and ~0.3 µm/sec at 8 minutes) following 5 µM salmeterol perfusion (Fig. 4.12).

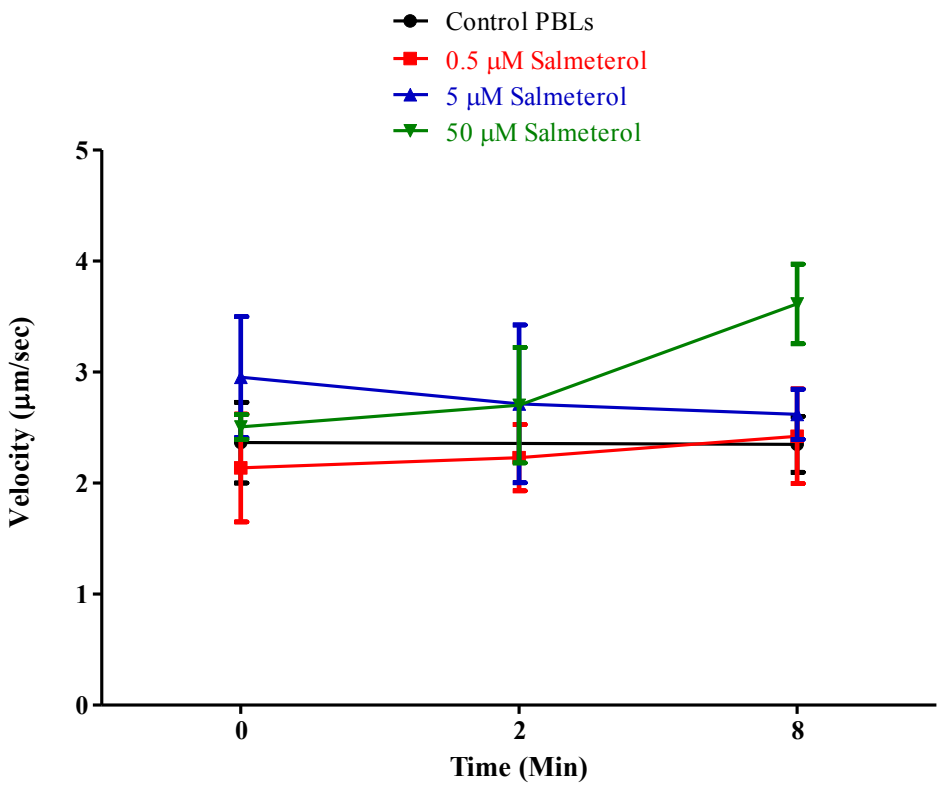



**Figure 4.12. Effect of different concentrations of salmeterol on the rolling velocity of lymphocytes recruited to VCAM-1 coated microslide from flow.** The data are the mean ± from five-six independent experiments. For specific conditions see Figure legend 4.10.

### 4.3.7 Effect of Epinephrine and Salmeterol on PBLs Adhesion and Rolling to 20 μg/ml VCAM-1

Although the changes seen support a role for increased rolling and decreased stationary cells in response to salmeterol, at different concentrations of salmeterol the changes remained relatively small. To address this, a higher concentration of VCAM-1 coated microslide (20 μg/ml) was used. In the next set of experiments we measured the effects of various salmeterol concentrations on the lymphocytes behaviour on 20 μg/ml VCAM-1 coated microslides.

We examined the effect of different concentrations of salmeterol (0-50 μM) on lymphocytes recruitment, behaviour and rolling velocity. A 4-minutes bolus of lymphocytes was perfused over 20 μg/ml VCAM-1 coated microslide at a wall shear stress of 0.05 Pa. Salmeterol at a specific concentrations was added at time 0 and their effect on PBLs behaviour was measured following 2 and 8-minutes of wash out respectively. As previously, our data show that the biggest increase in rolling and subsequently the biggest decrease in stationary PBLs occurred at the highest concentration of salmeterol (although in contrast to the previous data this increase was relatively small) (Fig. 4.13. A-B). Interestingly, at 0.5 μM salmeterol significantly decreased the percentage of PBLs rolling (~9.4% decrease; p=0.041 using paired sample t-test, p=0.069 using Wilcoxon signed rank test) and it subsequently increased the percentage of stationary PBLs. Overall, the changes in the percentage of rolling PBLs (Fig. 4.13. A) correspond to changes in the percentage of stationary adherent PBLs (Fig. 4.13. B), however increasing VCAM-1 concentration did not significantly improve the results.

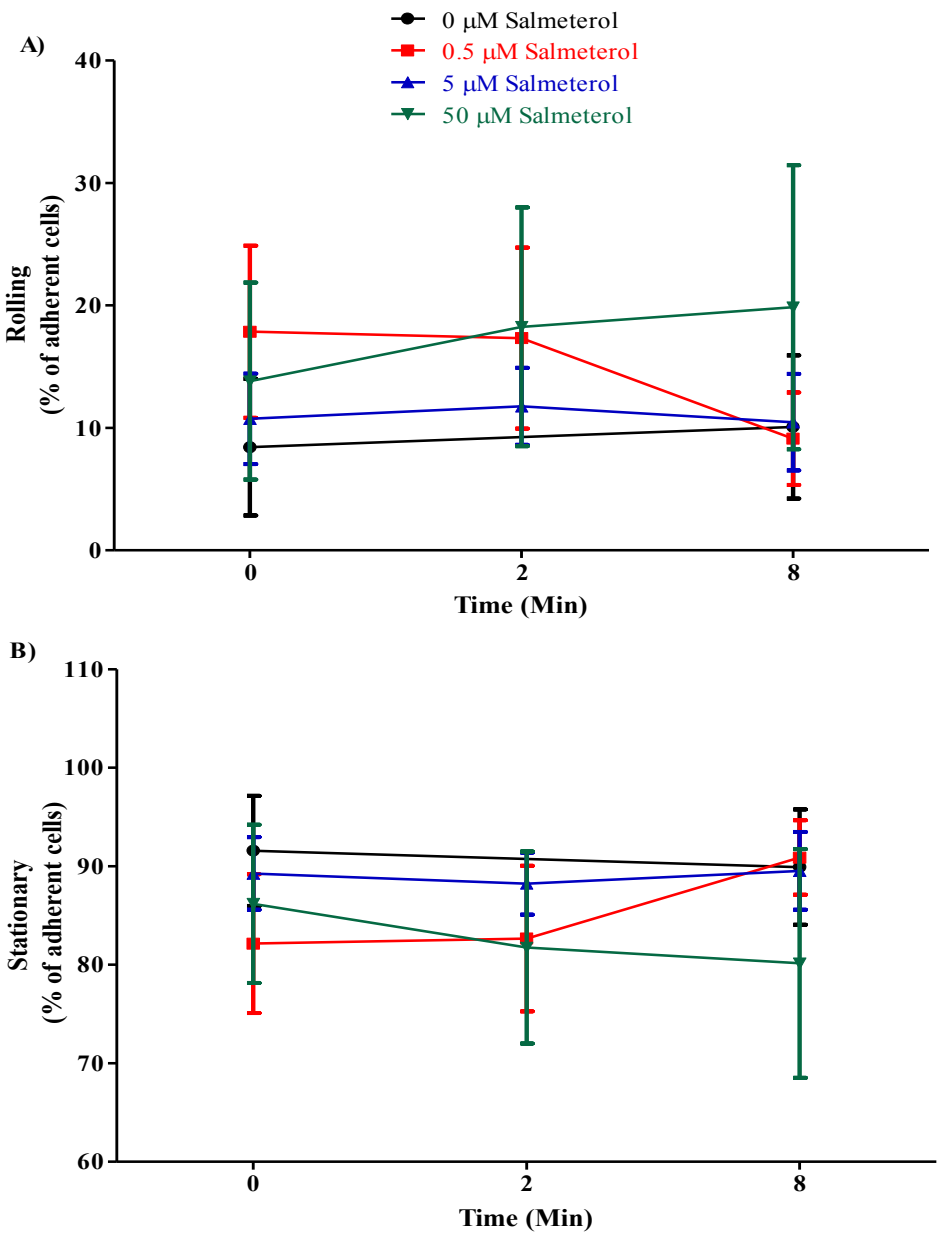



**Figure 4.13. Effect of different concentrations of salmeterol on lymphocytes recruitment to 20 µg/ml VCAM-1 coated microslide in a flow based assay.** A 4-min bolus of lymphocytes was perfused over 20 µg/ml VCAM-1 coated microslide at a wall shear stress of 0.05 Pa. Salmeterol was added at time 0 and their effect on PBLs behaviour ((A) rolling-(B) stationary) was measured following 2 and 8-min of wash out respectively. The data are the mean ± SEM from five-six independent experiments.

The number of PBLs recruited following perfusion of 50 µM salmeterol showed a significant ~33% decrease (p=0.027 using paired sample t-test; p=0.034 using Wilcoxon signed rank test) compared to the number of PBLs recruited to untreated control (Fig. 4.14.). This change remained at 2 minutes (p=0.040 using paired sample t-test; p=0.023 using Wilcoxon signed rank test) and 8 minutes (~35% decrease) of washout compared to the control, although borderline significance was observed at 8 minutes (p=0.056 using paired sample t-test; p=0.038 using Wilcoxon signed rank test). In addition, at baseline (0 minute) following washout salmeterol at 50 µM significantly decreased the number of total PBLs as compared to 0.5 µM (p=0.002 using paired sample t-test; p=0.014 using Wilcoxon signed rank test) and 5 µM salmeterol (p=0.007 using paired sample t-test; p=0.022 using Wilcoxon signed rank test) (Fig. 4.14.). Similarly at 8 minutes following washout, salmeterol at 50 µM, significantly decreased the number of total PBLs as compared to 0.5 µM (p=0.037 using paired sample t-test; p=0.022 using Wilcoxon signed rank test) and 5 µM salmeterol (p=0.009 using paired sample t-test; p=0.014 using Wilcoxon signed rank test). Over time (from 0 to 8 minutes) adhesion moderately increased in untreated and 0.5 µM salmeterol treated PBLs, and significantly increased in 5 µM salmeterol treated PBL (p=0.023 using paired sample t-test; p=0.022 using Wilcoxon signed rank test). In contrast total adhesion remained almost unchanged between 0-8 minutes after wash-out following treatment of cells with 50 µM salmeterol (Fig. 4.14.). Overall, these data are in agreement with our previous data whereby a 50 µM concentration of salmeterol decreased the recruitment of PBL to 10 µg/ml VCAM-1 coated microslide by ~45% (see Fig. 4.11.).

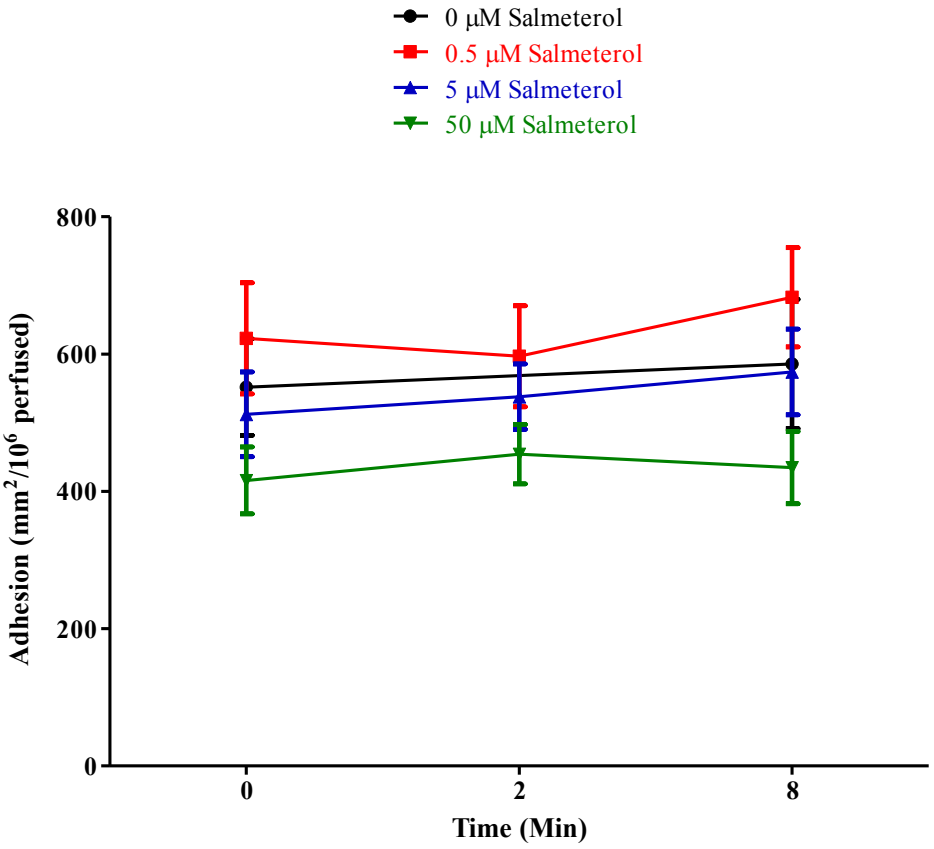



**Figure 4.14. Effect of different concentrations of salmeterol on lymphocytes adhesion to 20 µg/ml VCAM-1 coated microslide in a flow based assay.** The data are the mean ± from at least five independent experiments. For conditions see Figure legend 4.13.

The final factor to be investigated was the effect of different concentrations of salmeterol on the PBLs rolling velocity (Fig. 4.15.). As shown in this Figure, a salmeterol concentration of 50 μM, immediately increased (at 0 minute of washout) the PBLs rolling velocity by ~2.1 μm/sec compared to the untreated control (i.e. from ~2.2 μm/sec to ~4.0 μm/sec). However the rolling velocity decreased by 1.0 μm/sec (i.e. from ~4.0 μm/sec to ~3.0 μm/sec) following 2 and 8 minutes of washout as compared to the average PBLs rolling velocity immediately after salmeterol perfusion (at 0 minute) but increased by 1.0 μm/sec compared to the untreated control. Taken together these data indicate that salmeterol at concentrations of 50 μM moderately increased the PBLs averaged rolling velocity to 20 μg/ml coated VCAM-1 microslide. Almost no change in PBLs rolling velocity was observed at smaller concentrations of salmeterol.

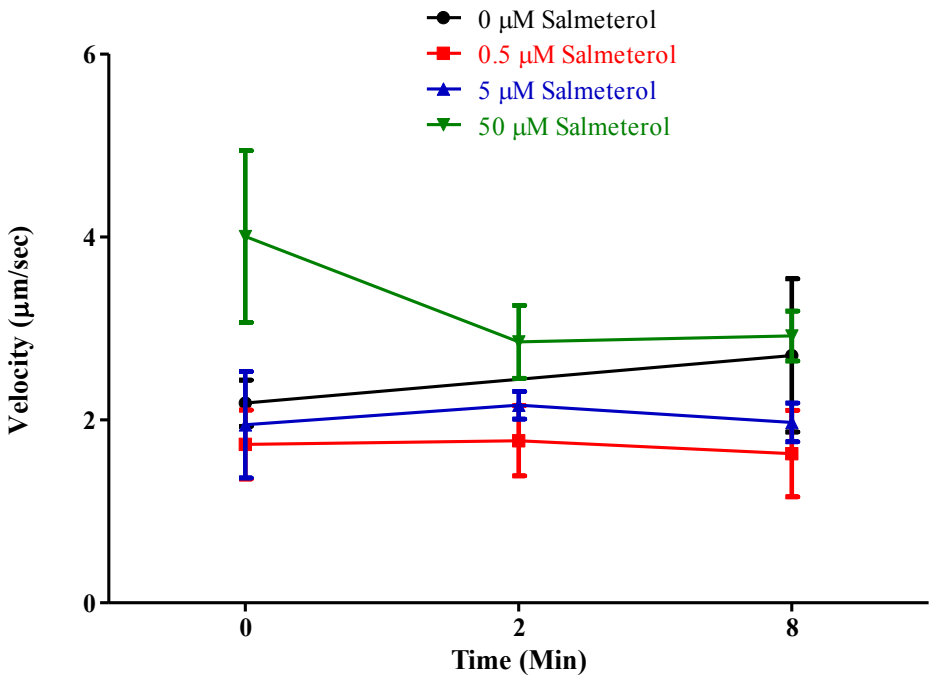



**Figure 4.15. Effect of different concentrations of salmeterol on the rolling velocity of lymphocytes recruited to 20 µg/ml VCAM-1 coated microslide from flow.** The data are the mean ± from five-six independent experiments. For conditions see Figure legend 4.13.

### 4.3.8 Effect of Different Concentrations of Salmeterol (0.5-50 μM) on the Detachment of CD8(+)CD28(-) T cells and NK cells from the 10ug/ml VCAM-1 Coated Microslide

Although the changes in total PBLs were generally small, we next wanted to determine whether salmeterol had any effects on the percentage of specific central memory/naive CD8(+)CD28(+) T cells (Arosa, 2002; Sallusto et al., 2004; Schirmer et al., 2002; Werwitzke et al., 2003), effector memory CD8(+)CD28(-) T cells (Arosa, 2002; Sallusto et al., 2004; Schirmer et al., 2002; Werwitzke et al., 2003) and NK cells (from adherence to 10 μg/ml VCAM-1 coated microslide) that have been shown to efflux in response to catecholamines (Dopp et al., 2000; Schedlowski et al., 1993a; Schedlowski et al., 1993b). These specific lymphocytes subpopulations were chosen to be assessed due to their expression of high levels of β2AR (Dimitrov et al., 2010). Immediately following the flow assay, PBLs were collected from the 10 μg/ml VCAM-1 coated microslides and cells were labelled with the respective antibodies. Flow cytometry analysis was used to determine the percentage of CD8(+)CD28(-), CD8(+)CD28(-) and NK cells (defined as CD3- and CD56+).

The data show that at concentration of 0.05 μM, 0.5 μM and 50 μM, salmeterol did not have any effect on the percentage of CD8(+)CD28(+) naive/central memory T cells and CD8(+)CD28(-) effector-memory T cells adherence to 10 μg/ml VCAM-1 coated microslide (Fig. 4.16. A). In contrast, at 5 μM concentration, more CD8(+)CD28(+) central memory/naive cells adhered (i.e. from ~73% to 89%) while more CD8(+)CD28(-) effector memory cells detached (i.e. from ~27% to 11%) compared to the control. This is in line with previous studies which show that stress mainly induces an increase in the effector memory cells (Anane et al., 2010; Benschop et al., 1996b; Dimitrov et al., 2010).

Salmeterol at concentration of 50 μM, significantly decreased the percentage of NK cells adherence to 10 μg/ml VCAM-1 compared to the control ($p=0.013$ using paired sample t-test;

p=0.054 using Wilcoxon signed rank test). In contrast, salmeterol at different concentrations (0.05-5 μM) used in this assay, did not have any significant effect on the percentage of NK cells adherent to 10 μg/ml VCAM-1 coated microslides (Fig. 4.16. B).

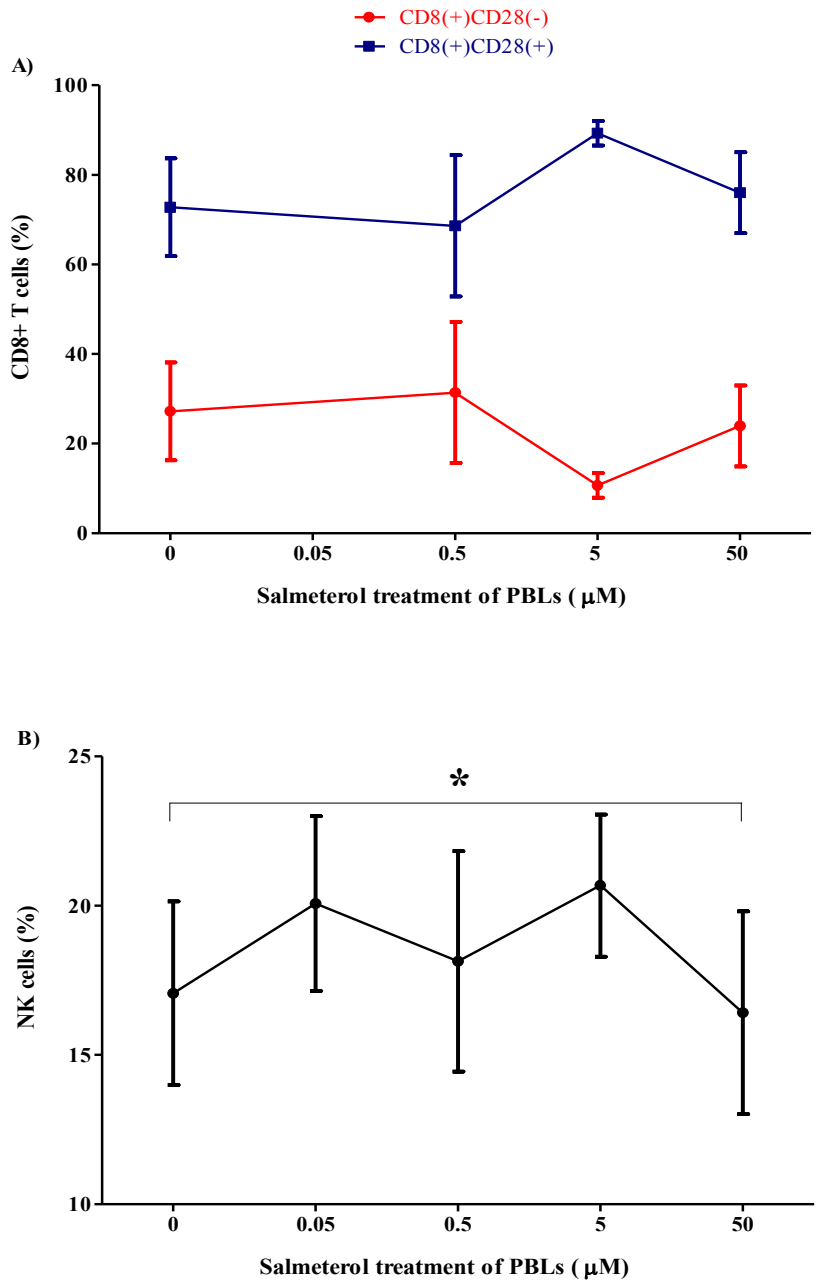



**Figure 4.16. Effect of salmeterol on the percentage of T cells and NK cells adherent to 10 µg/ml VCAM-1.** Cells were collected, labelled and analysed by flow cytometry to assess the percentage of CD8+ T (A) and NK cells (B) attached to the VCAM-1. In A, the CD8+ T cell population was subdivided into CD28 high expressing cells and CD28 low expressing cells. The data are the mean ± SEM from at least three independent experiments. Paired sample t test showed a significant difference *$p<0.05$ between salmeterol 50 µM and untreated (0) control regarding the percentage of adherent NK cells.

### 4.3.9 The Static Assay

Since neither salmeterol nor epinephrine had clear significant effects on lymphocytes recruitment, behaviour (stationary adherence) or lymphocytes rolling velocity over VCAM-1 in a flow based assay, we considered the possibility that salmeterol or epinephrine would affect lymphocytes behaviour by affecting other adhesion molecules in combination with VCAM-1 in a "non-flow" static assay model.

For our static assay model, HMEC-1 (human microvascular endothelial cell line-1) were used as a binding substrate; HMEC-1 express amongst others VCAM-1, ICAM-1 and E-selectin which being fast acting adhesion receptors are crucial to capturing and adhesion of lymphocytes to endothelial cells. These receptors are displayed on the surface of the endothelial cells and are upregulated in response to cytokines such as TNF-α, IFN-γ and IL-1 (McGettrick et al., 2009; Weinkopff and Lammie, 2011). In addition they are easily available, and grow easily.

### 4.3.10 Effect of Pre-treating PBLs with Epinephrine and Salmeterol on their Ability to Adhere and Migrate to Unstimulated HMEC-1

To determine whether epinephrine or salmeterol have any effect on the PBLs adhesion, migration or migration velocity to unstimulated HMEC-1, PBLs were incubated with salmeterol (0 μM-50 μM) or epinephrine ($5 \times 10^{-3}$ μM) for 30 minutes, washed and allowed to adhere to unstimulated HMEC-1 for 30 minutes. Non adherent cells were removed and adhesion (Fig. 4.17. A), transmigration (Fig. 4.17. B) and migration velocity (Fig. 4.17. C) were analysed by phase contrast microscopy. The results show that epinephrine (0.005 μM) and salmeterol at all concentrations used (0.05 μM-50 μM) caused a ~10.5 % increase in the percentage of adherent PBLs to unstimulated HMEC-1. This increase was significant for epinephrine 0.005 μM ($p=0.006$ using paired sample t-test; $p=0.022$ using Wilcoxon signed

rank test) and salmeterol at all concentrations, viz., 0.05 µM (p=0.006 using paired sample t-test; p=0.022 using Wilcoxon signed rank test), 0.5 µM (p=0.007 using paired sample t-test; p=0.022 using Wilcoxon signed rank test), 5 µM (p=0.028 using paired sample t-test; p=0.022 using Wilcoxon signed rank test) and 50 µM (p=0.018 using paired sample t-test; p=0.022 using Wilcoxon signed rank test). The highest increase in PBLs adhesion was observed at smaller concentrations of salmeterol (0.05-0.5 µM) suggesting that the optimal effect of salmeterol is observed at lower concentrations used in this assay. In contrast to PBLs adhesion, salmeterol at all concentrations (0.05-50 µM) and epinephrine (0.05 µM) did not affect the lymphocyte transmigration to unstimulated HMEC-1 compared with the untreated PBLs (Fig. 4.17. B). This lack of effect may be due to the high proportion (~88%) of untreated PBLs that had transmigrated following 30 minutes of incubation with HMEC-1. Furthermore, no difference in the lymphocytes transmigration was also observed between the different salmeterol concentrations. Salmeterol increased the migration velocity of PBLs at the lowest concentration of salmeterol 0.05 µM (~0.5 µm/sec) but not at the higher concentrations.

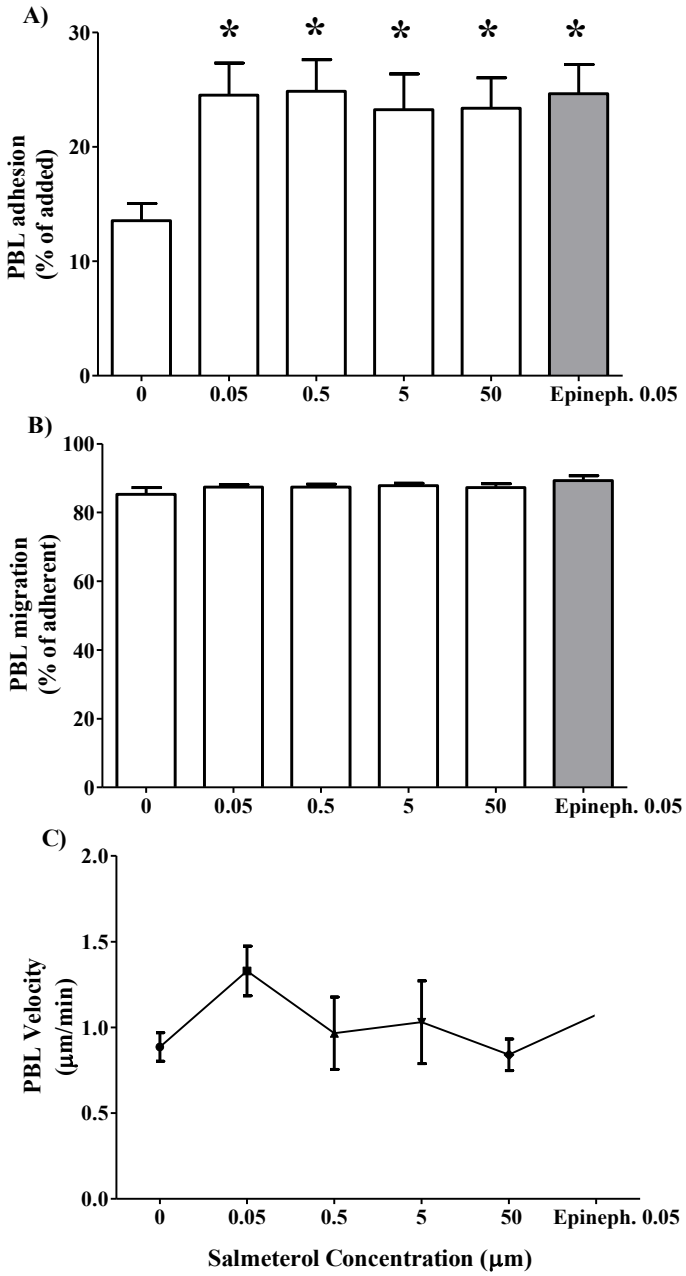



**Figure 4.17 Effect of pre-treating PBLs with β2AR agonists on their ability to adhere and migrate.** PBLs were incubated with salmeterol (0 µM-50 µM) or epinephrine (0.5 µM) for 30 min, washed and allowed to adhere to unstimulated HMEC-1 for 30 min. Non adherent cells were removed and adhesion (A), transmigration (B) and migration velocity were analysed at between 35 min – 42 min by phase contrast microscopy. The data are the mean ± SEM from five independent experiments. *$p<0.05$ using paired t-test and/or Wilcoxon signed rank test using untreated control as a reference.

### 4.3.11 Effect of Pre-incubating PBLs with Epinephrine or Salmeterol on the Percentage of CD4+, CD8+ T and NK Adhered to Unstimulated HMEC-1

To determine whether salmeterol has any effects on the percentage of adherent CD4+ T, CD8+ T and NK cells to HMEC-1 monolayer, following video recordings, adherent PBLs and HMEC-1 cells were detached from the culture plate with accutase. Adherent lymphocytes were labelled with a cocktail of fluorescent-labelled monoclonal antibodies, and results were analysed by flow cytometry to assess the percentage of T cells (Fig. 4.18. A) and NK cells (Fig. 4.18. B) adherent to HMEC-1 monolayer. Our data show that salmeterol at concentrations of (0.05-50 μM) and epinephrine (0.05 μM) caused a ~5% increase in the percentage of CD4+ T and a ~2.5% increase in the percentage of CD8+ T cells respectively. Similarly, the percentage of NK cells increased (by ~1.3%) upon different concentrations of salmeterol treatment or epinephrine. These results suggest that salmeterol and epinephrine increase the percentage of specific lymphocytes subpopulations; CD4+, CD8+ T and NK cells to unstimulated HMEC-1 monolayer, although these results were not significant (all p values from the paired t-test and Wilcoxon signed rank test were >0.05).

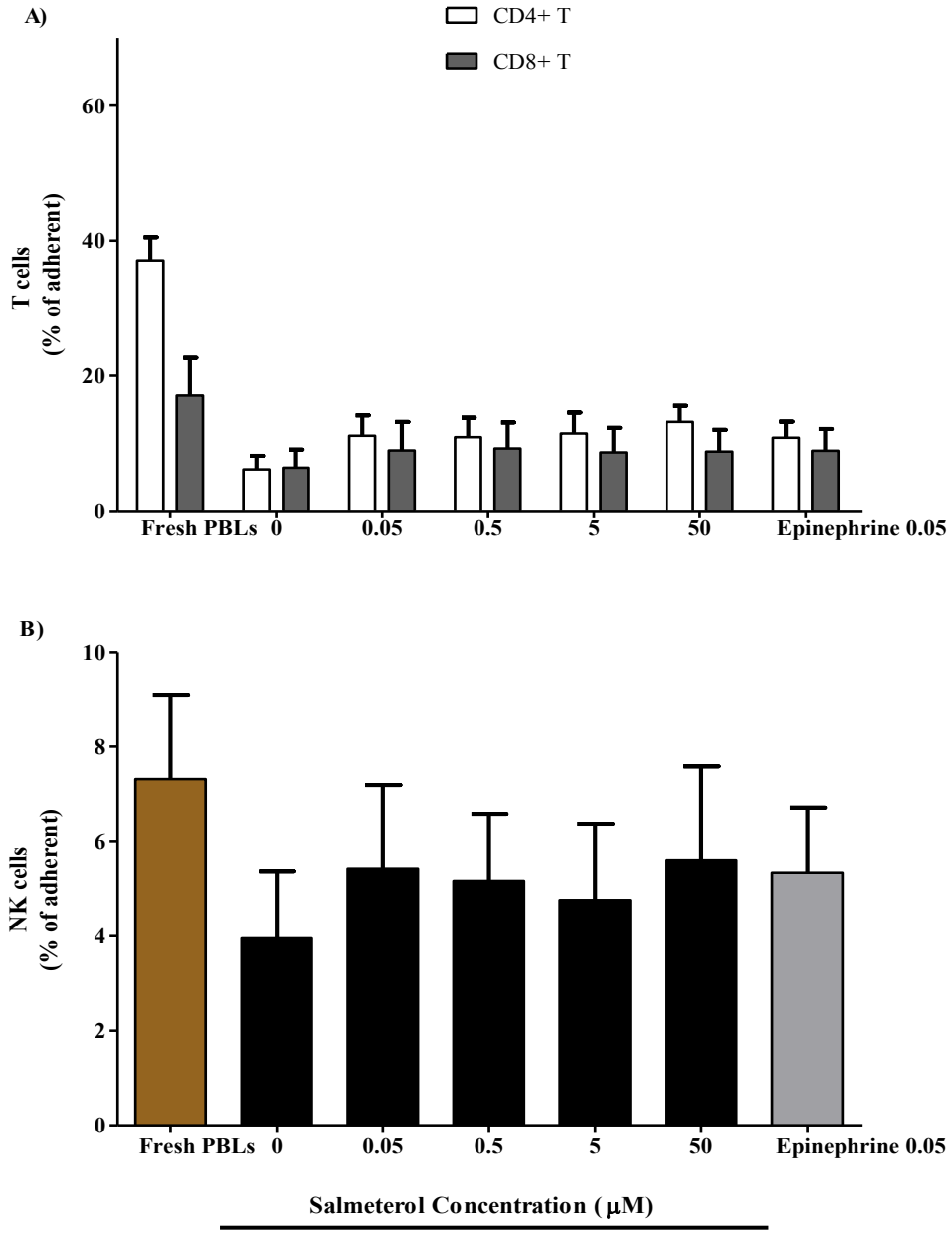



**Figure 4.18. Effect of β2AR agonists on the percentage of T cells and NK cells adherent to HMEC-1.** PBLs were incubated with salmeterol (0 μM-50 μM) or epinephrine (0.05 μM) for 30 min, washed and allowed to adhere to unstimulated HMEC-1 for 30 min. Cells were collected, labelled and analysed by flow cytometry to assess the percentage of T cells (A) and NK cells (B) adherent to HMEC-1 monolayer. The data are the mean ± SEM from three independent experiments.

We next wondered whether pre-incubation of PBLs with salmeterol or epinephrine would affect the PBLs adhesion, migration and migration velocity to cytokine treated HMEC-1 expressing ICAM-1, VCAM-1 and E-selectin. These receptors displayed on the surface of endothelial cells, are upregulated in response to cytokines such as TNF-α, IFN-γ and IL-1 (McGettrick et al., 2009; Weinkopff and Lammie, 2011). Therefore in our next experiments, it was important to set up an in vitro inflamed model, which would allow the upregulation of ICAM-1, VCAM-1 and E-selectin on the surface of endothelium and thus enable us to measure the effect of salmeterol on PBLs adhesion, migration and migration velocity. Various cytokines and cytokine combinations were tested to set up an in vitro inflamed model that would result in a high percentage of PBLs adhesion and migration to HMEC-1.

### 4.3.12 Determining Which Cytokine Combination Causes the Highest Adhesion and Migration of PBLs to HMEC-1

Initial experiments served to optimise the assay condition for using untreated HMEC-1 cells as a substrate. To further investigate the process in inflammatory conditions it was necessary to determine the optimal cytokine stimulation for HMEC-1 which would result in the highest adhesion of PBLs. Previous studies had indicated that the stimulation of endothelial cells (HUVEC) with a range of cytokines (IL-1, TNF-α, IFN-γ, or TNF-α and IFN-γ), caused modest increases in T cell adhesion and migration (McGettrick et al., 2009).

A cytokine stimulation period of 24 hours was required for optimal expression of VCAM-1 and ICAM-1 (through which the PBLs capture is possible), whereas E-selectin optimal expression was reached following 4 hours of endothelial cells stimulation with cytokines (Chen et al., 2002b; Ley and Tedder, 1995; Lindsey et al., 2000; Wong and Dorovini-Zis, 1995). For this reason in our initial experiments, HMEC-1 were stimulated with TNF-α (100

U/ml) or IFN-γ (10ng/ml) for 4 or 24 hours, alone or in combination. Following stimulation of HMEC-1 with cytokines, PBLs were allowed to adhere to HMEC-1 for 30 minutes, non adherent cells were washed off and the percentage of adhesion (Fig. 4.19. A) transmigration (Fig. 4.19. B) and the number of total PBLs adherent (Fig. 4.20.) were analysed immediately by phase contrast microscopy.

Results show that there was a significant ~11% increase ($p=0.012$ using paired sample t-test; $p=0.034$ using Wilcoxon signed rank test) in lymphocyte adhesion on TNF-α-24h and a significant ~12 % increase ($p=0.031$ using paired sample t-test; $p=0.034$ using Wilcoxon signed rank test) in lymphocyte adhesion to IFN-γ+TNF-α-24h-stimulated HMEC-1 compared to untreated control (Fig. 4.19. A). In contrast, when HMEC-1 were stimulated with TNF-α-4h or IFN-γ-24h alone, only a ~2.5% increase in the lymphocyte adhesion to HMEC-1 was observed compared to the unstimulated HMEC-1. Furthermore, stimulation of HMEC-1 with any cytokine combination only moderately increased the percentage (by an average of ~8%) of adherent lymphocytes that migrated through HMEC-1 compared to the unstimulated control (Fig. 4.19. B), and likewise, a small difference in lymphocyte migration was observed between different cytokine combination treatments. These results suggest that the percentage of adherent PBLs that migrated was not affected by cytokine treatments. From looking at the total number of adherent PBLs per $mm^2$, we observed that the proportion of PBLs adherent to HMEC-1 stimulated with IFN-γ+TNF-α-24h was ~86% significantly higher ($p=0.035$ using paired sample t-test; $p=0.034$ using Wilcoxon signed rank test) compared with unstimulated HMEC-1 (Fig. 4.20.). Treatment with TNFα-24h also significantly increased the proportion of PBLs adherent to HMEC-1 ($p=0.014$ using paired sample t-test; $p=0.034$ using Wilcoxon signed rank test), but since treatment with IFN-γ+TNF-α-24h cytokine combination supported the highest number of PBLs adherent to HMEC-1 than any other cytokine

treatment, it was decided to stimulate HMEC-1 with IFN-γ (10ng/ml) and TNF-α (100U/ml) for 24 hours.

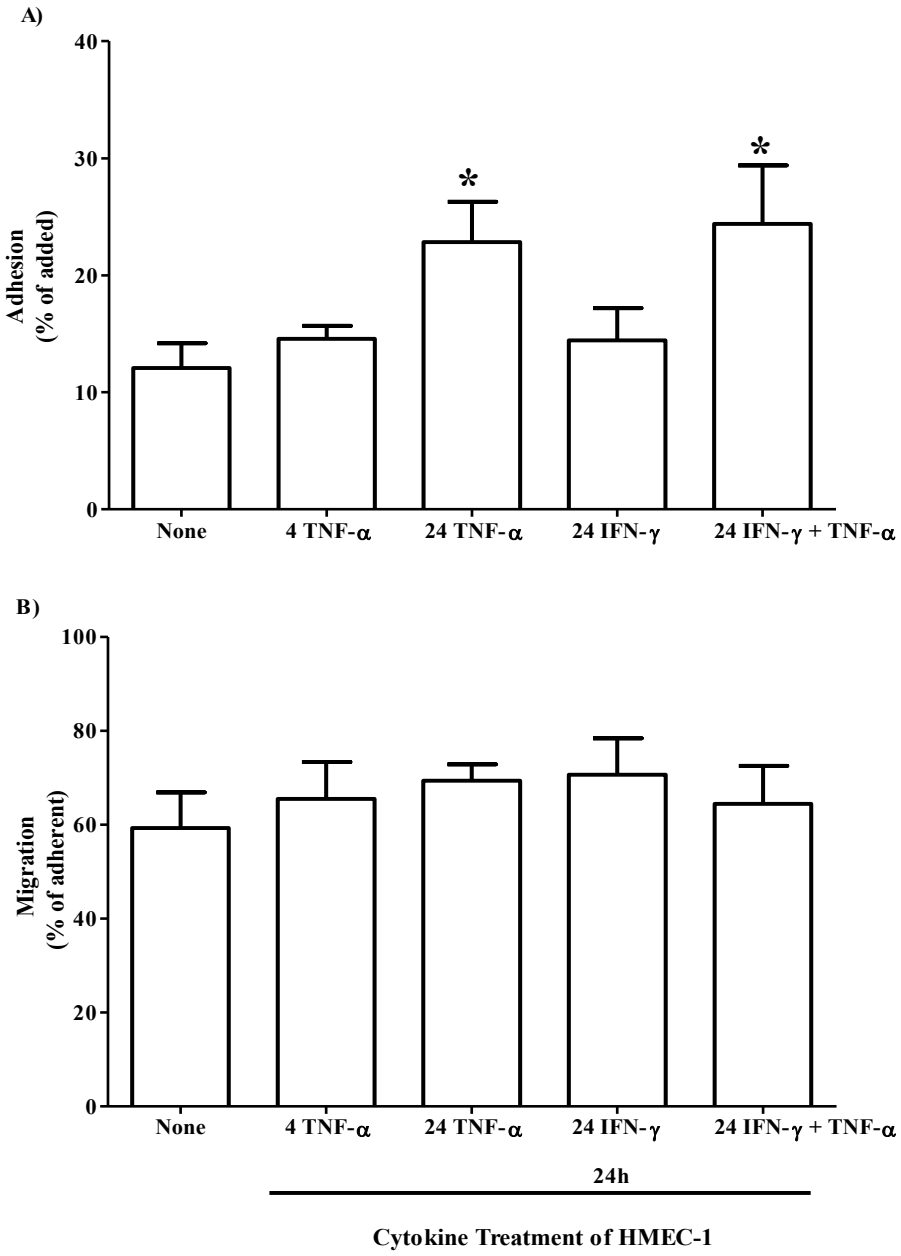



**Figure 4.19. Effect of cytokine treatment on HMEC-1 on PBLs adhesion and migration**. HMEC-1 were stimulated with TNF-a (100 U/ml) or IFN-γ (10ng/ml) for 4 or 24h, alone or in combination. At desired time, PBLs were allowed to adhere to HMEC-1 for 30 min, non adherent cells were washed off and adhesion (A) and transmigration (B) was analysed immediately by phase contrast microscopy. Data are the mean ± SEM from four independent experiments. *$p<0.05$ using paired t-test and/or Wilcoxon signed rank test using untreated control (None) as a reference.

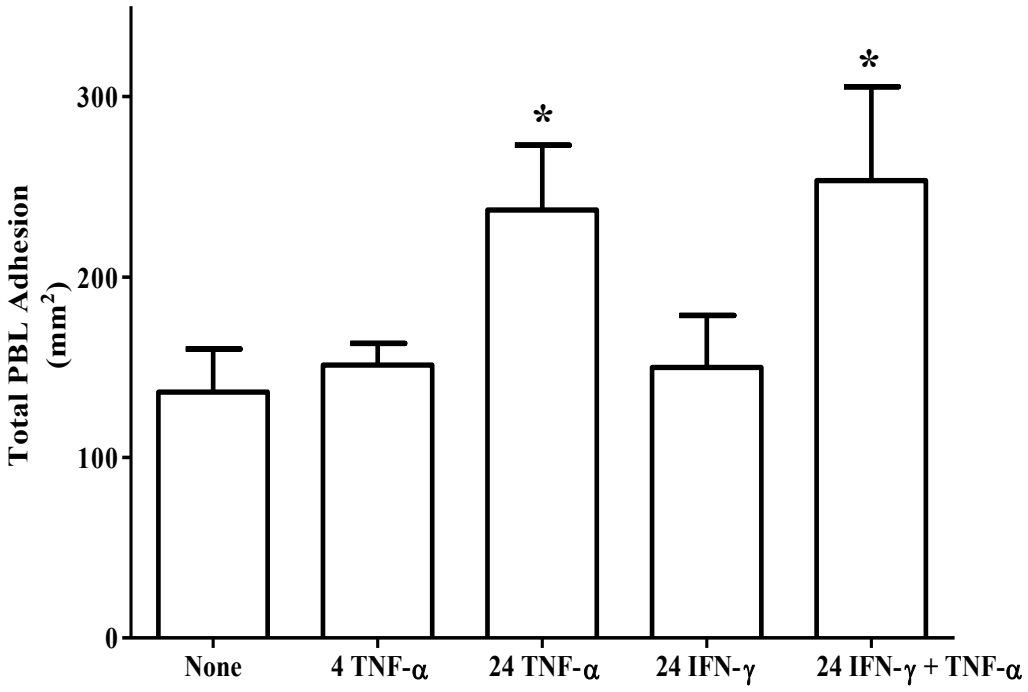



**Figure 4.20. Effect of cytokine treatment of HMEC-1 on total number of adherent PBLs**. Data are the mean ± SEM from four independent experiments. *p<0.05 using paired t-test and/or Wilcoxon signed rank test using untreated control (None) as a reference. For conditions see Figure legend 4.19.

### 4.3.13 Effect of Pre-incubating PBLs with Different Concentrations of Salmeterol on PBLs Adhesion, Migration and Migration Velocity to Cytokine Stimulated HMEC-1

To determine whether salmeterol has any effects on the PBLs adhesion, migration or migration velocity to IFNγ + TNF-α cytokine treated HMEC-1, PBLs were incubated with salmeterol (0 μM-5-50 μM) for 30 minutes, washed and allowed to adhere to stimulated HMEC-1 for 30 minutes. Non adherent cells were removed and adhesion (Fig. 4.21. A), transmigration (Fig. 4.21. B) and migration velocity (Fig. 4.21. C) were analysed by phase contrast microscopy. Our results show that salmeterol at concentration of 50 μM, increased (by~3.6%) the PBLs adhesion to cytokine treated HMEC-1 (Fig.4.21.A), whilst decreasing (by~6.2%) the migration of PBLs to cytokine stimulated HMEC-1 compared to the untreated control (Fig.4.21.B). Furthermore, the migration velocity was decreased in a dose dependent manner. A salmeterol concentration of 5 μM decreased the rolling velocity by ~0.4 μm/min whereas at 50 μM, salmeterol decreased the rolling velocity by ~1.9 μm/min (Fig. 4.21. C).

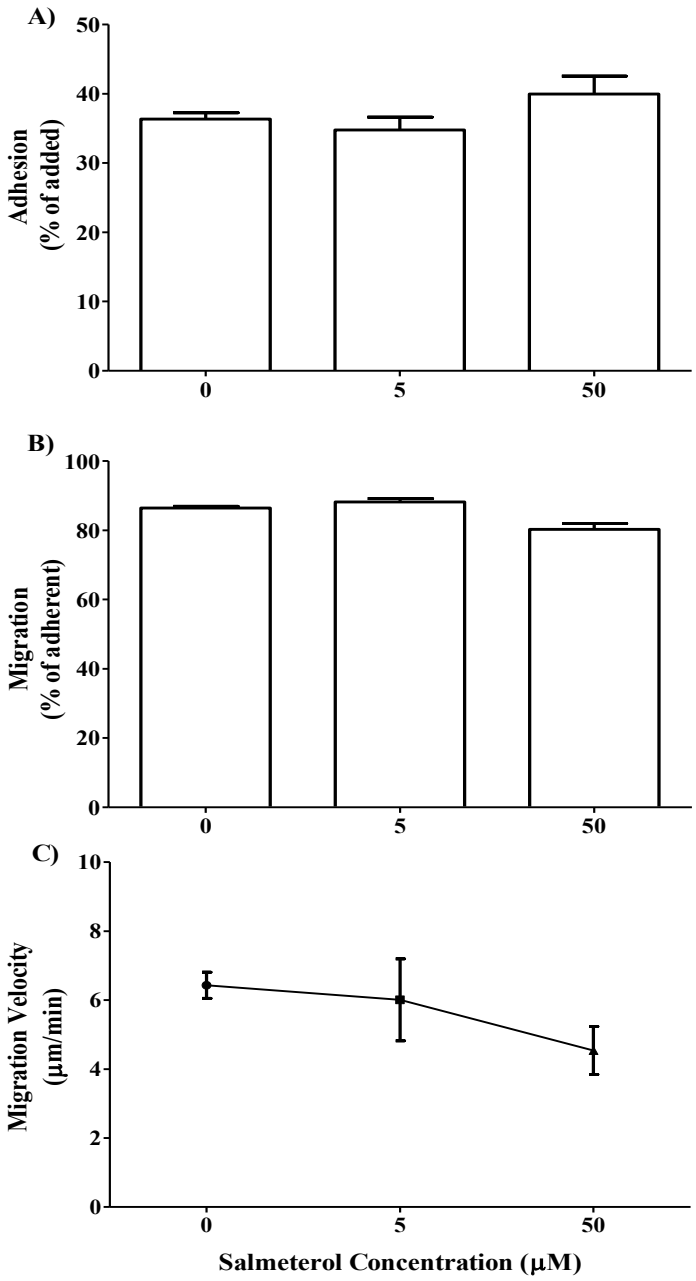



**Figure 4.21. Effect of pre-treating PBLs with salmeterol on their ability to adhere and migrate on cytokine treated HMEC-1.** PBLs were incubated with salmeterol (0 µM-50 µM) for 30 min, washed and allowed to adhere to IFN-γ + TNF-α treated HMEC-1 for 30 min. Non adherent cells were removed and adhesion (A), transmigration (B) and migration velocity were analysed at between 35 min-42 min by phase contrast microscopy. The data are the mean ± SEM from at least four independent experiments.

### 4.3.14 Effect of Pre-incubating PBLs with Different Concentrations of Salmeterol on the Percentage of CD4+, CD8+ T and NK Adhered to Cytokine Treated HMEC-1

To determine whether salmeterol has any effects on the percentage of adherent CD4+ T, CD8+ T and NK cells to cytokine treated HMEC-1 monolayer, following on from video recordings, adherent PBLs and HMEC-1 cells were detached from the culture plate with accutase. Adherent lymphocytes were labelled with a cocktail of fluorescent-labelled monoclonal antibodies, and results were analysed by flow cytometry to assess the percentage of T cells (Fig. 4.22. A) and NK cells (Fig. 4.22. B) adherent to HMEC-1 monolayer. The data shown in these Figures indicate that salmeterol at concentrations of (5-50 µM) increased the percentage of CD4+ T cells in a salmeterol dose dependent manner. At a concentration of 5 µM salmeterol the percentage of CD4+ T cells increased by ~2.4%, whereas at 50 µM a significant increase by ~7.9% ($p=0.049$ using paired sample t-test; $p=0.072$ using Wilcoxon signed rank test) was observed compared to the untreated control (see Fig. 4.22. A). Similarly, at 50 µM salmeterol increased (by~2.7%) the percentage of adherent CD8+ T cells, however a ~3.6% decrease in the percentage of CD8+ T cells adherent to HMEC-1 monolayer was observed at 5 µM, as compared to the untreated control. (This decrease was significant using using Wilcoxon signed rank test; $p=0.034$). Furthermore, salmeterol at 5 µM decreased the percentage of NK cells adherent by ~3.6% compared to the untreated control, but it further decreased (total average mean ~4.2%) the percentage of NK cells at a concentration of 50 µM (see Fig. 4.22. B). Similarly to the case above, this decrease proved to be significant when using Wilcoxon signed rank test whereby $p=0.034$ for both 5 uM and 50 uM salmeterol.

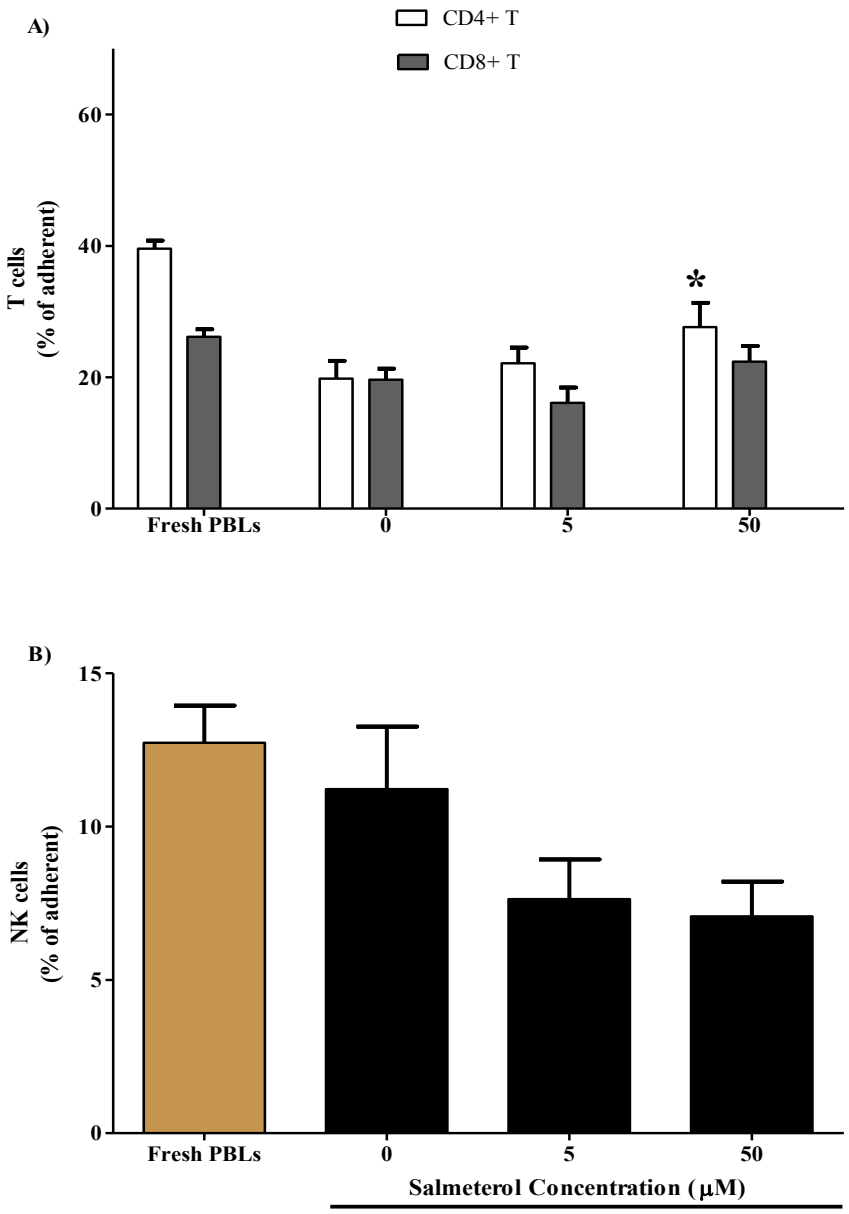



**Figure 4.22. Effect of salmeterol on the percentage of T and NK cells adherent to cytokine treated HMEC-1**. PBLs were incubated with salmeterol (0 μM-50 μM), washed and allowed to adhere to IFN-γ +TNF-α treated HMEC-1 for 30 min. Cells were collected, labelled and analysed by flow cytometry to assess the percentage of T (A) and NK cells (B) adherent to HMEC-1 monolayer. The data are the mean ± SEM from four independent experiments. *$p<0.05$ using paired t-test using untreated control (0) as a reference.

## 4.4 Discussion

The results in this chapter demonstrate for both the VCAM-1 flow assay and HMEC-1 static assay that catecholamines acting via the β2AR induce an increase in rolling behaviour and a decrease in stationary behaviour of PBLs. Moreover, the specific effect on memory CD8+ T cells and NK cells supports the previous findings that stress lymphocytosis leads to a selective increase in such cells (Anane et al., 2010; Benschop et al., 1996b; Campbell et al., 2009).

There are, however, caveats; most importantly the changes generally were small and typically did not reach statistical significance. This may have been due to the wide range of response in some experiments which may be indicative of the response of different donors. Most of the blood donors in this study were males, so gender may have not had any influence on the response to salmeterol. In addition, most of blood donors were among the mid-twenties and mid-thirties age group, which could explain why the effects are very small. It may also be that the most optimal conditions for analysing the effects of catecholamines on adhesion were not reached in these studies. VCAM-1 was selected as a target molecule based on previous studies with lymphocytes (Ley et al., 2007; McGettrick et al., 2009). However, VCAM-1 and its ligand $\alpha_4\beta_1$ (VLA-4) are particularly relevant to activated lymphocyte responses, (Hernandez-Caselles et al., 1996; Kurokawa et al., 1995) and while the cells released into the blood are memory cells they may utilise other receptors.

The initial aim was to study the effects of β2AR stimulation on migratory behaviour of CMV-specific T cells (see Chapter 3) and confirm the effect of salmeterol by using β2-antagonists; however, in light of the current effects showing the changes were small, studying such low-frequency cells and further extending the experiment by using β2-antagonists was deemed unrealistic.

Regardless, it may be that certain populations of memory T cells and NK cells will be affected more than others. It has been suggested that exercise demarginates older cells that are awaiting destruction in the spleen (Shephard, 2003). This could potentially now be addressed as memory T cells constitute a diverse phenotype that is just now being understood (Younes et al., 2011). Similarly, NK cells can be separated in to distinct populations based on licensing, i.e., whether they have been primed by HLA ligands (Yokoyama, 1998). Perhaps surprisingly, recent data has shown that it is unlicensed NK cells that control CMV infection (Orr et al., 2010). As licensing is determined by HLA genotype of each individual this could explain the differences seen in the current studies of NK migration. Regardless of these mechanisms, the results presented in this chapter consistently showed a pattern that would support the efflux of cells from the tissues to the blood due to catecholamine stimulation.

The association between catecholamines and adhesion molecule expression and function is complex. As stated, the numbers and phenotypes of circulating leukocyte subsets can change dramatically during and immediately following exercise or stress (Shephard, 2003). NK cell influx in humans in response to catecholamines did not associate with either increased adhesion molecules on NK cells or soluble adhesion molecules (Schedlowski et al 1996). In contrast however, Nagao et al. (2000) found a decrease in CD44 and CD18 on NK cells during exercise or administration of catecholamines. CD44 may be due to release from the endothelium via hyaluronic acid (Nagao et al., 2000). Therefore we need to look more closely at other adhesion molecules and migration.

The surface expression of adhesion molecules makes an important contribution to mobilisation responses by changing patterns of cell trafficking (Shephard, 2003). NK cells that are mobilised during exercise have been reported to express high levels of macrophage-1 antigen (Mac-1; CD11b), a marker associated with cellular activation (Huntington et al.,

2007; Shephard, 2003). Analogous changes can be induced by other forms of stress that release catecholamines or by catecholamine infusion, and responses are blocked by β2-blocking agents. Catecholamines also modify adherence and expression of other adhesion molecules *in vitro* (Shephard, 2003).

It is suggested that norepinephrine may play an endogenous neuroprotective role in the CNS in disorders where inflammatory events contribute to pathology (Feinstein et al., 2002; Marien et al., 2004; O'Sullivan et al., 2009; O'Sullivan et al., 2010; Zarow et al., 2003). Infiltration of peripheral immune cells into the brain via increased chemokines and cell adhesion molecules (CAMs) expression is known to exacerbate neuroinflammation and contribute in a number of neurodegenerative disease states (Ballestas and Benveniste, 1997; Brochard et al., 2009; Constantin, 2008; Lee and Benveniste, 1999; O'Sullivan et al., 2010; Weiner, 2009). In rats treated with the norepinephrine reuptake inhibitors (NRIs) desipramine and atomoxetine (agents that increase extracellular norepinephrine in the CNS), a suppression of interferon-inducible protein-10 (IP-10, CXCL-10) and regulated upon activation normal T-cell expressed and secreted (RANTES, CCL-5), and the CAMs, VCAM-1 and ICAM-1 is observed in cortex and hippocampus, following a systemic challenge with bacterial lipopolysaccharide (LPS) (O'Sullivan et al., 2010). The inhibitory action of NRIs on chemokines and CAMs expression was mimicked by *in vitro* exposure of cultured glial cells to norepinephrine, but not to the NRIs themselves (O'Sullivan et al., 2010) supporting the theory that norepinephrine has anti-inflammatory properties. In agreement with this finding, previous in vitro studies have demonstrated reduced RANTES release from lung epithelium and ICAM-1 expression in airway smooth muscles following administration of βAR agonists (Hallsworth et al., 2001; Kaur et al., 2008).

Adhesion of circulating monocytes to the vascular endothelium is one of the earliest steps in the development of atherosclerosis, mediated in part by $\beta_2$-integrins (Gahmberg et al., 1998; Greeson et al., 2009; Luster et al., 2005; Merched et al., 2010; Ross, 1999). In a study on the effects of stress, the Anger Recall Interview (ARI) was utilised and the expression of LFA-1 (CD11a), Mac-1 (CD11b) and p150/95 (CD11c) on circulating monocytes (CD14+) were monitored in 173 healthy, nonsmoking men and women (Greeson et al., 2009). The results showed that those individuals who showed the largest increases in norepinephrine during the ARI showed an increase in monocyte $\beta_2$-integrin expression (Greeson et al., 2009). Thus, heightened psychological and physiological stress responses induced phenotypic changes in monocytic expression of $\beta_2$-integrins that are consistent with the role of monocytes/macrophages in vascular inflammation (Luster et al., 2005; Merched et al., 2010; Ross, 1999; Takala et al., 1996).

Catecholamines can also influence on cytokine production and expression of adhesion molecules by human neutrophils (Chen et al., 2005; Kurokawa et al., 1995; Mills et al., 2002; Shephard, 2003; Wahle et al., 2005). Resting and LPS-activated neutrophils from healthy volunteers challenged with epinephrine showed decreased expression of ICAM-1 on activated neutrophils upon co-incubation with 1 mM EPI for 6 hrs (Wahle et al., 2005). Conversely, 1 mM EPI for 12 and 18 hrs decreased the spontaneous loss of ICAM-1 on resting neutrophils (Wahle et al., 2005). This data suggests that β2AR are functionally coupled to signalling cascades in human neutrophils, however the response may alter with time of incubation (Wahle et al., 2005).

Finally it is not just leukocytes that are influenced by catecholamines. Dermal fibroblasts migrate into a wound bed, proliferate, synthesize extracellular matrix proteins and contract the wound (Gabbiani et al., 1972; Grinnell, 1994; Pullar and Isseroff, 2006). Fibroblasts express

β2AR and cutaneous keratinocytes can synthesize catecholamines (Pullar et al., 2003; Pullar et al., 2006; Sivamani et al., 2009). Activation of β2AR on keratinocytes leads to a reduction in migration, while in dermal fibroblasts show increased migration, but inhibited collagen gel contraction (Chen et al., 2002a; Pullar et al., 2003; Pullar and Isseroff, 2006). Surgical stress can lead to increased catecholamine release and impair wound healing (Pullar et al., 2003; Pullar et al., 2006; Pullar and Isseroff, 2006).

In conclusion, we found that catecholamines induce a modest change in the adhesion of PBLs or substrate that supports the release of these cells from tissues into blood at times of stress as indicated by both, paired sample t- test and Wilcoxon signed ranked test. The efflux of specific populations of cells has been related to the high level of expression of β2AR, however the small size of changes suggest that other factors (specific cell types) have still to be identified or simply that the mechanisms studied here do not play a relevant role in stress-induced lymphocytosis. To confirm the involvement of the β2AR in this process, it was important to include β2AR–antagonist control as in the cytokie stimulation study (chapter 3), however due to the time in which salmeterol was passed over cells (2 minutes), the small responses of T cells to salmeterol, and the complexity of the assay itself, it wasn't possible to use an antagonist at this stage. As in the previous studies, one of the issues to address in chapter 4, is the dilution of salmeterol with ethanol which could have possibly influenced onto the effect of salmeterol on T cells. However, the fact that similar results were observed when epinephrine (diluted with water), was used as a control, provides confidence that the results are real and the data can be trusted. Yet, to eliminate any possibilities on the effect of ethanol on NK and T cells, the effect of ethanol on NK and T cells should be investigated in future short term studies.

# CHAPTER 5

# EFFECT OF ADRENERGIC STIMULATION ON THE CYTOTOXIC FUNCTION OF CD8+ T AND NK CELLS

## 5.1 Introduction

NK cells were discovered in the mid-1970s as large granular lymphocytes that had natural cytotoxicity against tumour cells, viruses, bacteria and parasites (Cooper et al., 2001; Evans et al., 2011; Loza et al., 2002; Paust and von Andrian, 2011; Tonnesen et al., 1989; Vivier et al., 2008). For this reason, they are considered potentially important for the immunosurveillance against cancer and virus infections (Hellstrand et al., 1985). NK cells also represent a bridge between the innate and the adaptive immune response due to their ability to secrete large amounts of cytokines that shape and drive adaptive immune response (Alter et al., 2004). One of the major effector lymphocytes of the adaptive immune response includes CD8+ T cells. Similarly to the NK cells, CD8+ T cells are involved in the recognition and lysis of infected and transformed cells. Both CD8+ T and NK cells express a high number of βAR, particularly of epinephrine-sensitive $\beta_2$ subtype (Anane et al., 2009; Anane et al., 2010; Benschop et al., 1996b; Hellstrand et al., 1985). Previous studies have demonstrated that CNS activities can broadly influence both CD8+ T and NK cells function (Elenkov et al., 2000; Padgett and Glaser, 2003; Schedlowski et al., 1996; Snijdewint et al., 1993; Van der Pouw-Kraan et al., 1992; Yang and Glaser, 2000). In effect, activation of the sympathetic nervous system under various stresses such as exercise, mental stress or loss of blood and surgery, are often associated with altered NK cell activity (Gan et al., 2002; Klokker et al., 1998). These effects may have clinical significance. It has, for example, been demonstrated that administration of β2 agonists *in vivo* increases tumour cell metastasis rates through reduction of NK cell activity (Shakhar and Ben-Eliyahu, 1998).

In this chapter we investigated the effect of β2 agonists (i.e. epinephrine and salmeterol) on the cytotoxic functions of both CD8+ T and NK cells *in vitro*. To address this aim, PBMCs were treated with epinephrine (0.05 μM) or salmeterol (5-50 μM) and were stimulated with either untransfected Chinese hamster ovary cells (CHO) or, MHC class I chain-related gene A (MICA*009) transfected Chinese hamster ovary cells (T-CHO). These transfected cells lack expression of MHC class I molecules, which mimics the conditions of some tumours and infected cells. The subsequent NK response is, in theory, amplified by the simultaneous expression of MICA on these cells. MICA are "stress-induced" ligands that are found on many tumour cells and are upregulated upon infection (Endt et al., 2007). This upregulation represents one of the signals by which CD8+ T cells and NK cells detect, and respond to by killing the target. MICA is recognized by the activating cell surface receptor NKG2D on NK cells and some cytolytic CD8+ T cells (Endt et al., 2007; Saito et al., 2011). This activation, which often combines signals from other activating receptors, (Lanier, 2008; Vivier et al., 2004) induces target cell death through the release of the cytotoxic granule contents, perforin and granzymes.

Lining the membrane of cytolytic granules is the lysosomal-associated membrane protein-1 (LAMP-1), otherwise referred to as CD107a (Alter et al., 2004). It has recently been demonstrated that CD107a is upregulated on the surface of CD8+ T and NK cells following stimulation together with a loss of perforin expression. CD107a is thus considered a marker of CD8+ T and NK cell activation and cytolytic function (Betts et al., 2003). For this reason the cytolytic activity of CD8+ T and NK cells in this study was assessed by measuring the percentage of CD107a positive cells by translocation to the surface of CD8+ T and NK cells, as assessed by flow cytometry.

To confirm the effect of epinephrine and salmeterol on the cytotoxic functions of CD8+ T cells and NK cells, PBMCs were treated (or not), with epinephrine (0.05 μM) or salmeterol (5-50 μM). Subsequently the cells were incubated with the K562 cell line (a highly undifferentiated human erythroleukemic cell line) and CD107a expression was determined. Similarly to T-CHO cells, K562 cell line lack the expression of MHC class I molecules and express MICA (which provides NK cells with the activatory signals they need by ligating NKG2D) (Gong et al., 2010). This model allowed us to mimic a condition of viral infection and transformation that occurs *in vivo*, which is also associated with the upregulation of MICA and the loss of the expression of MHC class I molecules. Both cell lines were tested because although K562 are readily killed by cytotoxic cells they are a human line and therefore other elements (in addition or apart from MICA) of NK and CTLs activation may be involved. By comparison, T-CHO are not human and only express human MICA through which they are readily killed. The clinical details of individuals used in this study are shown in Table 5.1.

**Table 5.1. Clinical Details of Individuals Studied for Degranulation Assay**

| Patient | Sex | Age |
|---|---|---|
| 31 | M | 24 |
| 32 | M | 38 |
| 33 | M | 55 |
| 34 | M | 48 |
| 35 | F | 25 |

**All individuals had a Caucasian background and were not under any medical treatment.** Individuals 31-35 were used for degranulation assay.

## 5.2 RESULTS

### 5.2.1 The Effect of β2 Agonists on the Cytolytic Potential of CD8+ T and NK Cells Following Exposure of PBMCs with MICA*009 Transfected Chinese Hamster Ovary Cells (T-CHO) for Six Hours

We investigated the effect of epinephrine (0.05 μM) and salmeterol (5-50 μM) on the cytolytic potential of CD8+ T and NK cells following exposure of PBMCs with T-CHO, by measuring CD107a upregulation, following flow cytometry analysis.

PBMCs were incubated with T-CHO cells at an effector to target ratio of 1:1. CD107-a-PeCy5 antibody was added directly to the cells at 5 μl of antibody/0.1ml of cell suspension**.** The cells were subsequently incubated for six hours at 37°C in 5 % $CO_2$ based on standard killing assays (Alter et al., 2004; Betts et al., 2003). Incubation of PBMCs with CHO served as a negative control. Following the above procedures, PBMCs were stained for surface CD8+ T and NK markers (see chapter 2, materials and methods) and cells were analysed by flow cytometry to assess the CD107a upregulation as a marker of degranulation of CD8+ T cells. The results represent the mean ±SEM data and positive p values from the paired sample t-test are highlighted. Our data show that surface expression of CD107a was low (~1%) on CD8+ T cells from unstimulated PBMCs (Fig. 5.1. A) and increased (2.3 fold) following incubation with CHO cells that served as a negative control. Stimulation of PBMCs with T-CHO did not increase the expression of CD107a on the surface of CD8+ T cells compared to negative control condition following stimulation of PBMCs with CHO cells (0.96 fold) (Fig. 5.1. A). Following incubation of PBMCs with CHO cells, Epinephrine (0.05 μM) decreased CD107a CD8+ TLs surface expression by 1.7 fold (Fig. 5.1. A-Fig. 5.2. A-C). However, neither epinephrine (0.05 μM) or salmeterol (5 μM) had an effect on the CD8+ T cells CD107a surface expression following stimulation of PBMCs with T-CHO (Fig. 5.1. A), although higher concentrations of salmeterol (50 μM) showed a significant 1.45 fold increase

(p=0.030 using paired sample t-test; p=0.022 using Wilcoxon signed rank test) following 6 hours incubation. Taken together these data indicate that at low concentration, salmeterol had no effect on the surface expression of CD107a on CD8+ T cells but it significantly increased the expression of CD107 on the surface of CD8+ T cells at 50 µM concentration.

Similarly to the CD8+ T cells, NK cells from freshly isolated PBMCs in this experiment, possessed a low expression of CD107a (~3.5%) that is moderately increased (1.4 fold) upon NK cell activation after incubation of PBMCs with T-CHO cells (Fig. 5.1. B). Incubation of PBMCs with CHO cells increased the NK cells CD107a surface expression by 2 fold compared to that observed in freshly isolated PBMCs, however there was no increase on NK cells CD107a surface expression following stimulation of PBMCs with T-CHO cells compared to stimulation with CHO cells. As observed in the case of CD8+ T cells, epinephrine decreased the NK cells surface expression of CD107a (by 1.4 fold), following incubation with CHO cells, however neither epinephrine nor salmeterol had any effect on the NK cell surface expression of CD107a upon stimulation of PBMCs with T-CHO cells.

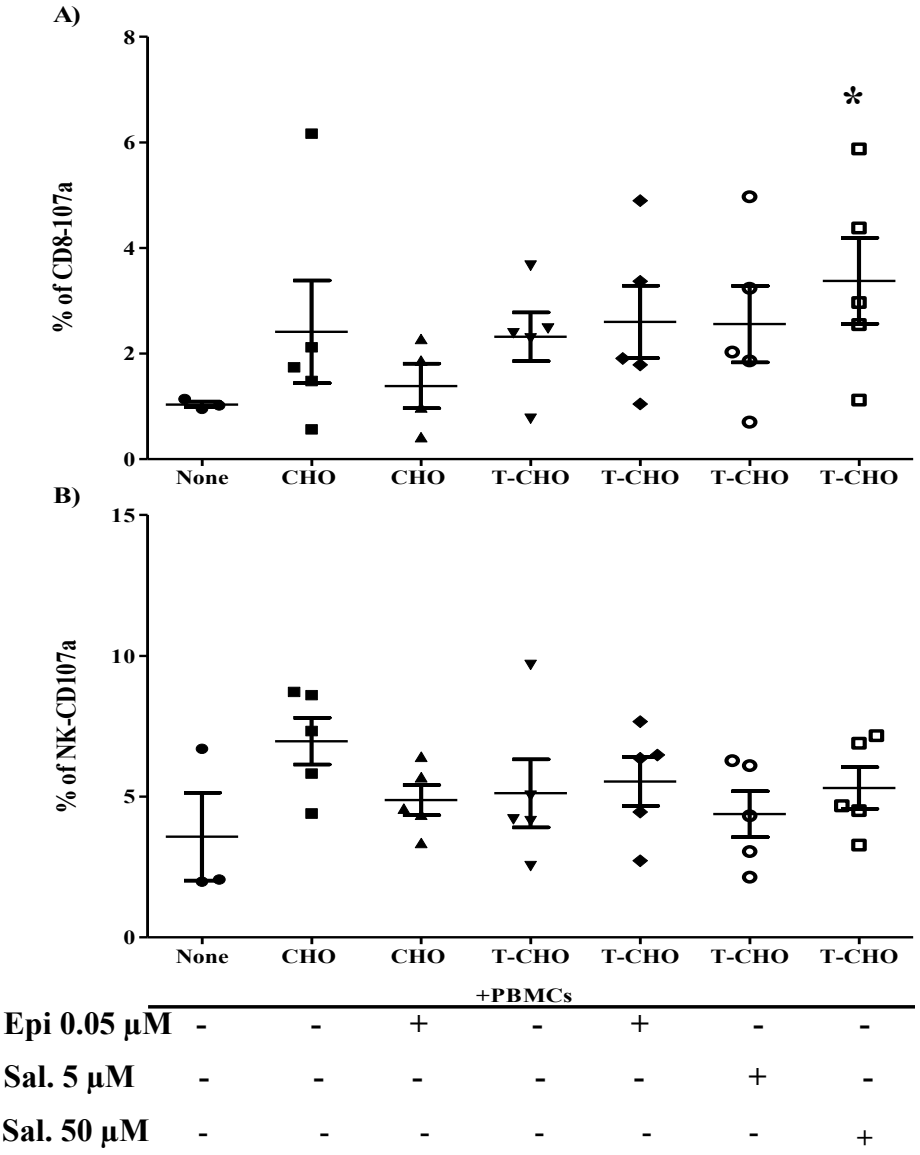



**Figure 5.1. The effect of $\beta_2$ agonists on the CD107a surface upregulation on CD8+ T (A) and NK cells (B) as a functional marker of CD8+ T and NK cell cytotoxicity.** PBMCs were isolated from fresh blood and incubated with epinephrine (0.05 µM) or salmeterol (5-50 µM) for 30 minutes at 37°C in 5 % $CO_2$. Following incubation, PBMCs were washed, and mixed with CHO (served as negative control) or MICA*009 transfected CHO (T-CHO) cells at an effector to target ratio of 1:1. To measure the percentage of CD107a upregulation on the surface of CD8+ T and NK cells, following mixing, cells were immediately labelled with CD107-a-PeCy5 antibody and incubated for 5 hours at 37°C in 5 % $CO_2$. Subsequently, PBMCs were stained for surface CD8+ T and NK markers and cells were analysed by flow cytometry to assess the percentage of CD107a upregulation. The data are the mean ± SEM from five independent experiments. Paired sample t test and/or Wilcoxon signed ranked test showed a significant effect of salmeterol (50 µM) on the cytotoxicity of the CD8+ T cells; *$p<0.05$ compared with positive control (PBMCs+T-CHO). Flow cytometry data of a representative sample (n=1) is shown in Fig. 5.2-5.5.

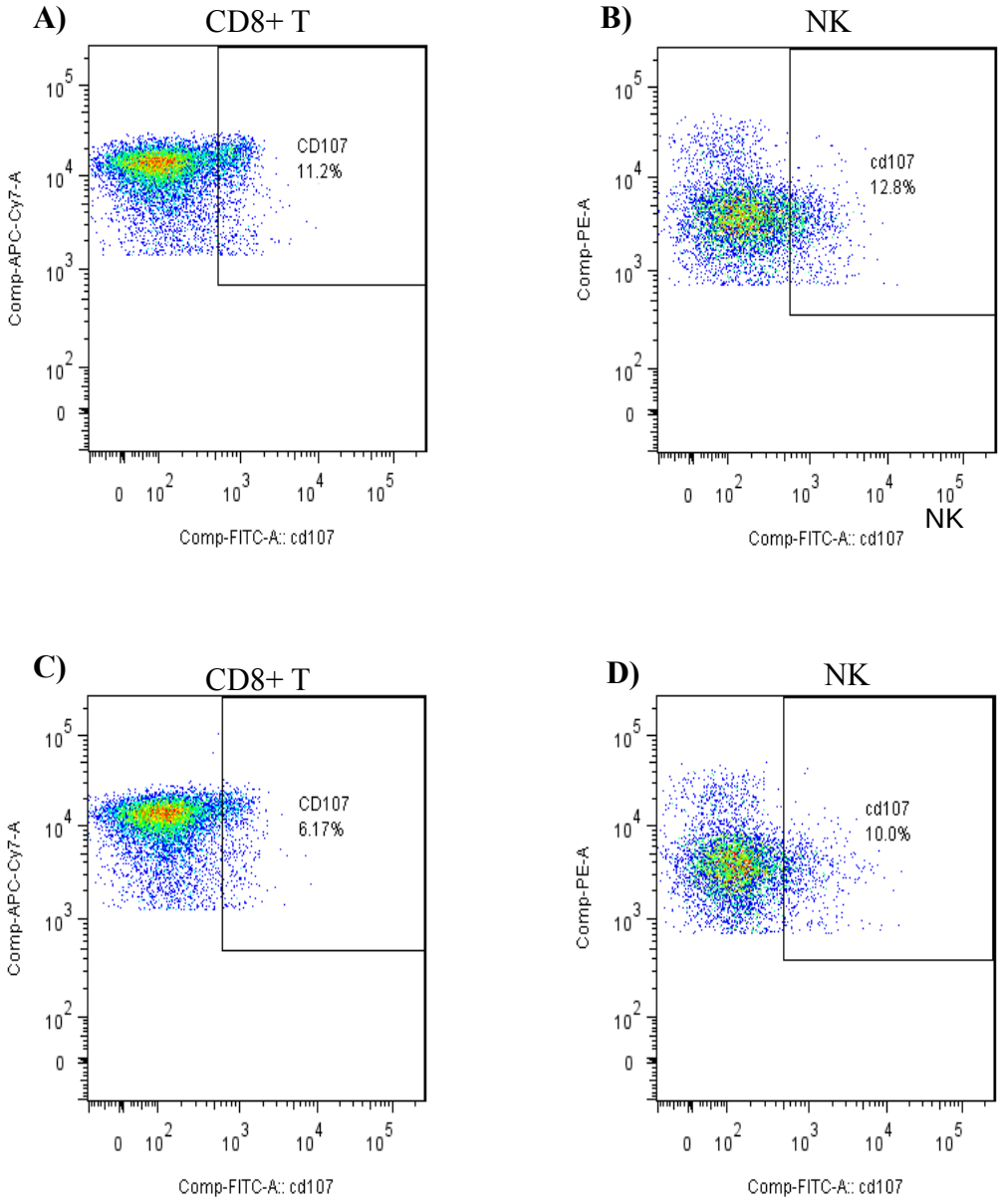



**Figure 5.2. (A-D). Effect of epinephrine (0.05 µM) on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without epinephrine and subsequent incubation with CHO cells for 6 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in CHO stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with epinephrine and incubated with CHO cells. N=1.

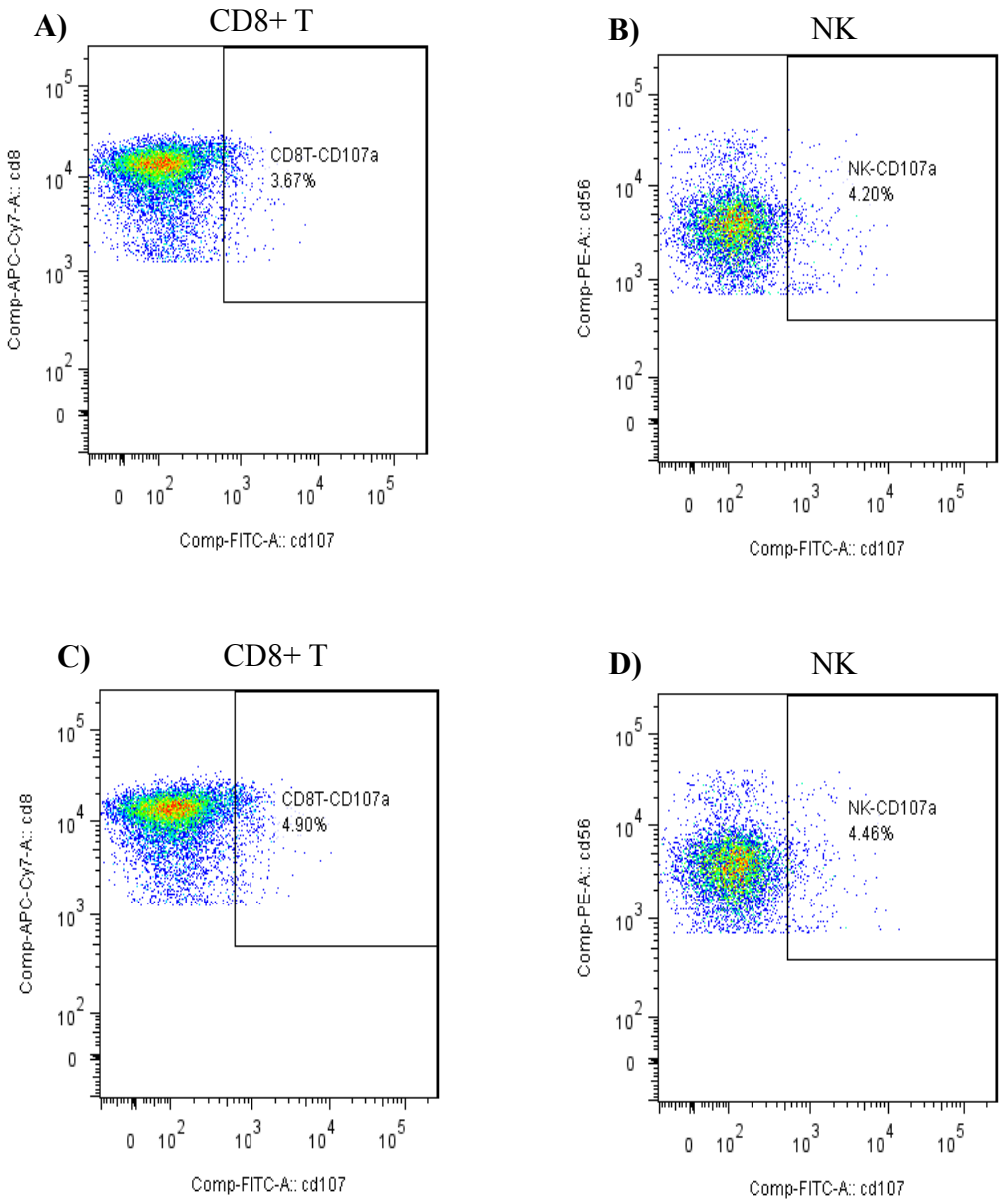



**Figure 5.3. (A-D). Effect of epinephrine (0.05 µM) on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without epinephrine and subsequent stimulation with T-CHO cells for 6 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in T-CHO stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with epinephrine and stimulated with T- CHO. N=1.

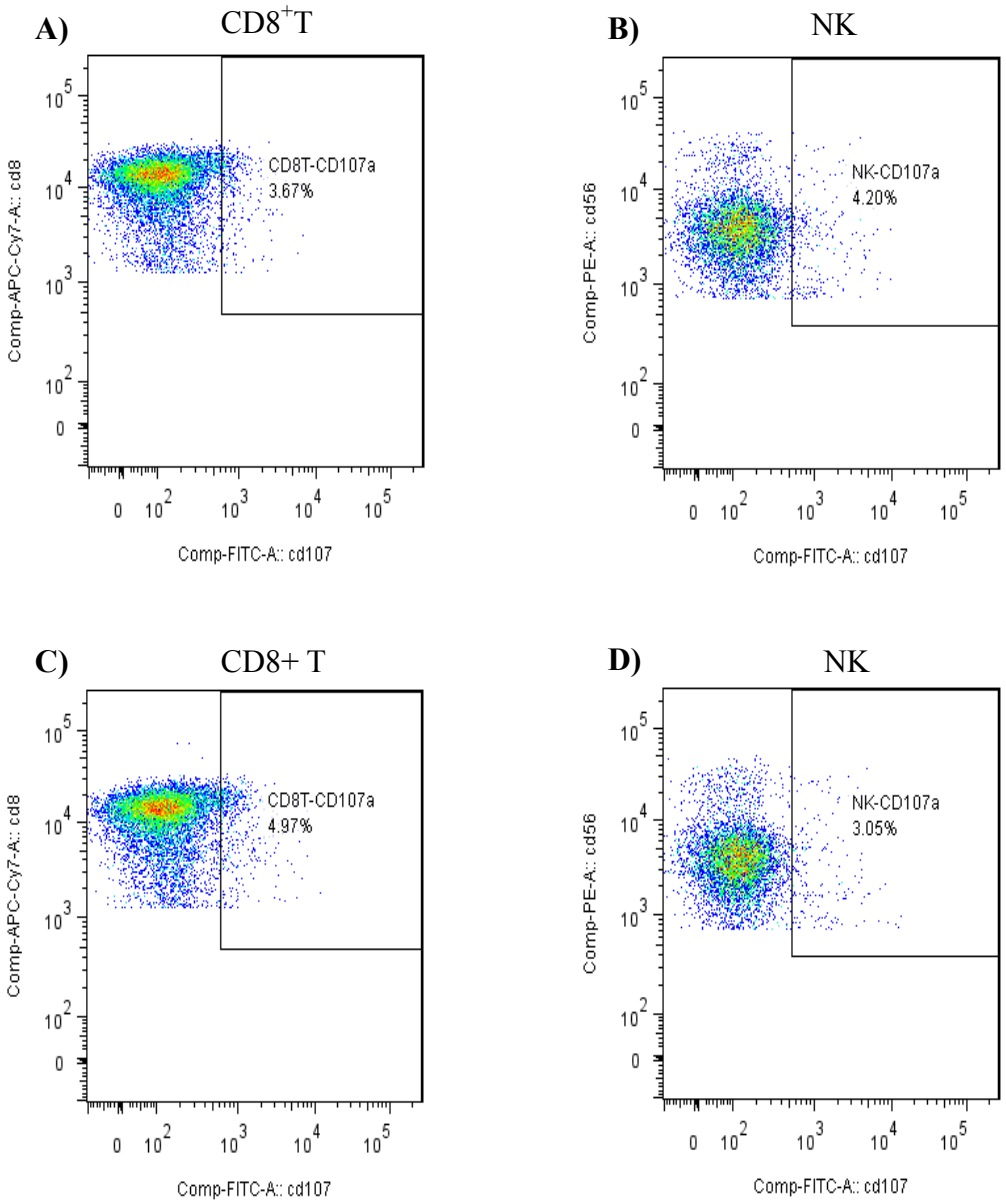



**Figure 5.4. (A-D). Effect of salmeterol (5 µM) on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without salmeterol (5 µM) and subsequent stimulation with T-CHO cells for 6 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in T-CHO stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with salmeterol (5 µM) and stimulated with T-CHO. N=1.

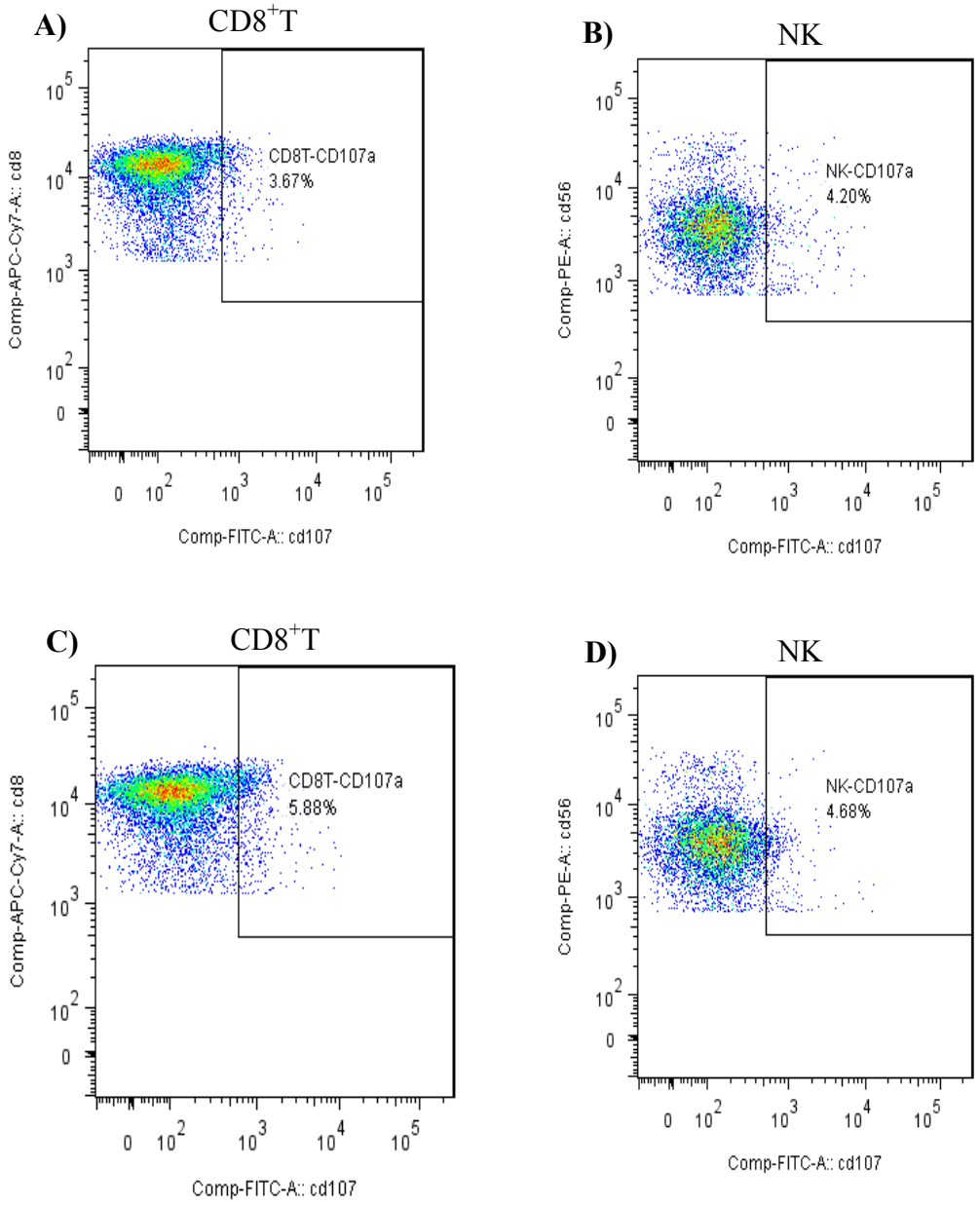



**Figure 5.5. (A-D). Effect of salmeterol (50 µM) on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without salmeterol (50 µM) and subsequent stimulation with T-CHO cells for 6 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in T-CHO stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with salmeterol (50 µM) and stimulated with T-CHO. N=1.

### 5.2.2 The Effect of β2 Agonists on the Cytolytic Potential of CD8+ T and NK cells Following Exposure of PBMCs with T-CHO for Three Hours

Since stimulation of PBMCs with T-CHO cells did not upregulate the expression of CD107a on the surface of CD8+ T and NK cells, there was the possibility that we had missed the time point at which the CD107a was optimally expressed. For this reason, in the next experiments, PBMCs were stimulated with T-CHO for a shorter period (3 hours). We again investigated the effect of epinephrine (0.05 µM) and salmeterol (5-50 µM) on the cytolytic potential of CD8+ T, and NK cells following stimulation of PBMCs with T-CHO.

Surprisingly, our results indicated that expression of CD107a on the surface of CD8+ T cells was high (~11%) in unstimulated PBMCs alone (Fig. 5.6. A) and comparable (~10%) following incubation of PBMCs with T-CHO cells, which served as a positive control. Incubation of PBMCs with T-CHO increased CD8+ T cells surface expression of CD107a (1.16 fold) comparable to that of PBMCs incubation with CHO cells (Fig. 5.6. A). Both epinephrine (0.05 µM) and salmeterol at concentration of 5 µM caused a significant decrease ((1.46 fold; p=0.010 using paired sample t-test; p=0.014 using Wilcoxon signed rank test) and (1.53 fold; p=0.049 using paired sample t-test; p=0.028 using Wilcoxon signed rank test) respectively)), in the CD107a expression on CD8+ T cells incubated with T-CHO, thereby suggesting that epinephrine and salmeterol decrease the CD8+ T cell cytotoxicity (Fig. 5.6. A). Furthermore, at 50 µM salmeterol also decreased the expression of CD107a on CD8+ T cells incubated with T-CHO (by 1.3 fold), although this change was not significant.

Fig. 5.6. B shows that, similarly to the CD8+ T cells, the CD107a expression on the surface of NK cells in unstimulated freshly isolated PBMCs (represented by None in the graph) was high (~13%) and incubation of PBMCs with CHO cells did not upregulate the CD107a expression. However, there was 1.4 fold increase on the CD107a NK cells surface expression following stimulation of PBMCs with T-CHO (Fig. 5.6. B).

Fig. 5.6. B shows that the effect of salmeterol and epinephrine on the CD107a NK cell surface expression was comparable to that observed in CD8+ T cells. Both epinephrine (0.05 uM) and salmeterol at concentrations of 5 µM and 50 µM reduced the CD107a NK cell surface expression by an average of 2 fold (Fig. 5.6. B**)**. Wilcoxon signed rank test only, showed that epinephrine (0.05 uM) and salmeterol (50 µM) significantly reduced the CD107a NK cell surface expression as in both cases p=0.014.

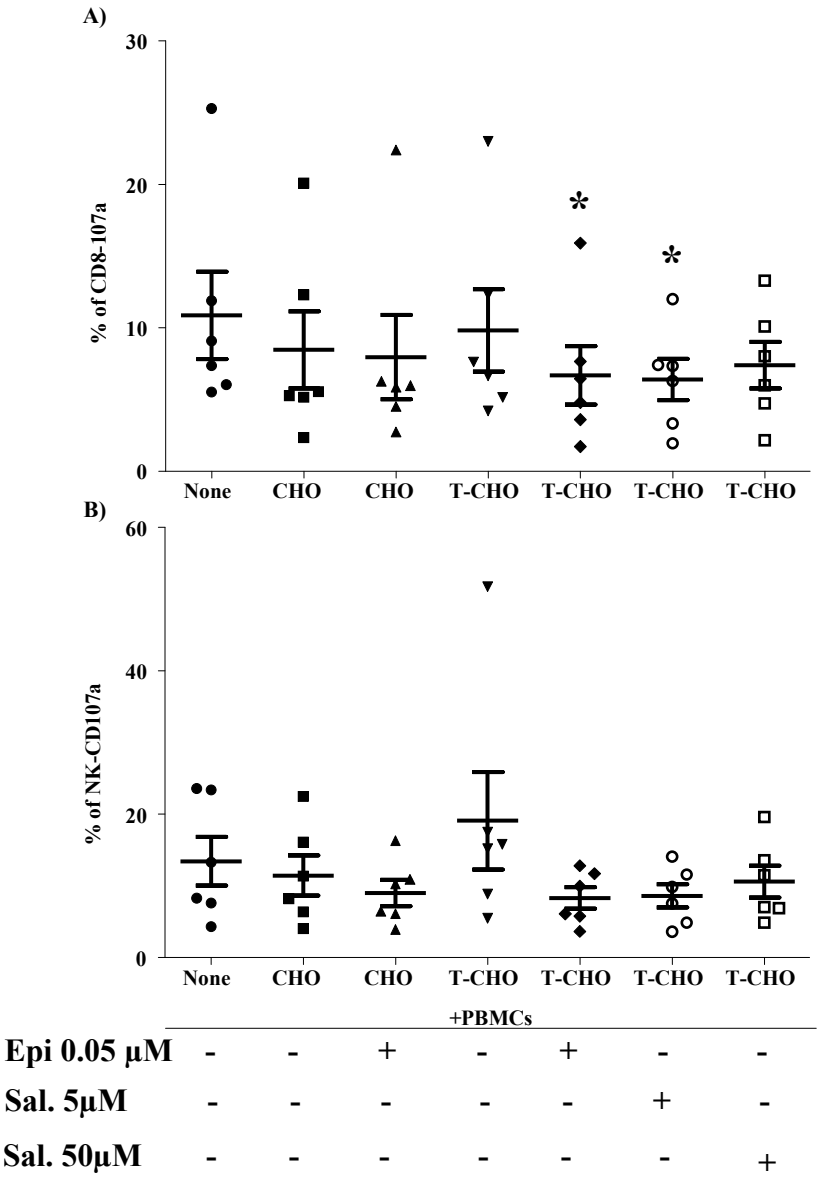



**Figure 5.6.The effect of $\beta_2$ agonists on the CD107a surface upregulation on CD8+ T (A) and NK cells (B) as a functional marker of CD8+ T and NK cell cytotoxicity.** PBMCs were isolated from fresh blood and incubated with epinephrine (0.05 µM) or salmeterol (5-50 µM) for 30 minutes at 37°C in 5 % $CO_2$. Following incubation, PBMCs were washed, and mixed with CHO (served as negative control) or T-CHO cells at an effector to target ratio of 1:1. To measure the percentage of CD107a upregulation on the surface of CD8+ T and NK cells, following mixing, cells were immediately labelled with CD107-a-PeCy5 antibody and incubated for 3 hours at 37°C in 5 % $CO_2$. Subsequently, PBMCs were stained for surface CD8+ T and NK markers and cells were analysed by flow cytometry to assess the percentage of CD107a upregulation. Horizontal lines represent mean ± SEM from five independent experiments. Paired sample t test and/or Wilcoxon signed rank test showed a significant effect of epinephrine (0.05 µM) and salmeterol (5 µM) on the cytotoxicity of the CD8+T cells; *$p<0.05$ compared with positive (PBMCs+T-CHO) control. Flow cytometry data of a representative sample (n=1) is shown in **Fig. 5.7-5.10**.

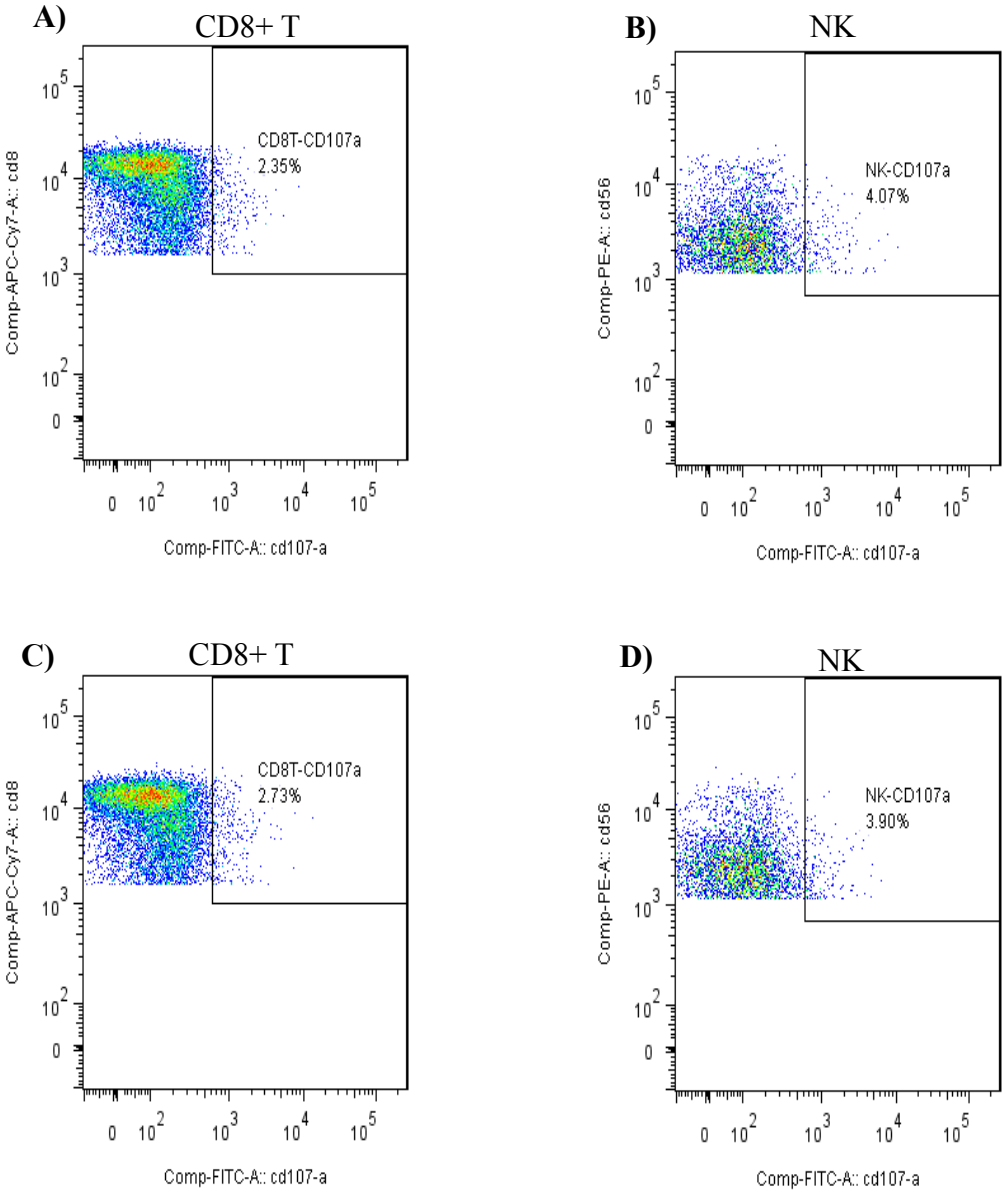



**Figure 5.7. (A-D). Effect of epinephrine on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without epinephrine (0.05 µM) and subsequent incubation with CHO cells for 3 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in CHO stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with epinephrine and incubation with CHO. N=1.

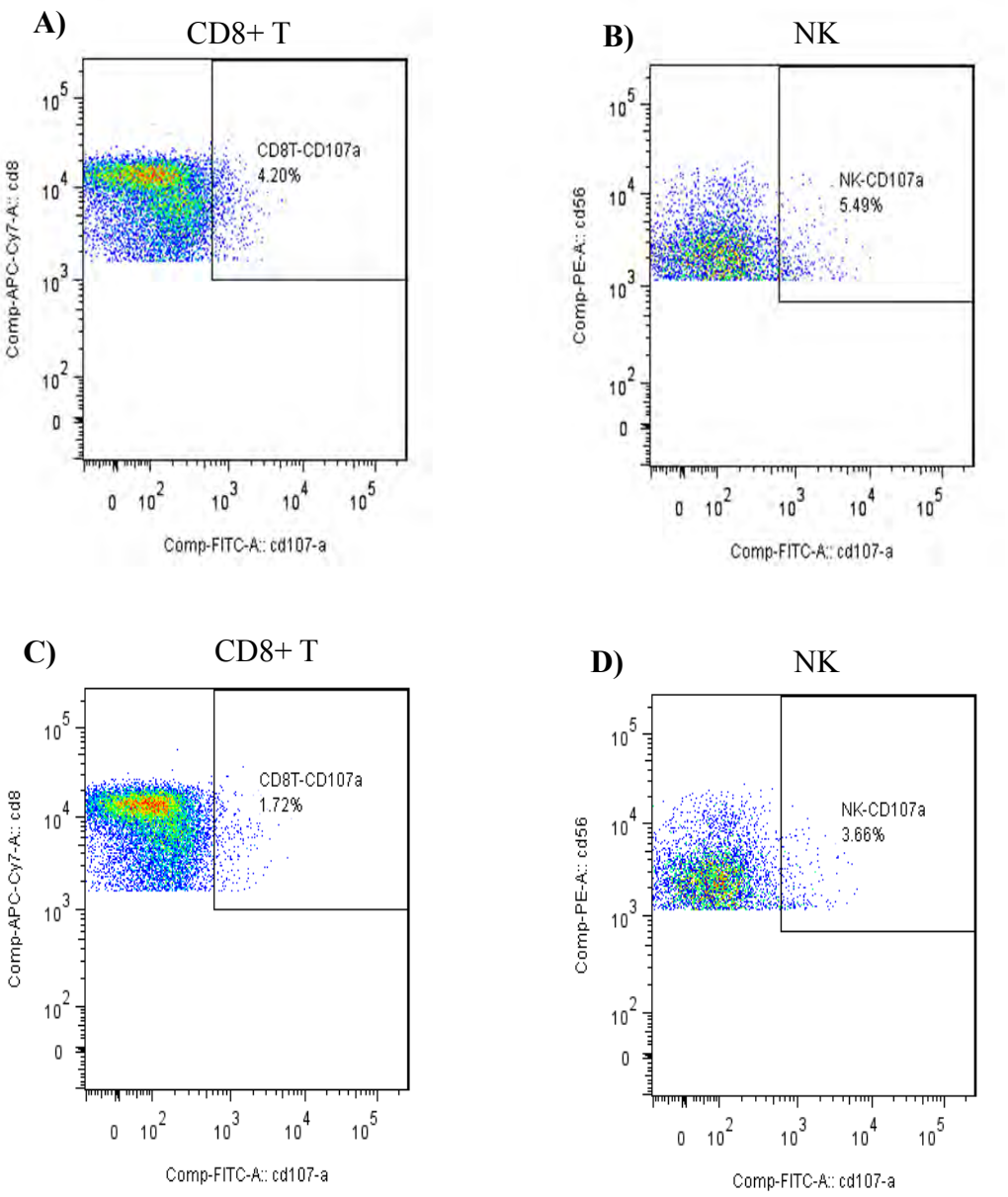



**Figure 5.8. (A-D). Effect of epinephrine on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without epinephrine (0.05 µM) and subsequent stimulation with T-CHO cells for 3 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in T-CHO stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with epinephrine and stimulated with T-CHO. N=1.

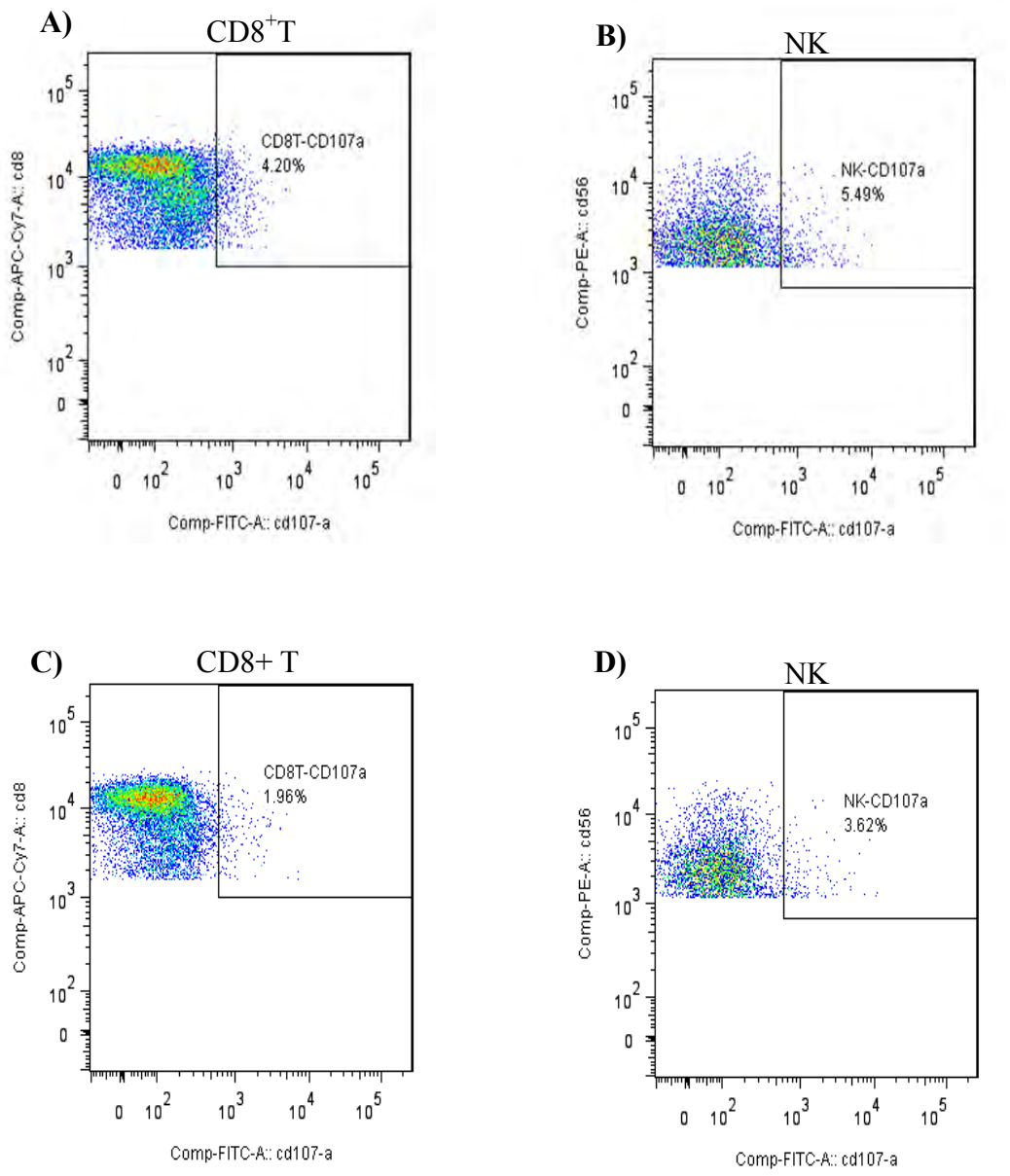



**Figure 5.9. (A-D). Effect of salmeterol on CD107a expression on surface of CD8+ and NK cells following treatment of PBMCs with or without salmeterol (5 µM) and subsequent stimulation with T-CHO cells for 3 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in T-CHO stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with salmeterol and stimulated with T-CHO. N=1.

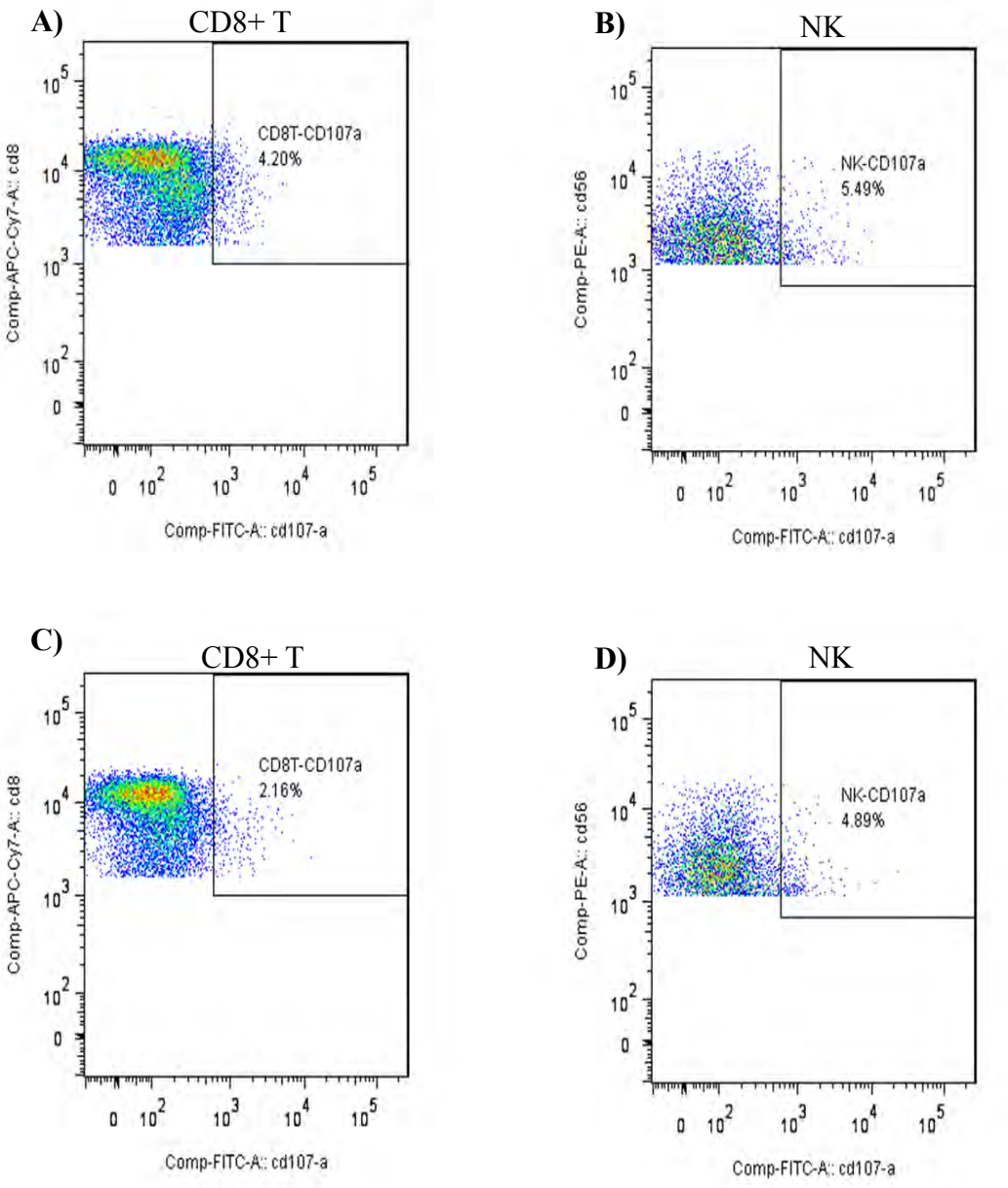



**Figure 5.10. (A-D). Effect of salmeterol on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without salmeterol (50 µM) and subsequent stimulation with T-CHO cells for 3 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in T-CHO stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with salmeterol and stimulated with T-CHO. N=1.

### 5.2.3 The Effect of β2 Agonists on the Cytolytic Potential of CD8+ T and NK Cells Following Exposure of PBMCs with Human Erythroleukemic Cell Line (K562) for Three Hours

To confirm the effect of epinephrine (0.05 µM) and salmeterol (5-50 µM) on the cytolytic potential of CD8+ T and NK cells, PBMCs were stimulated for three hours with K562 cells that lacked the expression of MHC class I. Similarly to the experiments with T-CHO cells, PBMCs were stimulated with K652 cells at an effector to target ratio of 1:1, and following staining with CD107a antibody, PBMCs + K562 were incubated for three hours at 37°C in 5 % $CO_2$. Incubation of PBMCs with medium alone, served as a negative control. Following incubation, PBMCs were stained for surface CD8+ T and NK markers and cells were analysed by flow cytometry to assess the percentage of CD107a upregulation as a marker of activated CD8+ T and NK cells.

The data show that expression of CD107a on the CD8+ T cell surface was low (~4%) in unstimulated PBMCs (Fig. 5.11. A) and this did not increase following stimulation of PBMCs with K562 cells. Furthermore, in contrast to the reducing effect of salmeterol on the CD107a CD8+ T cells surface expression following stimulation of PBMCs with T-CHO cells for 3 hours, neither salmeterol nor epinephrine had any effect on the CD8+ T cells surface expression of CD107a upon stimulation with K562 cells (Fig. 5.11. A).

As shown in Fig. 5.11. B, CD107a expression on the surface of NK cells in unstimulated freshly isolated PBMCs was 7.5% (Fig. 5.11. B). In contrast to CD8+ T cells CD107a expression, the expression of CD107a on the surface of NK cells increased significantly ($p=0.024$ using paired sample t-test; $p=0.037$ using Wilcoxon signed rank test) by 1.6 fold following stimulation of PBMCs with K562 cells. Both epinephrine (0.05 µM) and salmeterol

(5 µM and 50 µM) decreased the surface expression of CD107a on NK cells by an average of 1.4 fold (Fig. 5.11. B). This decrease was significant at 50 uM concentration of salmeterol (p=0.030 using paired sample t-test; p=0.037 using Wilcoxon signed rank test).

Overall, the data suggest that catecholamines did not affect the CD8+ T cell cytotoxicity, as observed following stimulation of PBMCs with K562 cells for 3 hours. However they do decrease the cytotoxicity of NK cells.

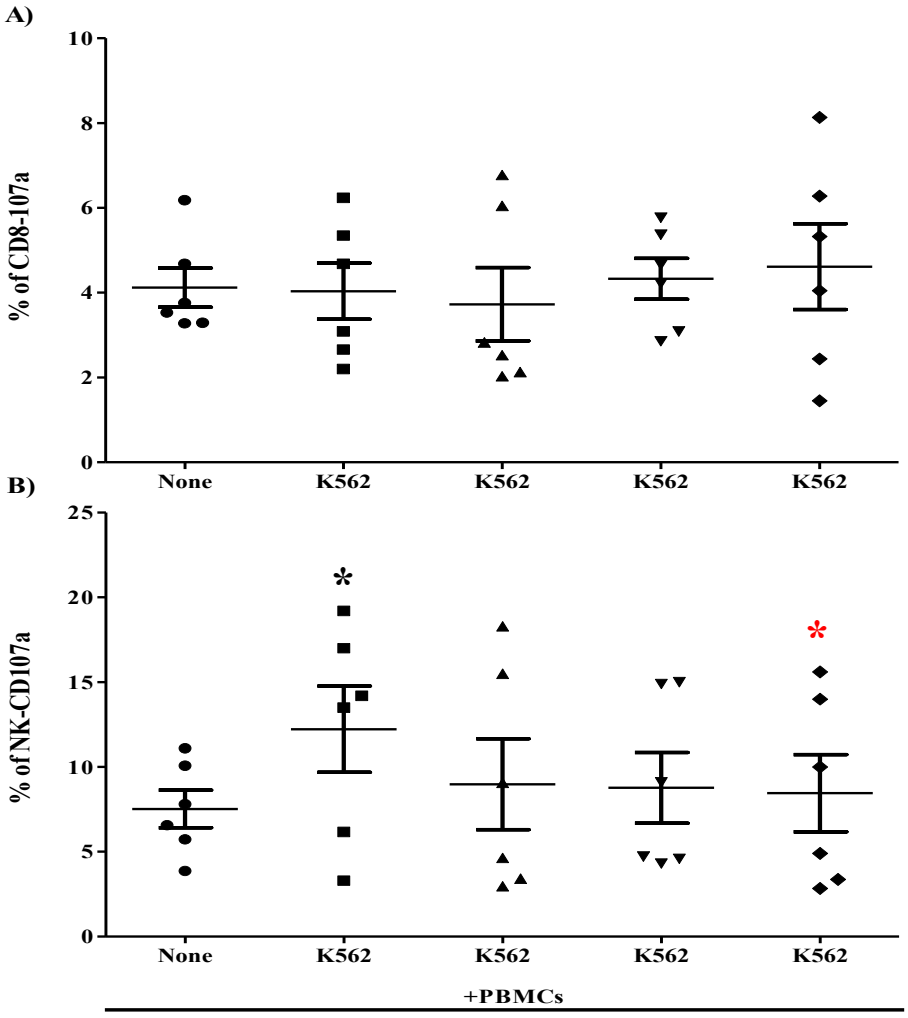



**Figure 5.11.The effect of $\beta_2$ agonists on the CD107a surface upregulation on CD8+ T (A) and NK cells (B) as a functional marker of CD8+ T and NK cell cytotoxicity.** PBMCs were isolated from fresh blood and incubated with epinephrine (0.05 µM) or salmeterol (5-50 µM) for 30 minutes at 37°C in 5 % $CO_2$. Following incubation, PBMCs were washed, and mixed with K562 cells at an effector to target ratio of 1:1. To measure the percentage of CD107a upregulation on the surface of CD8+ T and NK cells, following mixing, cells were immediately labelled with CD107-a-PeCy5 antibody and incubated for 3 hours at 37°C in 5 % $CO_2$. Subsequently, PBMCs were stained for surface CD8+ T and NK markers and cells were analysed by flow cytometry to assess the percentage of CD107a upregulation. Data are mean ± SEM from six independent experiments. *$p<0.05$ by paired sample t-test and/or Wilcoxon signed rank test compared to untreated (None) control. *$p<0.05$ by paired sample t-test compared to positive (PBMCs+ K562) control. Flow cytometry data representative of 1 example (n=1) is shown in **Fig. 5.12-5.14.**

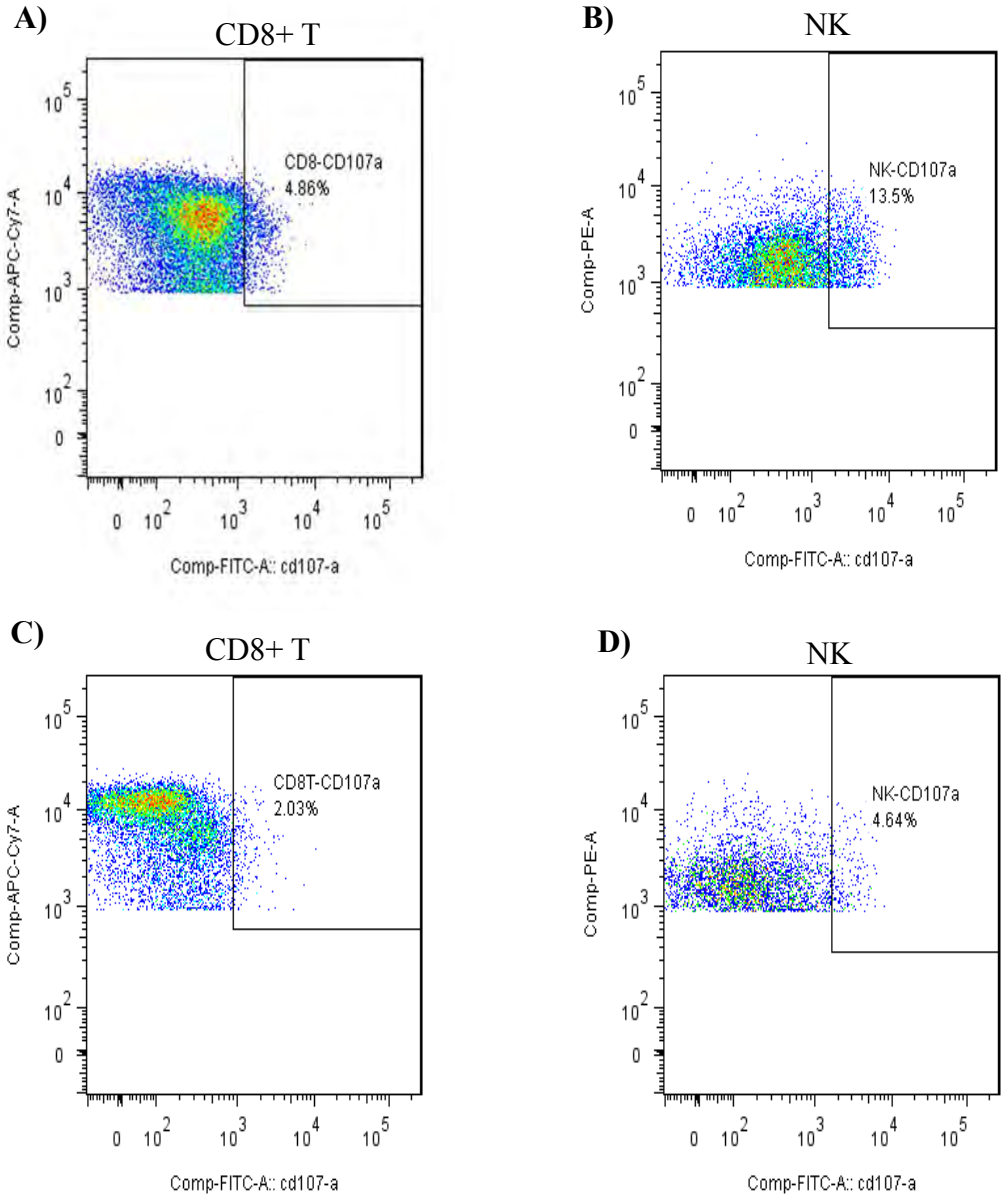



**Figure 5.12. (A-D). Effect of epinephrine (0.05 µM) on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without epinephrine (0.05 uM) and subsequent stimulation with K562 cells for 3 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in K562 stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with epinephrine and stimulated with K562 cells. (N=1).

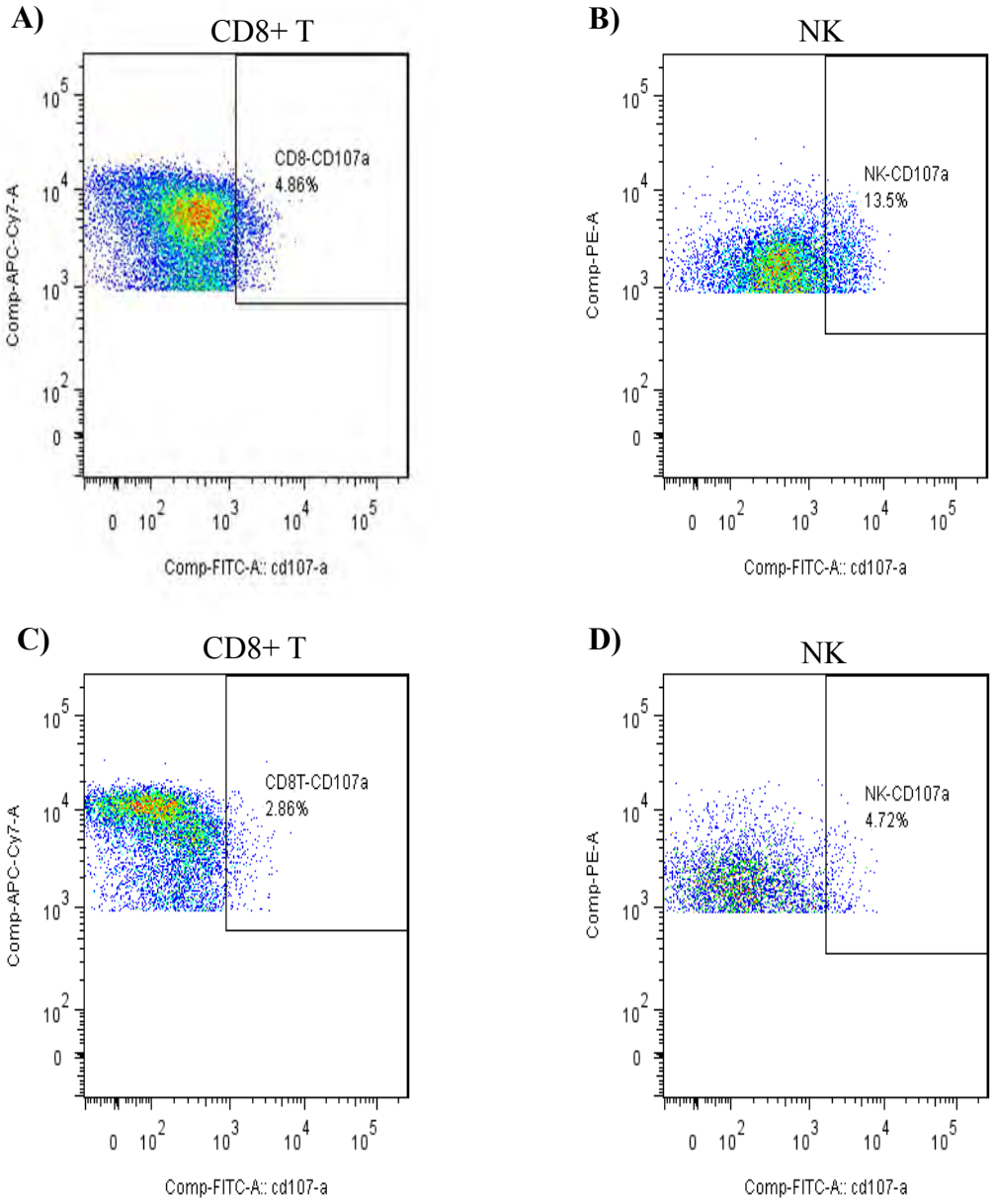



**Figure 5.13 (A-D). Effect of salmeterol on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without salmeterol (5 µM) and subsequent stimulation with K562 cells for 3 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in K562 stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with salmeterol (5 µM) and stimulated with K562 cells. (N=1).

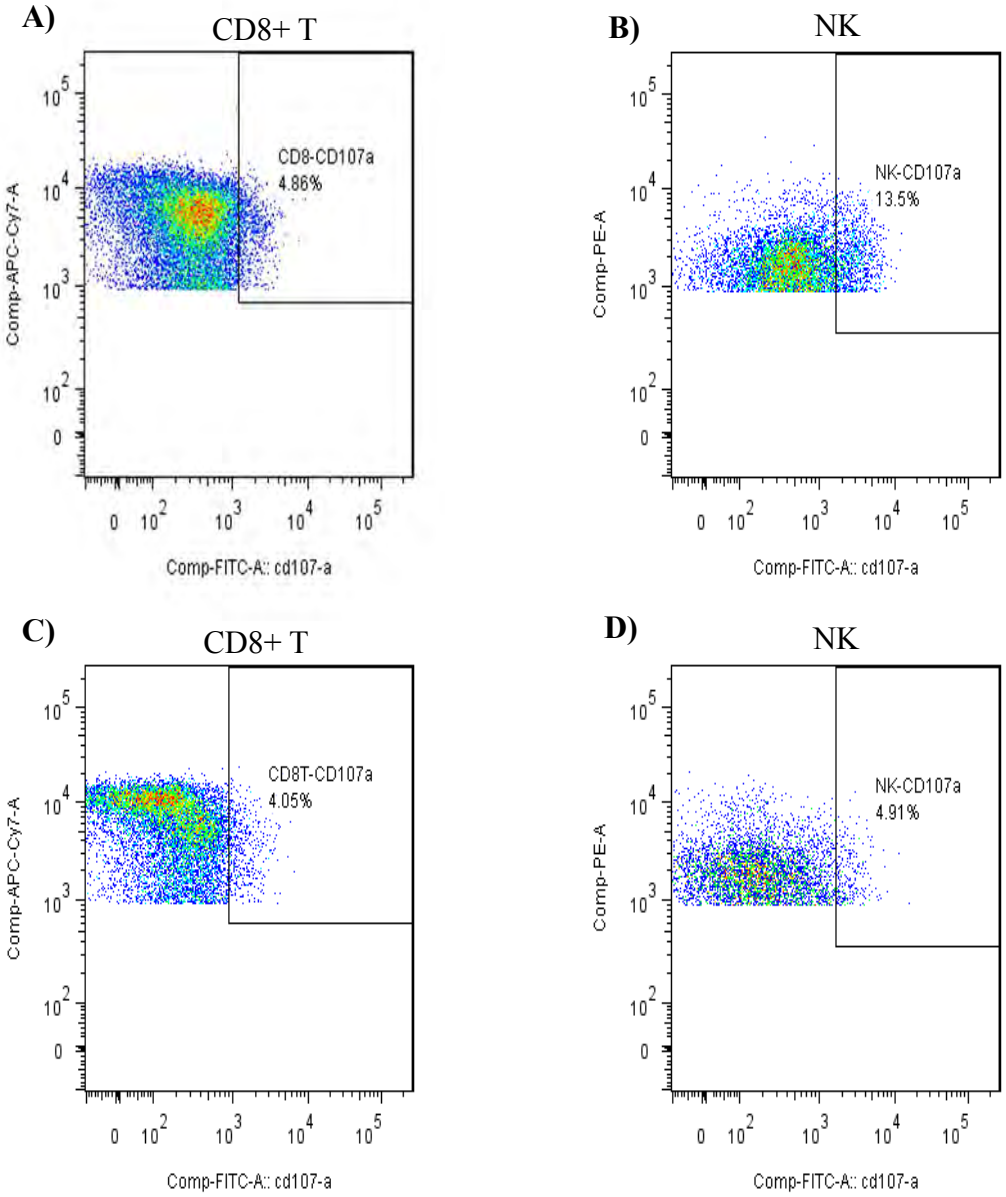



**Figure 5.14 (A-D). Effect of salmeterol on CD107a expression on surface of CD8+ T and NK cells following treatment of PBMCs with or without salmeterol (50 µM) and subsequent stimulation with K562 cells for 3 hours.** The CD107a-CD8+ T and CD107a-NK cell surface expression were gated within the CD8+ T and NK population respectively. The percentage of CD107a-CD8+ T (A) and CD107a-NK cells (B) in K562 stimulated PBMCs were compared to the percentage of CD107a-CD8+ T (C) and CD107a-NK cells (D) in PBMCs treated with salmeterol and stimulated with K562 cells. (N=1).

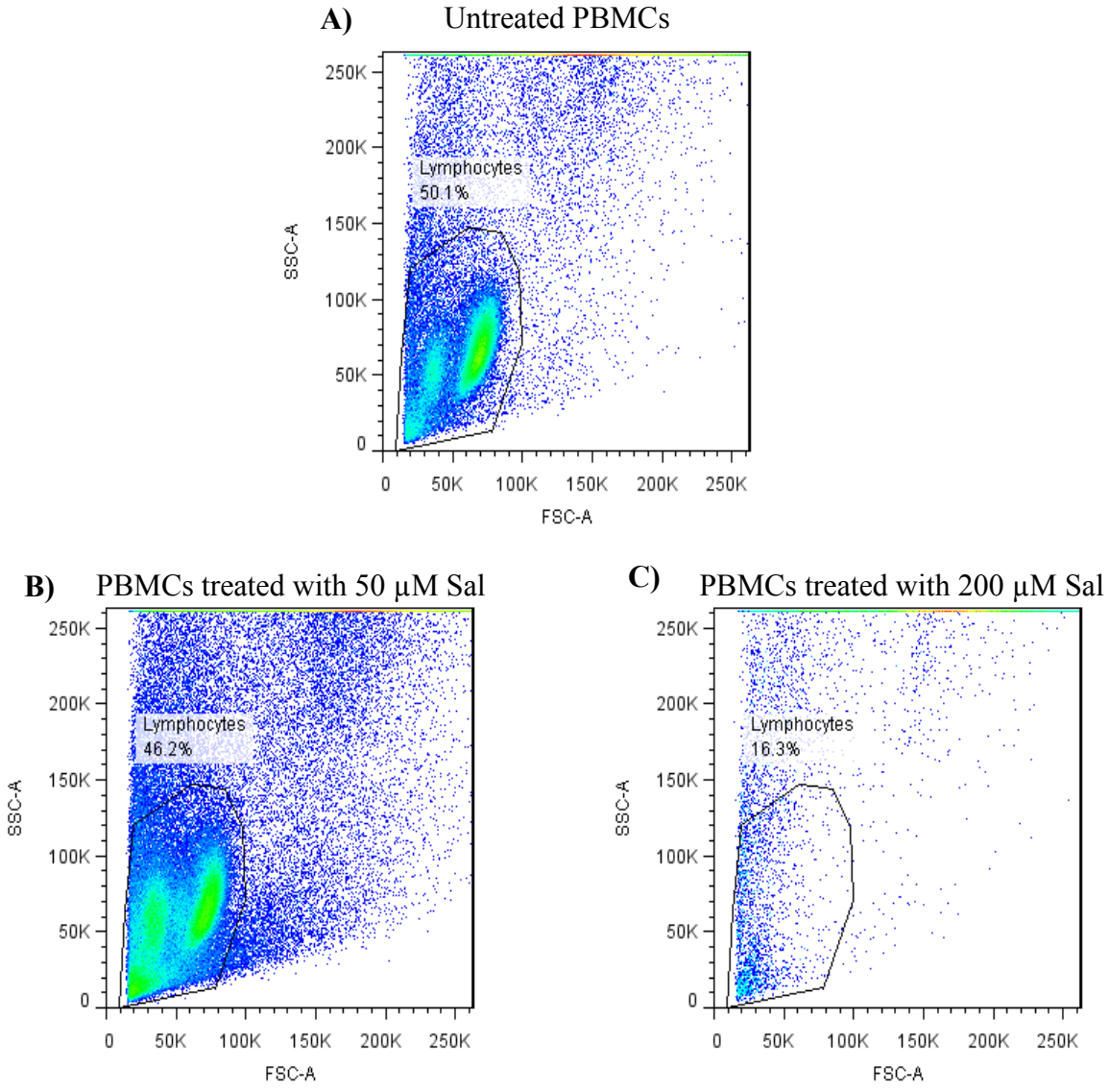



**Figure 5.15 (A-C). 50 µM concentration of salmeterol is not toxic to population of lymphocytes.** To confirm that 50 µM salmeterol was not toxic to PBMCs, PBMCs were treated with either no salmeterol (A); with 50 µM salmeterol (B) or with 200 uM salmeterol (C). The percentage of lymphocytes in untreated PBMCs (A) were compared with the percentage of lymphocytes in the 50 µM salmeterol treated PBMCs (B) and 200 µM salmeterol treated PBMCs (C).

## 5.3 Discussion

CD8+ T cells and NK cells are cytotoxic lymphocytes potentially important for the immunosurveillance against cancer and virus infections. Both express a high number of βAR, particularly of epinephrine-sensitive $\beta_2$ subtype (Anane et al., 2009; Anane et al., 2010; Benschop et al., 1996b; Campbell et al., 2009; Hellstrand et al., 1985) and for this reason, they are "stress sensitive" (Gan et al., 2002). The data presented in this chapter support the concept that catecholamines inhibit killing of target cells by NK cells and to a lesser extent by CD8+ T cells.

Previous studies have demonstrated that activities of the central nervous system can have an impact on both T cells and NK cells function (Elenkov et al., 2000; Madden et al., 1995; Padgett and Glaser, 2003; Yang and Glaser, 2000). However, the precise effect of various psychological or physical stresses on the cytotoxicity of CD8+ T and NK cells remains controversial. Some studies link stress with an increased NK cell activity and others report inhibition of CD8+ T and NK cell cytotoxicity. In previous studies, CD8+ T and NK cell cytotoxicity was measured through the standard chromium release and flow-based killing assays (Betts et al., 2003; Brunner et al., 1968; Liu et al., 2002; Sheehy et al., 2001; Slezak and Horan, 1989). For example, Brunner et al. (1968) incubated the pre-labelled radioactive chromium ($^{51}$CR) allogeneic target cells (tumour allograft) with sensitized lymphoid cells (from spleen cells of mice) and measured the target cell lysis by lymphoid cells by release of radioactive chromium ($^{51}$CR) from pre-labelled target cells. More specifically, the cytotoxic effect of the sensitized lymphoid cells, was expressed by the difference in chromium release in the presence of the immune lymphoid cells (experimental release) and chromium release in the presence of normal lymphoid cells (spontaneous chromium release from labelled target cells alone) (Brunner et al., 1968; Chahroudi et al., 2003). Thus the release of chromium from

direct target cells represents a direct quantitative measurement of target cell lysis and the lytic activity of the effector cells in the lymphocytes population. However, the Cr release assay has a number of disadvantages. For example, the high non-specific spontaneous release of chromium might influence the effector cell population. Moreover, there are problems with labelling certain cell types, the low sensitivity of the assay itself, as well as health risks problems associated with gamma irradiation (Chahroudi et al., 2003; Sheehy et al., 2001). For these reasons investigators began to research other methods that could eliminate problems associated with chromium assay release (Sheehy et al., 2001; Slezak and Horan, 1989).

One of the recently used methods to determine the targeted cell lysis is the fluorometric assessment of T lymphocyte antigen specific lysis assay (FATAL) (Sheehy et al., 2001). This is a non-radioactive assay which employs two fluorescent markers to recognise and evaluate the target population. The fluorescent markers utilised are: PKH-26 (a lipid associated dye) and 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE). The latter is an uncharged fluorescein derivative which diffuses through the (target) cell membrane and cleaves to produce a charged form (Sheehy et al., 2001). Following incubation of CTL effectors with the PKH-26þ labelled targets, the CFSE dye is instantaneously lost from the target cells as the membrane of the target cells is damaged (due to the lytic activity of CTLs). Thus this assay measures the target cell death by means of measuring the loss of CFSE in the PKH-26þ targets, following incubation with CTL effectors (Chahroudi et al., 2003). It is important to note that this assay is totally dependent on the measurement of target cell membrane destruction, which is a late-consequence of the apoptotic process, and thus in certain circumstances whereby the CTL-mediated apoptosis may not result in actual cell destruction until for a long period of time, even this assay can be semi-sensitive (Chahroudi et al., 2003). Furthermore, it is important to note that both of these assays do not examine the

CD8+ T and NK cells that mediate killing directly. Instead, their examination of the CD8+ T and NK effector cell function is an indirect interpretation of target cell death by means of measuring chromium release (in the chromium assay) or the CFSE release (in the FATAL assay) following incubation of target cells with these effector cells (Betts et al., 2003). For these reasons we decided to use the CD107a assay to measure target cell killing.

In this chapter we have investigated the effect of epinephrine and salmeterol ($\beta_2$ agonist), on the cytotoxic functions of both CD8+ T and NK cells *in vitro*, using a novel assay that directly measures degranulation, and thus functional capacity, of the responding CD8+ T and NK cells per se (Alter et al., 2004; Betts et al., 2003) but does not measure killing. Since CD107a has been shown a marker for the cytotoxicity of both CD8+ T and NK cells (as its expression was associated with the loss of perforin following antigen stimulation), we quantified the CD8+ T lymphocyte and NK cell activity by determining the percentage of CD107a expression on the surface of CD8+ T and NK cells.

The results show that pre-treatment of PBMCs with epinephrine or salmeterol does not appear to have a general effect on CD8+ T, or the NK cell activity, following stimulation of PBMCs with T-CHO at six hours (although pre-treatment with 50 μM salmeterol significantly increased the CD107a CD8+T cell surface following stimulation of PBMCs with T-CHO). As CD107a expression on the surface of CD8+ T and NK cells was only slightly upregulated following stimulation of untreated PBMCs with T-CHO for 6 hours comparable with unstimulated PBMCs alone, and did not upregulate in comparison with stimulation of PBMCs with CHO, this may have missed the appropriate time point. By comparison, CD107a expression on the surface of NK cells was increased following stimulation of untreated PBMCs with T-CHO for 3 hours compared with untreated PBMCs alone and PBMCs treated with CHO. This was not observed with CD8+ TLs. Thus, our data suggest that the optimal

expression of CD107a occurs following 3 hours of PBMCs' stimulation with T-CHO, and that after 3 hours the CD107a surface expression, might start to degrade from the surface of both CD8+ T and NK cells. In contrast to the findings in our study, other studies by Betts et al. (2003) and Alter et al. (2004) assessed the percentage of CD107a expression following stimulation of PBMCs with K562 cell-line for six hours. The reason for these differences is not clear. Both epinephrine and salmeterol decreased the cytolytic potential of CD8+ T and NK cells following incubation of PBMCs with T-CHO target cells for three hours. The decrease on the cytolytic potential of CD8+ T cells was significant following incubation of PBMCs with epinephrine (0.05μM) ($p=0.010$) or salmeterol (5μM) ($p=0.049$). Our findings are in agreement with Shakhar et al. (1998) who demonstrated that administration of β2 agonists *in viv*o, increased tumour cell metastasis rates through reduction of NK cell activity. In addition, Oya et al. (2000) have reported that the cytotoxicity induced by NK cells was suppressed following exposure of mice to stress, although other stress responses such as cortisol may have played a role. Direct *in vivo* administration of epinephrine in mice decreased the NK cytotoxicity in liver mononuclear cells (MNC) of mice (24 hours after injection of epinephrine) as determined following incubation of MNC against mouse lymphoma (YAC-1 cell line) (Oya et al., 2000). Furthermore, addition of epinephrine ($10^{-6}$ M) directly to lymphocytes/target cell mixtures *in vitro* has been shown to inhibit the NK cell cytotoxicity (Hellstrand et al., 1985).

In contrast to this and our data it has been demonstrated that pre-treatment of lymphocytes, with low concentrations of epinephrine ($10^{-7}$-$10^{-9}$ M) enhanced the NK cells activity via lymphocyte βAR (Hellstrand et al., 1985). Tonnesen et al. (1989) showed that excessive release of catecholamines in a case study in a patient undergoing surgical removal of phaeochromocytoma (a small vascular tumour of the adrenal medulla) induces NK cell

cytotoxicity *in vivo*. In line with this, administration of epinephrine in patients (n=30) undergoing partial laminectomy (a surgical procedure in which the posterior arch of a vertebra is removed) under general anaesthesia increased the NK cell activity (Nomoto et al., 1994). In this study, arterial blood samples were obtained from each patient after administration of premedication, after induction of anaesthesia, before administration of epinephrine and 15, 30, 60 and 120 minutes following administration of an average dose of 2μg $kg^{-1}$ epinephrine (comparable with the epinephrine concentration found during major surgery). After the taking of blood samples, the NK cell activity was measured in a chromium-51 release assay following incubation of mononuclear cells from patients for use as effector cells against pre-labelled $^{51}$CR K-562 target cells (Nomoto et al., 1993; Nomoto et al., 1994). This epinephrine -induced increase in NK cell activity might be direct, as result of epinephrine directly activating the lymphoid β2AR. It is also possible that this increased NK cell activity can be explained by an increase in the number of circulating NK cells following the release of epinephrine, as opposed to the increased NK cells activity at a single NK cell level per se (Nomoto et al., 1994). For example, Shephard and Rhind. (1994) reported an increased in NK cell numbers and total NK cell activity during an acute bout of exercise. However, since the total increase in NK cell number was bigger than the total increase in the NK cytolytic activity such result could possibly reflect a suppression of NK cell activity at a single NK cell level. Yet, the effect of exercise on NK cytolytic activity unfortunately remains conflicting; with other studies showing a decreased in NK cell activity following exercise (Peters et al., 1994; Rincon et al., 1996).

In the second part of this chapter we showed that β2 agonists did not affect the cytolytic potential of CD8+ T cells, but decreased the cytolytic potential of NK cells following stimulation of PBMCs with K562 cells for three hours. This decrease in NK cell cytotoxicity

was significant at higher concentration of salmeterol (50 uM) (p=0.030). Importantly, whereas K562 cells have proved to be very good stimulators of NK cell cytotoxicity (p=0.024), it did not stimulate the CD8+ T cell cytotoxicity compared to the negative control. Thus, the effect of epinephrine and salmeterol on the cytotoxic function of CD8+ TLs stimulated with K562 cells remains ambiguous.

If we had to consider the variation between CD8+ T and NK cells cytotoxicity responses to epinephrine and salmeterol, we could explain such results by variations in density of sensitivity of lymphocytes β2AR. More specifically, since NK cells express higher levels of $\beta_2$AR (Wahle et al., 2001), and are more sensitive to stress response compared to CD8+ TLs, the stimulation of more $\beta_2$AR by salmeterol and epinephrine can have a greater effect on the transmembrane signalling process of catecholamines. Indeed, the heterogeneity of the CD8+ TLs population may have stronger effects on particular subsets expressing higher levels of β2AR on their surface (Dimitrov et al., 2010). Our intention was to use CMV-tetramer positive EM cells to address this but the use of a much smaller population of cells would have been difficult to measure.

Another possible explanation for such results is that CD8+ TLs require antigen recognition for full activation compared to NK cells and that stimulation period of 3 hours may not be sufficient to see the response in CD8+ T cells following stimulation of PBMCs with K562 cell line. Indeed, Penack et al. (2005) showed that optimal expression of CD107a occurs between 3 and 6 hours post stimulation. Moreover, the degranulation in central memory and effector memory CD8+ T cells specific for the lymphocytic choriomeningitis virus (LCMV) obtained from mice increased up to 4 hours after stimulation with glycoprotein peptides amino acids 33-41 (gp33–41) (Wolint et al., 2004). Similar degranulation kinetics have been shown for human T cell responses whereby the maximum CD107a CD8+ T cell surface expression

occurred following 6 hours of stimulation with anti-CD3 when nearly every perforin expressing cell had degranulated (Betts et al., 2003). Recent work from Shafi et al. 2011 have also reported that interindividual variation in lymphocyte stress surveillance in response to MICA could be shown in NK cells but not for CD8+ T cells using the CD107a assay, and such findings may impact on the data presented in this thesis.

It is important to address that initially, it was our intention to extend the experiment by using a β2-antagonist in combination with salmeterol (to treat PBMCs), in order to confirm that the reductive effect of salmeterol on PBMCs was due to the stimulation of β2-ARs. Unfortunately due to time constraints of the project, this was not achieved and remains to be investigated in the future studies.

One of the issues to address in this study is that in contrast to epinephrine concentration (at nanomole (nM) levels), which is consistent with the physiological concentration of epinephrine in peripheral blood (Gan et al., 2002), the concentrations of salmeterol used in this study are in micromole levels (uM). However, since primary and secondary lymphoid tissues are innervated by adrenergic and noradrenergic nerve fibres, which are in direct (synaptic) contact with the immune competent cells, it is assumed that the concentrations around the nerve terminals or between synaptic gaps should be higher than the concentration of epinephrine in the peripheral blood (Gan et al., 2002). Importantly, such concentrations of salmeterol were not cytotoxic to immune cells as compared to untreated PBMCs (Fig. 5.15. A-C). In contrast, PBMCs treated with concentrations of salmeterol (200 μM) (too high to be used in this study) proved to be cytotoxic (Fig. 5.15. A-C). Thus, although this needs to be confirmed by future studies, it could be that the equivalent micromole level of catecholamine could be physiological, in the first site where epinephrine is released (Gan et al., 2002). Furthermore, the dilution of salmeterol with ethanol as in previous studies, does not for

certain eliminate the possibility that some effect of salmeterol could also be due to small effect of ethanol. However, the fact that the data presented in this chapter are in strong agreement with what has been observed by Seema et al., 2011, provides confidence that the effects are due to salmeterol alone and that they are real. Yet, to eliminate any discrepancies, it is important that the effect of ethanol on NK, and T cells, is investigated in future studies.

In conclusion, our data suggest that epinephrine and salmeterol inhibit the cytotoxic function of CD8+ T and NK cells, following stimulation of PBMCs with T-CHO for 3 hours. Moreover, the effect of epinephrine and salmeterol tends to be dependent upon the specific stimulus used to stimulate PBMCs and the timing of stimulation. No effect of epinephrine and salmeterol was observed in CD8+ TLs, following stimulation of PBMCs with K562 cells, although both decreased the cytotoxic function of NK cells following stimulation of PBMCs with K562 cells. Exactly how epinephrine and salmeterol inhibit the CD8+ T and NK cells cytotoxicity, is not investigated in this study. However one possibility could involve the down regulation of the NKG2D receptor by epinephrine and salmeterol. This receptor is expressed on all NK cells and on activated CD8+ T cells and has been implicated as a key trigger for some forms of NK cell mediated cytotoxicity (Billadeau et al., 2003). It has been reported that decreased NKG2D expression correlates with an impaired function of CD8+ T and NK cells (Saito et al., 2011; Wiemann et al., 2005). The inhibition of CD8+ T lymphocytes and NK cells cytotoxicity is thought to have clinical relevance for the ability of NK cells to fight infectious diseases, to survey against cancer cells and prevent tumour metastasis. Understanding the mechanisms by which CD8+ T and NK cells' cytotoxicity is inhibited by β2 agonists could help in finding treatment for cancer and other infectious diseases.

# CHAPTER 6

# GENERAL DISCUSSION

## 6.1 The Effect of β2AR Stimulation on the IFN-γ Production from CD8+ and CD4+ T Cells

The data collected in Chapter 3 extended earlier findings and provided evidence that β2-agonists inhibit the IFN-γ producing CD4+ and CD8+ T cells (Betz and Fox, 1991; Sanders et al., 1997; Snijdewint et al., 1993; Van der Pouw-Kraan et al., 1992). The effect of salmeterol on the IFN-γ producing CD4+ and CD8+ T cells was a result of salmeterol having both, a direct and an indirect effect on T cells. In the latter case salmeterol would affect T cells indirectly by firstly affecting other immune cells expressing β2AR. Indeed, it has been reported that both, epinephrine and norepinephrine *in vitro* and *in vivo* may downregulate inflammatory responses particularly by targeting monocytes/macrophages and T lymphocytes, decreasing the excessive synthesis of pro-inflammatory cytokines (i.e. TNF-α, IL-1B, IL-12) and elevating levels of anti-inflammatory cytokines (i.e. IL-10, IL-1) via a βAR dependent mediated pathway (Hasko et al., 1998a; Hasko et al., 1998b; Platzer et al., 2000; van der Poll et al., 1996; Van der Poll and Lowry, 1997). In line with these findings, immunodepression after brain injury, neurosurgery, major injury and trauma is thought to be associated with high levels of IL-10, as result of catecholamine mediated IL-10 *de novo* synthesis by monocytes *in vitro* (Woiciechowsky et al., 1998). Furthermore, myocardial infarction is linked with a temporary attenuation of the ability of monocytes to present antigen and release TNF (Platzer et al., 2000; Shibata et al., 1997). Grebe et al. (2009) suggest that norepinephrine released from the sympathetic nervous system (SNS) during infection, interacts with β2AR on dendritic cells, and alters them to mediate their costimulatory capacity. It should be noted that the modulation of immunity can also be controlled by neurotransmitters other than epinephrine and norepinephrine. One of these neurotransmitters is dopamine, which exerts its

effects by binding to dopamine receptors (DARs) found on the surface of immune cells including T cells, DCs and other immune cells (Besser et al., 2005; Pacheco et al., 2009; Sarkar et al., 2006; Watanabe et al., 2006). There are two different types of DARs. Type 1 DARs (D1 and D5) are coupled to $G_{as}$ with subsequent production of cAMP, type 2 DARs (D2, D3, D4) are normally coupled to $G_i$ and subsequent inhibition of cAMP synthesis (Pacheco et al., 2009; Sibley et al., 1993). Studies in human and murine T cells have shown that the stimulation of D1 and D5 receptors impairs the function of T cells as result of increasing levels of cAMP. Moreover, stimulation of D1 not only impairs the function of cytotoxic CD8+ T cells (Saha et al., 2001) but also diminishes the function and differentiation of regulatory T cells (Tregs) that play crucial roles in the control of immune homeostasis (Cosentino et al., 2007; Pacheco et al., 2009). Immune deficiency and dysregulation is also observed in older individuals (Gouin et al., 2008; Graham et al., 2006; Segerstrom et al., 2008). Older individuals have a high proportion of "aged" late-differentiated CD8+ T cells, which are thought to be the most stress and adrenergic sensitive. Indeed, immunosenescence is characterized by an accumulation of late differentiated CD8+ T cells, a reduced response to recall antigens, an increased sensitivity to infections, predicting thereby morbidity and mortality in old individuals (Caruso et al., 2009; Pawelec et al., 2006; Sansoni et al., 2008).

It should be pointed out that the data collected in this thesis shows a large variation in response between different donors. One possibility to explain such variations could be linked to inter-individual differences in unstimulated baseline hormone level involved in shaping individual's response. Elenkov et al. (2008) reported a link between adrenomedullary epinephrine and innate cytokine profiles in humans, demonstrating that the activity of cytokine-secreting cells toward relatively high or low pro-inflammatory cytokine profiles in

rodents and humans, is conditioned by relatively low or high baseline levels of epinephrine respectively.

Questions remain on how exactly catecholamines induce immunosuppression of the immune system. Although not directly investigated in our study, salmeterol probably reduces the proportion of the IFN-γ CD4+ and CD8+ T cells via mechanisms that involve increased levels of cAMP. Consistent with this possibility, studies by Raskovalova et al. (2007) have reported that cytokine production and cytolytic activity of human anti-melanoma specific CD4+ T and CD8+ T lymphocytes can be inhibited by Adenosine-Protein kinase A type 1 signalling. Adenosine is an endogenous adenine nucleoside (that is formed from ATP, ADP, and AMP) and mediates inhibition of the IFN-γ, IL-2, TNF-α, IL-13, macrophage inflammatory protein (MIP-1α) production via a process that leads to stimulation of cAMP production. Stimulation of cAMP leads to the activation of protein kinase A (PKA) (Lokshin et al., 2006; Raskovalova et al., 2007). More specifically, it has been reported that the suppressive effects of adenosine in human T cells are thought to be a result of the activation of PKA I regulatory subunit of the PKA (Raskovalova et al., 2007). In line with these findings, other studies have reported that the inhibitory effect of cAMP elevating agents on the functional activity of the T cells is the result of cAMP-mediated release of the regulatory PKA I subunit and is independent of the catalytic activity from of the PKA (Lokshin et al., 2006; Raskovalova et al., 2007).

Exactly how cAMP affects the downstream molecular signalling of IFN-γ production is yet to be elucidated. One possible mechanism could involve inhibition of the transcription factor signal transducer and activation of transcription (STAT-1) activity. To be able to exert its antiviral and immunomodulatory activities, IFN-γ needs to bind to the type II interferon

receptor widely expressed in peripheral blood cells and bone marrow hematopoietic progenitors (Platanias and Fish, 1999). The binding of cytokines to their respective receptors results in receptor homo- or hetero-dimerization and triggering of intracellular signals such as the protein tyrosine kinases, Janus kinases (Jaks) (Ihle, 1995; Pelletier et al., 2003; Platanias and Fish, 1999; Schindler and Darnell, 1995). In the case of IFN-γ, Jak-1 and Jak-2 are the two tyrosine kinases associated with the interferon-γ receptor 1 and 2 (IFNGR1) and (IFNGR2). Upon activation, (as result of ligand binding) these tyrosine kinases are activated and phosphorylate IFNGR1 providing docking sites for STAT-1 transcriptional activator (Greenlund et al., 1994; Greenlund et al., 1995). The ability of STAT-1 to act as a substrate for Jak tyrosine kinase activity, allows it to undergo tyrosine phosphorylation. Following tyrosine phosphorylation, STAT-1 proteins form homodimers that translocate to the nucleus to regulate gene transcription by binding to the IFN-γ-activation site elements (GAS) in the IFN-γ regulated gene promoters. Ivashkiv et al. (1996) reported inhibition of STAT-1 expression or DNA-binding activity of STAT-1 following treatment with cAMP and immediate activation with phytohaemagglutinin (PHA) and IFN-γ in human mononuclear T cell cultures and T cells. Other studies have reported that extracellular signal-regulated kinase (ERK) signalling is required for maximum IFN-γ synthesis (Baumgarth et al., 1997) and that cAMP inhibits the activation of ERK1/2 pathways in primary human T cells (Grader-Beck et al., 2003). Similarly, Treg activity is reported to be correlated with activation of the ERK1/2 signalling pathway and dopafivee suppresses Treg activity by possible inhibition of ERK1/2 (Kipnis et al., 2004).

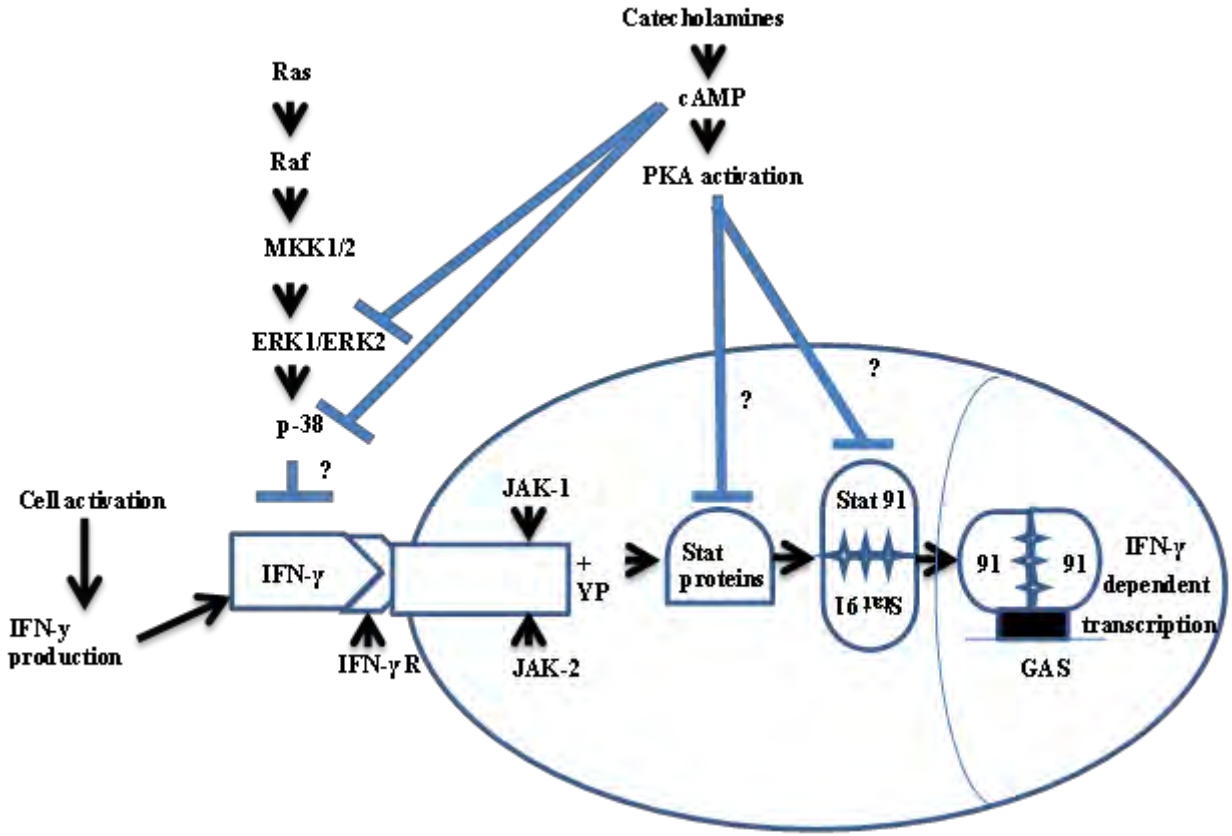



**Figure 6.1. Possible mechanism by which cAMP might inhibit IFN-γ production.** MKK1/2: mitogen activated protein kinase kinase 1/2; ERK: extracellular signal-regulated kinase; JAK: janus kinase; Stat: signal transducer and activation of transcription; GAS: IFN-γ-activation site elements; Y-P: tyrosine phosphorylation; : regions of Stat proteins containing SH2-phosphotyrosine.

## 6.2 Influence of Catecholamines on Lymphocytes Mobilisation

It has been repeatedly demonstrated that acute physical exercise and mental stress increases immune cells in peripheral circulation, with the biggest increases observed in NK cells and CD8+ cytotoxic T cells (Anane et al., 2009; Anane et al., 2010; Atanackovic et al., 2006; Benschop et al., 1996b; Campbell et al., 2009; Dhabhar et al., 1995; Dimitrov et al., 2010; Silberman et al., 2003; Simpson et al., 2007; Viswanathan and Dhabhar, 2005). It is known that the effects of catecholamines on immune cells are caused by binding to β2AR on PBMCs with high levels of β2AR expressed on NK cells and intermediate levels on T cells (Anane et al., 2009; Anane et al., 2010; Benschop et al., 1996b; Campbell et al., 2009). The increased number of immune cells could be a result of reduced margination of lymphocytes to endothelial cells lining venules. This means that cells which would normally be adhered to endothelial cells prior to migration into tissues are kept in general circulation (Carlson et al., 1996). In Chapter 4, we demonstrated that catecholamines modulate lymphocyte migration by altering lymphocyte-endothelium cell adhesion in static and lymphocyte-VCAM-1 cell adhesion in flow assays. Adhering NK and CD8+ T cells lose adherence to monolayers of HMEC-1 or VCAM-1 coated on microslides after the addition of catecholamines. Although the changes were not statistically significant and only occurred at higher concentrations of salmeterol, these results show a consistent trend in that catecholamine-induced rolling and detachment of PBLs from endothelial cells or VCAM-1 coated microslide is mediated via β2AR. Since both NK cells as well as endothelial cells express β2AR, the effect of catecholamines on the adhesion of NK and CD8+ T cells supports their influx into blood under conditions of stress.

Moreover, the effect of catecholamines is linked to a specific profile of adhesion molecule and chemokine receptor expression. Stress mobilized cells with low expression of CD62L and

high expression of integrin LFA-1 such as effector CD8+ T cells and $CD16^{+}CD56dim$ cytotoxic NK cells, have been reported. Effector memory cells are also characterised by their high expression of inflammatory chemokine receptors (such as CCR5, CXCR1, CXCR3, and CX3CR1). In addition, epinephrine selectively mobilized cells with cytotoxic effector function including $CCR7^{-}CD45RA^{+}CD8^{+}$ effector T cells (EFF), $CD4^{-}CD8^{-}$ γ/δ T cells, $CD3^{+}CD56^{+}$ NKT-like cells, $CD16^{+}CD56^{dim}$ cytotoxic NK cells, and $CD14^{dim}CD16^{+}$ proinflammatory monocytes all of which are characterized by high expression of CD11a and CX3CR1 (Dimitrov et al., 2010). It is thought that PBMCs are attached to endothelium through CD11a via CX3CR1-induced signalling, and that these cells are immediately released into the circulation on epinephrine binding. Furthermore, the same subset of cytotoxic effector cells showed preferential adhesion to activated endothelium *in vitro* and this effect was blocked by epinephrine (Dimitrov et al., 2010). Thus, it could be possible that epinephrine mediates its effect by inhibiting CX3CR1 -CD11a binding. Whether catecholamines alter the affinity of adhesion molecules interactions, or downregulate the expression of adhesion molecules on endothelial cells, such as VCAM-1, which is reported to mediate lymphocyte adherence to cytokine activated culture human endothelial cells (Carlos et al., 1990), should be the basis of future work.

### 6.3 Effects of Catecholamines on the Cytotoxicity of NK Cells and CD8+ T Cells

The effect of catecholamines on the CD8+ T and NK cell cytotoxicity was tested in two different cell lines. Firstly T-CHO which are non-human cells transfected to express human MICA, and K562 a human cell line, both of which lack the expression of MHC class I. In both assays salmeterol inhibited killing in a dose-dependent manner, although it was clear that NK cells were more sensitive to catecholamine treatment. It has previously been reported that

one of the ways by which NK and T cell lose their cytotoxicity is through the downregulation of NKG2D. The release of TGF-β by tumour cells downregulates expression of NKG2D in CD8+ T and NK cells and diminishes MICA and ULBP2 transcript expression and surface protein levels on malignant glioma cells (Gonzalez et al., 2008; Lee et al., 2004; Ljunggren, 2008). In addition, it has also been hypothesised that the suppression of cytotoxic T cell activity by TGF-β is also partially mediated by upregulation of inhibitory receptors, such as CD94/NKG2A (Lee et al., 2004). However, NKG2D downregulation can not be the only factor to account for the lost cytotoxicity in CD8+ T and NK cells. Guerra et al. (2008) demonstrated that NKG2D deficient mice developed normally and were characterized by normal numbers and proportions of lymphocytes. Furthermore, the expression of other activation and inhibitory receptors was similar to that in wild mice and these NK cells retained NKG2D-independent cytotoxicity against different tumour lines and responsiveness to direct crosslinking of activation receptors other than NKG2D (Ljunggren, 2008). Importantly, in the K562 human cell line other elements of NK and CTL activation may be involved. For example, catecholamines could inhibit/reduce the expression of (TNF)-family death ligand on the surface of NK cells, suppressing their interaction and engagement with respective receptors on target cells and thus leading to inhibition of target cells apoptosis. Moreover, it has been reported that a CD40-dependent pathway is involved in the induction of cytotoxicity of T cells and on human NK cells (Carbone et al., 1997; Sartorius et al., 2003). CD40, known as a member of the tumour necrosis factor receptor (TNF-R) family, is a surface receptor expressed on APCs such as B cells, dendritic cells, and monocytes and has the capacity to initiate multifaceted activation signals in normal B cells and dendritic cells (DCs). CD40 interacts with CD40 ligand (CD40L) expressed on activated T cells and NK cells. It is this CD40L-CD40 interaction which is important for induction of T and NK cell

cytotoxicity (Carbone et al., 1997; Sartorius et al., 2003; Schoenberger et al., 1998). Thus, another possibility by which catecholamines could inhibit the cytotoxicity of T cells and NK cells would be by inhibiting this interaction i.e. through downregulation of CD40 receptor on the surface of APCs or downregulation of the CD40L in activated T or NK cells. Consistent with this suggestion, Tamura et al. (2004) reported that adrenergic agonists, norepinephrine, epinephrine, isoproterenol and the β2 agonists terbutaline and salbutamol inhibited the expression of CD40 on monocytes in mixed lymphocyte reaction (MLR). Interestingly ligation of the CD40 triggers a complex signalling cascade that also results in the phosphorylation of p38 mitogen activated protein kinase which in turn leads to downstream NFKβ upregulation and cytokine production (Chakrabarti et al., 2007; Vogel et al., 2004). Thus as mentioned in Chapter 3 one of the ways by which salmeterol could inhibit cytotoxicity may also involve inhibition of p38, a member of the MAP kinase subfamily that is involved in regulating cellular responses to inflammation. Furthermore, Whalen and Bankhurst. (1990) reported that forskolin (an agent that elevates cAMP levels) inhibited the cytotoxicity of human NK cells by inhibiting the effector cell-target cell (NK-K562 tumour cell-line conjugation). On the other hand, Loshkin et al. (2006), reported that inhibition of the cytotoxic activity of activated NK cells by adenosine was mediated via cAMP-dependent activation of the regulatory subunits of PKA I. Regardless of how the cytotoxic activity of CD8+ T and NK cells is inhibited, the reduction in the CD8+ T and NK cytotoxicity upon catecholamine release coincides with a rapid mobilisation of CD8+ T and NK cells into peripheral circulation possibly via multiple mechanisms including shear stress due to a substantial increase in peripheral blood flow or downregulation of adhesion molecule expression (Nagao et al., 2000; Timmons and Cieslak, 2008). It has also been reported that during prolonged exercise (more than 3 hours) the counts of circulating NK cells may return

to pre-exercise levels (Gannon et al., 1997; Timmons and Cieslak, 2008) or can even drop below pre-exercise levels possibly due to the entry of cells at sites of muscle damage (Gannon et al., 1997; Timmons and Cieslak, 2008).

Since epinephrine mostly affect a specific subpopulation of CD8+ T and NK cells, the non-significant effects of catecholamines in our study could be due to the use of whole populations (PBMCs) that might not show the whole picture and thus we would need to isolate CD8+ T and NK cells subsets separately to see significant results. In addition, it was planned to incorporate CMV-specific T cells to analyse migration (Chapter 4) and killing (Chapter 5), however due to the relatively small changes adding small numbers of these cells was regarded unrealistic. Furthermore, it should be pointed out that specific populations of memory T cells and NK cells would be affected more than others. Most cell researchers define memory cells on the basis of their surface markers and not on function. Indeed, it is just now being understood that memory cells may be a more diverse population that had been considered, and that only relatively few of these cells may have been aroused by exposure to foreign antigens (Younes et al., 2011). Recent studies have shown that in response to high lymphocytic choriomeningitis virus (LCMV) infection it were naive cells that responded more efficiently compared to the memory population (West et al., 2011). Interestingly, NK cells can also be divided in to distinct population based on licensing, i.e. whether or not they have been primed by HLA ligands (Yokoyama, 1998). Orr et al. (2010) reported that in normal mice, a significant number of phenotypically mature NK cells lack the inhibitory receptors to recognise self-MHC class I, rendering them hyporesponsive and unable to acutely reject MHC class I deficient bone marrow. Similarly, the expression of the inhibitory KIRs recognising self-HLA receptors on the surface of human NK cells render them more responsive than NK cells that lack self-reacting inhibitory KIRs following *in vitro* stimulation with antibodies

against their activating receptors (Orr et al., 2010). Since licensing is governed by HLA genotype of each individual, this could account for the differences in the studies of NK migration and killing (Chapter 5).

The question raised by the current studies is what is the biological meaning of catecholamine-induced immune changes?

Upon epinephrine release during psychological stress, exercise, trauma, heat, shock, these subpopulations of cytotoxic effectors cells mobilise in circulation in order to build up an acute defense system to allow efficient surveillance of tissues and rapid accumulation at sites of injury and infection (Bouchama et al., 1992; Dimitrov et al., 2010; Karandikar et al., 2002; Simpson et al., 2007). However, these catecholamines also appear to inhibit cytotoxic effector cells' functional capabilities including IFN-γ production and cytotoxicity.

At least two possible mechanisms are suggested by these data. One that the increase in the number of CD8+ T and NK cells in circulation could also be a compensatory mechanism to account for the loss of cytotoxicity in the effector cells and thus a reduced ability to defend against pathogens, or that as memory CD8+ T cells and NK cells are full of toxic effector molecules migration into the blood must be mediated by decreased function.

**6.4 Summary and Future Research**

The studies presented in this thesis provided evidence that catecholamines decrease the cytotoxic activity of CD8+ T and NK cells, and by inhibition of IFN-γ production on CD4+ T and CD8+ T cells. The highest inhibition was observed with the IFN-y producing CD8+ T cells, possibly as result of higher β2AR expressed on the surface of these cells. The effect of catecholamines on the CD8+ T and NK cell cytotoxicity was also assessed using a novel assay that directly measures degranulation, and thus functional capacity, of the responding CD8+ T and NK cells per se (Alter et al., 2004; Betts et al., 2003). We concluded that

catecholamines decrease the percentage of CD107a on both CD8+ T and NK cells possibly involving the downregulation of the NKG2D activating receptor expressed on effector CD8+ T and NK cells. It is also reported that the cytotoxic CD8+ T and NK cells lose their preference to adhere to VCAM-1 coated microslide or endothelial cells upon treatment with higher concentrations of salmeterol. While the studies presented in this thesis brought to light a number of new facts, several fundamental questions remain to be resolved.

Future research activities should determine the cellular mechanisms of catecholamine induced lymphocytosis. Questions such as what are the exact migration pathways of the affected cell population after catecholamine administration; need to be analysed and answered. Emphasis should be given on specific adhesion molecules required for leukocytes-endothelial cell interaction as well as on the mechanisms of modification of adhesion such as the adhesion structure. The intracellular mechanism of how catecholamines modify the adhesion and signal transduction pathways of CD8+ T and NK cells need also to be elucidate.

Another fundamental question is perhaps the clinical relevance of stressed induced leukocytes mobilisation. While, several suggestions have been proposed, these remain largely speculative. Furthermore, it would be interesting to know whether responses of the immune system to catecholamines could be used as predictors for future development of disease.

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# APPENDIX

**Table A.1: List of adhesion receptors involved in leukocytes recirculation and their counter ligands expressed on the endothelium**.

| Lymphocytes | Endothelium | Function |
|---|---|---|
| L-Selectin | GlyCAM-1<br>MadCAM-1 | tethering/rolling |
| CLA, SL-1 | E-selectin | tethering/rolling |
| PSGL-1 | P-selectin | tethering/rolling |
| CD44 | hyaluronate | tethering/rolling |
| αLβ2 (LFA-1) | ICAM-1,-2, -3 | firm adhesion |
| α4β1 (VLA-4) | VCAM-1 | rolling and firm adhesion |
| α4β7(LPAM-1),<br>CD49d/CD- | MadCAM, VCAM-1 | rolling and firm adhesion |
| CD31 | CD31 | transmigration |
| | | |

GlyCAM: glycosylation cell adhesion molecule; MadCAM: mucosal addressin cell adhesion molecule; CLA: cutaneous leukocyte antigen; SL-1: E-selectin monospecific ligand; PSGL: P-selectin glycoprotein ligand; LFA: leukocyte functional associated antigen; ICAM: intracellular cell adhesion molecule; VLA: very late antigen 4 and VCAM: vascular cell adhesion molecule. Adapted from (Fabbri et al., 1999; Springer, 1995).

**Table A.2: Properties of the selectin family**

| Family molecule - Selectin | Selectin Expression | Ligand/counter receptor | Ligand Expression | Function |
|---|---|---|---|---|
| E-selectin (CD62E) (ELAM-1) | Cytokine-activated endothelium | Sialylated fucosylated lactosamines (i.e., sLeX, sLeA), Lselectin, PSGL-1<br>ESL-1<br>CLA | Leukocytes<br><br>Skin homing T cell | Leukocyte localization at sites of inflammation<br><br>Leukocytes homing to the skin |
| P-selectin (CD62P) (PADGEM, GMP140) | Weibel-palade bodies of endothelial cells<br><br>α-granules of platelets | Sialylated fucosylated lactosamines (i.e., sLeX, sLeA),<br>L-selectin<br>PSGL-1<br>CD24 | Leukocytes | Leukocyte localization at sites of inflammation |
| L-selectin (CD62L) (LAM-1, MEL-14) | All circulating leukocytes except effector and memory subgroups | GlyCAM-1<br>CD34<br>PNAd,<br>MAdCAM-1 | HEV in peripheral lymphoid tissues, mucosal and mesenteric tissues | Homing of lymphocytes to lymphoid tissues (i.e. peripheral lymph nodes)<br><br>Leukocyte localization at sites of inflammation |

PSGL-1: P-selectin glycolipid ligand-1; ESL-1: E-selectin ligand-1; CLA-cutaneous lymphocyte antigen; GlyCAM-1: glycosylation-dependent cell adhesion molecule-1; PNAd: peripheral node addressin; HEV: high endothelial venules and MAdCAM-1: mucosal addressin cell adhesion molecule-1.Adapted from (Butcher and Picker, 1996; Crockett-Torabi, 1998; Mackay, 1993; Penberthy et al., 1997; Springer, 1995).

**Table A.3: Properties of the integrin family**

| Family molecule - Integrin | Leukocyte Expression | Ligand/counter receptor | Ligand Distribution |
|---|---|---|---|
| $\alpha_1\beta_1$ (VLA-1) | Activated T cells | LN-1, CN | ECM |
| $\alpha_2\beta_1$ (VLA-2) | PMN | LN-1, CN | ECM |
| $\alpha_4\beta_1$ (VLA-4) | Lymphocytes | VCAM-1, MAdCAM-1, FN | ECM, activated EC, HEV |
| $\alpha_5\beta_1$ (VLA-5) | PMN and memory T cells | VCAM-1, FN | ECM, activated EC |
| $\alpha_6\beta_1$ (VLA-6) | PMN and lymphocytes | LN | ECM |
| $\alpha_9\beta_1$ (VLA-9) | PMN | VCAM-1, osteopontin | Activated EC, ECM, bone marrow stroma, osteoblasts, mesothelium |
| $\alpha_L\beta_2$ (LFA-1) | All leukocytes | ICAM-1, ICAM-2, ICAM-3, ICAM-4, ICAM-5, JAM-1 | PMN, monocytes, macrophages, EC, platelets |
| $\alpha_M\beta_2$ (MAC-1) | PMN | ICAM-1, ICAM-2, ICAM-3, iC3b, fibrinogen | Lymphoid cells, monocytes, macrophages, neutrophils |
| $\alpha_V\beta_3$ | PMN | PECAM-1, FN | EC, PMN |
| $\alpha_4\beta_7$ | Lymphocytes | MAdCAM-1, VCAM-1, fibronectin | HEV, lamina propria, spleen |

VLA: very late antigen; MAC-1: macrophage-1 antigen; PMN: neutrophils; LN: laminin; CN: collagen; FN: fibronectin; MAdCAM-1: mucosal addressin cell adhesion molecule-1; VCAM: vascular cell adhesion molecule; ICAM: intercellular cell adhesion molecule; JAM: junctional adhesion molecule; PECAM-1: platelet-endothelial cell adhesion molecule-1; ECM: extracellular matrix; EC: endothelial cells and HEV: high endothelial venules. Adapted from (Larson and Springer, 1990; Luscinskas et al., 1995; Ostermann et al., 2002; Springer, 1994, 1995).

**Table A.4: CC- chemokines**

| Chemokines | Cellular Distribution | Chemokine Receptor | Function |
|---|---|---|---|
| MCP-1, 3 and 4 | M | CCR2 | Migration of memory T cell in inflammation; Migration of ($T_{H1}$) effector T cells; |
| Eotaxin-1, 2, and 3; RANTES; MCP-2, 3, and 4, HCC-2, MIP-5 | Eo, Bs, T | CCR3 | Migration of ($T_{H2}$) effector T cells |
| TARC, MDC-1 | T, P | CCR4 | Migration of memory T cell to the skin; Migration of ($T_{H2}$) effector T cells |
| RANTES, MIP-1α, MIP-1β, MCP-2 | T, M, MØ, DC | CCR5 | Migration of memory T cell in inflammation; Migration of ($T_{H1}$) effector T cells |
| SLC, ELC, MIP-3β | T, B, DC | CCR7 | Naïve T cell migration to lymph nodes and Peyers patches; Migration of memory T cells to lymphoid tissues |
| I-309 | M, Thymus | CCR8 | Migration of ($T_{H2}$) effector T cells |
| TECK | T, Thymus | CCR9 | Migration of memory T cells to the gut; Migration of pro-thymocytes to thymus |

MCP: macrophage chemoattractant protein; RANTES: regulated on activation of normal T cell expressed and secreted; HCC: human CC chemokine; MIP: macrophage inhibitory protein; TARCC: thymus and activation regulated chemokine; MDC: macrophage derived chemokine; TECK: thymus expressed chemokine; M: monocyte/macrophage; Eo: eosinophil: Bs: basophil; T: T-lymphocyte; P: platelet; MØ: macrophage; DC:dendritic cell; and B: B-lymphocyte.Adapted from (Moser and Loetscher, 2001; Moser et al., 2004; Murdoch and Finn, 2000; von Andrian and Mackay, 2000).

**Table A.5: CXC- chemokines**

| Chemokines | Cellular Distribution | Chemokine Receptor | Function |
|---|---|---|---|
| CXCR1 | N, M, T, NK, Bs, Ms, En | Interleukin-8, GCP-2 | Recruitment of neutrophils in inflammation |
| CXCR2 | N, M, T, NK, MS, As, Nn, Ms, En | Interleukin-8, Groα, Groβ, Groγ, Nap-2, GCP-2, ENA-78 | Recruitment of neutrophils in inflammation |
| CXCR3 | Activated T, | IP-10, Mig, I-TAC, | Migration of ($T_{H1}$) effector T cells |
| CXCR4 | Myeloid, T, B, Ep, En, DC | SDF-1α | Migration of naïve T cells within lymphoid tissues, Migration of ($T_{H2}$) effector T cells, Neutrophils homing to bone marrow |

N: neutrophil; M: monocyte/macrophage; T: T-lymphocyte; NK: natural killer cell; Bs: basophil; Ms: mast cell: En: endothelial cell; As: astrocyte; Nn: neurone; B: B-lymphocyte; Ep: epithelial cell; DC: dendritic cell; GCP-2: granulocyte chemotactic protein-2; GRO: melanoma growth-stimulating activity; Nap-2: neutrophil-activating peptide-2; ENA78: epithelial derived neutrophil attractant-78 amino acids; IP-10: interferon-γ-inducible protein 10; Mig: monokine induced by interferon-γ; ITAC: interferon-inducible T cell alpha 1 and SDF-1α: stromal-derived factor 1α. Adapted from (Engelhardt et al., 1998; Moser et al., 2004; Murdoch and Finn, 2000; von Andrian and Mackay, 2000).

**Table A.6: Regulation of NK function by cytokines**

| Cytokines | Effect on NK cells |
|---|---|
| IL-15 | NK cell proliferation and differentiation; major mediator of NK cell homeostasis. |
| IL-2 | Potent immunoregulator of NK cells; supports survival and proliferation; may aid local immunity to infections by stabilizing or inducing cytotoxic granules. |
| IL-12 | Stimulates the production of IFN-γ; induces cytotoxicity; induces expression of MIP-1α when in synergism with IL-15; stabilizes or induces cytotoxic granules, |
| IFNα/β | Induces NK cytotoxic activity and proliferation in vivo; inhibits IL-12 production. |
| IFN-γ | Major source of NK cell production; eliminates viral infected cells, stressed cells and tumour cells; promotes maturation of DCs by NKs; facilitates Th1 response; induces blastogenesis of NK cells in vivo. |
| IL-1α, β | Stimulates production of IFN-γ. |
| IGIF | Induces cytotoxicity; stimulates IFN-γ production when in synergism with IL-12. |
| IL-10 | Inhibits production of IL-12 and IFN-γ; downmodulates NK cell responses. |
| TGF-β | Stops proliferation and cytotoxicity; Inhibits production of IFN-γ and IL-12. |

Adapted from (Anfossi et al., 2006; Biron et al., 1999; Colucci et al., 2003; Conigliaro et al., 2011; Cooper et al., 2001; Lodoen and Lanier, 2006; Martin-Fontecha et al., 2004; Qian et al., 2011; Suzui et al., 2004; Vivier et al., 2008)

**Table A.7: Regulation of NK function by chemokines**

| Chemokines | Effect on NK cells |
|---|---|
| MIP1-α | Induces chemotaxis in culture and following in vivo infection. |
| MIP-1β | Induces chemotaxis in culture. |
| MCP-1, 2, 3 | Induces chemotaxis in culture. |
| RANTES | Induces chemotaxis in culture. |

(Ranson et al, 2003; Reviewed by Colucci et al, 2003; Reviewed by Cooper et al, 2001; Natuk and Welsh, 1987; Reviewed by Vivier et al, 2008; Biron et al, 1999; Mariel Levy et al, 2010; Lodoen and Lanier, 2006; Conigliaro et al, 2011; Suzui et al, 2004; Yang et al, 2011; Loza et al, 2002, Anfossi et al 2006; Martin-Fontecha et al, 2004; Qian et al, 2011).