Expression of $\alpha_1$-Adrenergic Receptors on Immune Cells During Inflammation

Thesis is presented for the Honours degree in Biomedical Science at Murdoch University

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Murdoch University

by

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BSc Biomedical Science
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2015
Declaration

I declare this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any tertiary institute.

Harry Nguyen
Abstract

The immune system is a complex network of interacting cells and proteins that are constantly protecting the host from pathogens. Lymphoid and myeloid cells are the cellular mediators of the immune system, and the functions of these cells are partly controlled by the nervous system, however the mechanisms are not understood. The sympathetic nervous system may play a role through a family of receptors called adrenergic receptors (AR). Adrenergic receptors are G-protein coupled receptors that mediate the actions of adrenaline and noradrenaline. These receptors are split into the α- and β- subtypes, which can be further divided into subtypes. While β-ARs have been well-studied, less is known about α-ARs and their roles in immune regulation. The α-ARs can be divided into the α₁- and α₂- subtypes: this project focussed on the α₁-ARs as there is evidence to suggest that they play a role in inflammatory diseases. However, relatively little is known about which immune cell types express α₁-ARs, and how they vary during inflammation.

The major Aim of this project was to examine the expression of α₁-ARs on lymphoid and myeloid cells in mouse spleen and establish whether simulating an acute inflammatory response (bacterial lipopolysaccharide: LPS) in splenocytes would alter the protein expression of α₁-ARs. Protocols were optimised for the multi-parameter labelling and analysis of mouse spleen lymphoid and myeloid cells by flow cytometry, and the optimisation of specific α₁-AR surface protein staining using BODIPY-prazosin. Normal mouse lymphoid and myeloid populations were found to express varying levels of α₁-ARs, and that all except plasmacytoid dendritic cells (pDC) showed an unexpected reduction in α₁-AR protein expression after exposure to LPS in vitro. The results of this project provide preliminary evidence that except for pDC,
lymphoid and myeloid cell populations within the spleen undergo downregulation of \( \alpha_1 \)-ARs during an acute inflammatory response.
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<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Allergic Asthma</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
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<tr>
<td>AD</td>
<td>Adrenaline</td>
</tr>
<tr>
<td>AD4</td>
<td>Arginine deiminase 4</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AR</td>
<td>Adrenergic receptor</td>
</tr>
<tr>
<td>ARC</td>
<td>Animal resource centre</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>ANS</td>
<td>Anatomical nervous system</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BPr</td>
<td>BODIPY-prazosin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional dendritic cell</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Con-A</td>
<td>Concanavalin A</td>
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<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence minus one</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
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</table>
iT<sub>reg</sub> Induced regulatory T cell
JRA Juvenile rheumatoid arthritis
LPS Lipopolysaccharide
MAPK Mitogen activated protein kinase
mAB Monoclonal antibody
MFI Mean fluorescence intensity
MHC I Major histocompatibility complex I
MHC II Major histocompatibility complex II
mRNA Messenger RNA
NA Non-allergic asthma
NAD Noradrenaline
NK Natural killer
nT<sub>reg</sub> Natural regulatory T cell
PAA Periarterial adventitial
PAMP Pathogen associated molecular pattern
PBMC Peripheral blood mononuclear cell
pDC Plasmacytoid dendritic cell
PI Propidium iodide
PKC Protein kinase C
PLC Phospholipase C
PNS Peripheral nervous system
PSNS Parasympathetic nervous system
RA Rheumatoid arthritis
RT-PCR Reverse transcription polymerase chain reaction
SD Standard deviation
SEM Standard error of the mean
SNS Sympathetic nervous system
SSC Side Scatter
T<sub>c</sub> Cytotoxic T cell
TCR T cell receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>Th1</td>
<td>Helper T cell 1</td>
</tr>
<tr>
<td>Th2</td>
<td>Helper T cell 2</td>
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<tr>
<td>Th9</td>
<td>Helper T cell 9</td>
</tr>
<tr>
<td>Th17</td>
<td>Helper T cell 17</td>
</tr>
<tr>
<td>Tfh</td>
<td>Follicular Helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>α1-CHO</td>
<td>Transfected α1-AR CHO cells</td>
</tr>
</tbody>
</table>
Acknowledgements

What a year. I am ready and set to go onto a new journey, hopefully to something more relaxing like a holiday perhaps. Before this year and this honours project is over, I would like to give thanks to the many people who have helped me along the way. To my supervisors, Phil and Peter who are on top of my list, I cannot thank you both enough for all the help you have given me along the way with this honours project. Thanks for being able to bear with me as I embarked on this journey as an honour student and providing me with more freedom than I could have ever asked for.

Besides my main supervisors, there are also my unofficial supervisors that I would like to thank as well. To Chelsea and Linda, my lab buddies and to the people I went to when I needed help the most, thank you so much for sticking with me and tolerating me for the entire year. They have definitely seen me at my best and at my worst and I just can’t thank them enough for all the help and guidance they have given me throughout the year, be it from flow cytometry to cell culturing techniques, they were ready to assist. Thank you so much. To Irma, the flow specialist at the CMCA, thank you for the help you given me. From teaching me how to work the flow cytometer to just general advice on how to construct my antibody panels. Also, thank you to the Animal Resource Centre who supplied all the mice I needed for my experiments.

Special mention and thanks to my dad and mum, thanks for hearing me out, providing me support and listening to every single one of my issues. To my sisters, Helen and Hera, thanks for supporting me throughout this journey of mine. Thank you for tolerating me at 4 in the morning when all you could hear was my keyboard and music.

To my friends. Geez guys we’ve been through a lot. Four years of hell we call university. In no particular order, Alex, Angelica, Jesse, Nick, Nicole, Nicki, Narelle,
Saba, Tisha and Tabitha. We’ve been through the ringer, back, and thanks for supporting me in ways I could never have imagined or thought I needed until the time came. Whether you listened to my rants, helped me in the lab, hung out with me on my breaks, you guys are absolute legends!!

Lastly, to my office members, Jess, Nat, Kirstie, Margaret and Jane, thank you for hearing me out during the hardest moments of this honours project and just bearing with me in general.

Thanks to all of you for being here with me on my journey. You guys are all legends!!
1. Introduction

The primary function of the immune system is to protect the host from infectious microbes in the external environment (Chaplin, 2010). The pathogens encountered have a large spectrum of pathologic mechanisms. The immune system therefore uses a complex array of protective mechanisms to control and eliminate the pathogens (Chaplin, 2010). The protective mechanisms can be divided into two classes: innate immunity and adaptive immunity.

Inflammation is an essential immune response utilised by the host in response to detrimental insults such as microbial infection, tissue damage or other harmful stimuli (Ahmed, 2011). It involves a physiological process where immune cells are recruited and activated at compromised sites to remove any harmful stimuli and initiate the healing process (Ahmed, 2011). It can become pathological resulting in a chronic inflammatory state due to complications in the removal of the harmful stimuli or development of an inappropriate immune response such as allergies or auto-immune diseases (Medzhitov, 2010). Compared to the acute inflammation which is considered to be part of the innate immune response, chronic inflammation is mainly considered to be mediated by adaptive immunity with lymphocytes and antigen presenting cells being the predominant cell types (Medzhitov, 2008).

The innate and adaptive immune systems interact with the central nervous system (CNS) and autonomic nervous system (ANS) (Pongratz and Straub, 2014). Noradrenaline (NAD) and adrenaline (AD) act as neurotransmitters in the CNS and sympathetic nervous system (SNS) (Marino and Cosentino, 2013). The stimulation of the SNS brings the body into a state of raised activity and attention, usually called the
‘fight or flight’ response (Marieb and Hoehn, 2010). The effects of AD and NAD are mediated by adrenergic receptors (ARs) (Marino and Cosentino, 2013).

Adrenergic receptors are a class of G-protein coupled receptors expressed in virtually all tissues (Marino and Cosentino, 2013). They are divided into two major subsets, α-ARs and β-ARs (Hein and Kobilka, 1997). The α-ARs and β-ARs can be further divided into two subsets α₁- and α₂-ARs with their own subtypes and β-ARs can be divided into three subsets β₁-, β₂-, β₃-ARs (Michelotti, Price, and Schwinn, 2000).

Adrenergic receptors are expressed on immune cells and appear to have a function in the modulation of their activity (Reviewed in Marino and Cosentino, 2013). There is also evidence to suggest that the SNS may also modulate the inflammatory process (Pongratz and Straub, 2014; Basbaum and Levine, 1991).

The impact and expression of β-ARs on immune cells and inflammation has been well studied. In contrast, there is limited knowledge about the expression of α-ARs, specifically α₁-ARs on immune cells and their possible functions (Grisanti, Perez, and Porter, 2011).

This project will attempt to establish the baseline expression of α₁-ARs in normal mouse lymphoid tissue and compare this to the expression of α₁-ARs during an inflammatory state.
1.1. The Immune Response and Inflammation

1.1.1. Overview of the Immune response

The immune system utilises a complex array of protective mechanisms to eliminate pathogenic microbes to protect the host (Chaplin, 2006). These protective mechanisms can be divided into three components: anatomic and physiological barriers, the innate immune system and the adaptive immune system (Figure 1.1).

1.1.2. Anatomical and Physiological Barriers

The anatomical and physiological barriers are the first line of defence against pathogens. These barriers include the skin, vigorous mucociliary mechanisms, low stomach pH and bacteriolytic lysozyme in tears, saliva and other secretions (Turvey and Broide, 2010).

Figure 1.1 The integrated human immune system. The human immune system is split into three divisions. (1) The anatomical and physiological barriers, (2) Innate immunity, (3) Adaptive immunity (Adapted from Turvey and Broide, 2010).
The epithelium provides a physical barrier between the internal milieu and the pathogens that inhabit the external environment. The epithelium comprises the skin and the lining of the internal tubular structures including the gastrointestinal, respiratory and urogenital tracts. The internal epithelia are known as mucosal epithelia and secrete fluids called mucus which contains glycoproteins called mucins (Murphy, 2011). Mucus has a number of protective roles. Microorganisms which are coated in mucus are prevented from adhering to the epithelium and expelled outwards by the cilia found on the mucosal epithelium (Hsieh et al., 1993).

1.1.3. Innate Immune System

The innate immune system augments the protection given by the anatomical and physiological barriers (Janeway and Medzhitov, 2002). The innate immune system relies on a few invariant receptors called toll-like receptors (TLRs), but compensates by targeting conserved microbial components that are shared by a large group of pathogens (Turvey and Broide, 2010). A defining characteristic of the innate immune system is that within minutes of pathogen exposure, an inflammatory response can be generated. Additionally the innate system plays an important role in activating the adaptive immune system.

The main mediators and cells involved in the innate immune system come from both haematopoietic and non-haematopoietic origin. Haematopoietic cells involved in the innate immune response include macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, natural killer (NK) cells and NK T cells. In addition to these cells are the non-haematopoietic cells, the epithelial cells (Turvey and Broide, 2010). The primary responses, mediated collectively by these cells include opsonisation,
phagocytosis, immune cell recruitment and inflammation (Janeway et al., 2001; Abrutyn et al., 1977; Gin, Brottier, and Aubertin, 1984; Turvey and Broide, 2010).

While the innate immune response is rapid, it is limited by its inability to combat rapidly mutating pathogens such as viruses (Turvey and Broide, 2010). However, the innate immune system has evolved to recognise microbial components that are essential for the viability or virulence of the pathogen called pathogen-associated molecular patterns (PAMPs) by the use of TLRs (Turvey and Broide, 2010). TLRs allow for recognition and response to diverse microbial epitopes, which allows for the innate immune system to distinguish between groups of pathogens and to induce the appropriate cascade of effector adaptive responses (Turvey and Broide, 2010). PAMPs such as lipopolysaccharide (LPS) and ssRNA are recognised by TLR 4 and 8 respectively (Turvey and Broide, 2010).

1.1.4. Adaptive Immune System

The adaptive immune system reinforces the protection initiated by the innate immune system. Although both immune systems are activated by detection of an antigen, the adaptive immune system is much more specific and provides a broader and fine-tuned ability to recognise self and non-self antigens. The adaptive immune response is initiated by antigen presenting cells (APCs) which include DCs, macrophages and B cells (Bonilla and Oettgen, 2010; Chaplin, 2010). These APCs traffic the antigen from where it has been detected to secondary lymphoid tissues such as lymph nodes and the spleen (Bonilla and Oettgen, 2010). The adaptive immune system is mediated by T and B lymphocytes, which facilitate pathogen-specific immunologic effector pathways such as cytotoxicity and immunological production, generate immunological memory and
regulate immune homeostasis (Bonilla and Oettgen, 2010; Medzhitov, 2007). Compared to innate immune responses, the adaptive immune responses take longer, ranging from hours to days, to initiate due to the time required for the proliferation and differentiation of antigen-specific T and B lymphocytes (clonal expansion) (Bonilla and Oettgen, 2010).

1.2. Overview of the Inflammatory Response

Inflammation is a protective strategy in higher organisms which responds to detrimental insults such as microbial infection, tissue damage and other harmful situations (Ahmed, 2011). Immune cells are recruited and activated which enables the removal of harmful stimuli as well as the healing of damaged tissue (Ahmed, 2011). The involvement of immune cells leads to the five classical symptoms of inflammation which include redness, swelling, pain, heat and the disturbance of function (Medzhitov, 2008). The inflammatory response consists of four components: inflammatory inducers, the sensors that detect them, the inflammatory mediators and the target tissue that is affected by the inflammatory mediators. These components can vary such that depending on the type of infection (bacterial, viral or parasitic), an appropriate inflammatory response can be generated (Medzhitov, 2010).

In injuries where infections are absent, acute inflammation assists in the healing and repair of the injury site by promoting tissue repair and assisting in the prevention of colonisation by opportunistic pathogens (Medzhitov, 2010). The tissue damage is detected by tissue-resident macrophages, which induce inflammatory and healing responses, and pain receptors (nociceptors) that enable pain detection and sensation in the affected area (Medzhitov, 2010).
The acute inflammatory response is normally terminated when the triggering stimuli are eliminated, the infection is cleared and the damaged tissue has been repaired (Serhan and Savill, 2005). The termination of inflammation and the transition to the homeostatic state is a highly active and highly regulated process. A key event in the resolution of inflammation is the switch from the secretion of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα to anti-inflammatory cytokines such as IL-10, TGF-β and glucocorticoids. This broadly causes a transition from the recruitment of inflammatory cells such as neutrophils to the recruitment of monocytes which results in the clearance of debris and the initiation of tissue repair at the damage site (Medzhitov, 2010; Ahmed, 2011).

If the resolution of inflammation or the elimination of the triggering stimuli such as a persistent infection or chronic cellular injury fails for any reason, then the acute inflammation can transition into a state of chronic inflammation (Ahmed, 2011). The chronic inflammatory stage is not beneficial to the host, with the majority of chronic inflammatory conditions being pathogenic in nature and the majority of the damage is mediated by the immune response. Some of the common chronic inflammatory diseases include atherosclerosis, asthma, type 2 diabetes, rheumatoid arthritis and cancer (Capellino and Straub, 2008; Ahmed, 2011; Pelaia et al., 2003; Locksley, 2010).
1.3. Cells and Cytokines Involved in the Inflammatory Response

1.3.1. Lymphocytes

The lymphocyte population is a major contributor to both the innate and adaptive immune system. The lymphocyte population is mainly comprised of the thymus-derived cells (T-cells), bone-marrow derived cells (B-cells), and the natural killer cells (NK cells). T-cells mediate cellular immunity, along with B-cells which mediate humoral immunity and provide what is collectively known as the adaptive immune system (Luckheeram et al., 2012).
1.3.1.1. T cells

The majority of the T-cells are comprised of two major types: Helper T-cells (T\(_H\) cells) and cytotoxic T-cells (T\(_C\) cells).

1.3.1.1.1. Helper T cells

T\(_H\) cells are otherwise known as CD4\(^+\) T cells because they express the CD4 glycoprotein on their cell surface (Luckheeram et al., 2012). T\(_H\) cells play a critical role in immune protection. Following activation via binding of T cell receptor (TCR) to antigenic peptides expressed on major histocompatibility complex class II (MHC II) on the surface of APCs, T\(_H\) cells differentiate into distinct effector T\(_H\) cell subtypes which produce cytokines and chemokines (Luckheeram et al., 2012). These cytokines and chemokines have the capacity to help B-cells make antibodies, induce macrophages to develop enhanced microbicidal activity, and to recruit neutrophils, eosinophils and basophils to sites of infection and inflammation (Zhu and Paul, 2008).

The naive T\(_H\) cells can be differentiated into a number of subtypes with the major subtypes being the T-helper 1 (T\(_H\)1) and T-helper 2 (T\(_H\)2 cells). The other subtypes include T-helper 17 (T\(_H\)17), follicular helper T-cells (T\(_F\)H), ‘natural’ T-regulatory cells (nT\(_{reg}\)), induced T-regulatory cells (iT\(_{reg}\)), type 1 T-regulatory cells, T-helper 9 (T\(_H\)9) (Figure 1.2) (Kaplan, Hufford, and Olson, 2015; Luckheeram et al., 2012; Roncarolo et al., 2001; Schmitt and Williams, 2013).
### 1.3.1.1.1.1. Helper 1 T cell

T\textsubscript{H} cells play an important role in inflammation by secreting cytokines and chemokines that can activate and/or recruit other cell types (Zhu and Paul, 2008). T\textsubscript{H}1 cells mediate the host immune response against viral and microbial pathogens. T\textsubscript{H}1 cells primarily secrete interferon-\textgamma (IFN-\textgamma), IL-2 and the pro-inflammatory cytokine lymphotoxin-\alpha (LT-\alpha) in response to intracellular pathogens (Figure 1.2). Of importance is IFN-\textgamma which is important in activating macrophages to increase their microbicidal activity and IL-2 which plays an important role in both T\textsubscript{H} cell memory and T\textsubscript{c} cell memory (Kopf et al., 1993; Williams, Tynzink, and Bevan, 2006; Zhu and Paul, 2008).

![Figure 1.2. Summary of T\textsubscript{H} cells functions and cytokines produced.](image)

Each subset produces a unique profile of cytokines and is responsible for mediating a range of adaptive responses (Adapted from Zhu and Paul, 2008).
T\textsubscript{H}1 cells also play an important role in the activation and class-switching of immunoglobulin G (IgG) producing B cells. T\textsubscript{H} cells provide two types of co-stimulatory signals, CD40 ligand (CD40L) and cytokines (Zhu and Paul, 2008). The interaction between CD40L to CD40, a receptor expressed on B cells, causes the activation of B cells (Noelle et al., 1992). Under the influence of IFN-\(\gamma\), B cells preferentially induce the class-switch from IgM to IgG (Murphy, 2011). IgGs are capable of neutralising virus particles and toxins and activate the complement system (Vidarsson, Dekkers, and Rispens, 2014).

1.3.1.1.1.2. Helper 2 T cell

T\textsubscript{H}2 cells mediate the host defence against extracellular parasites and play an important role in the induction and persistence of allergic diseases (Zhu and Paul, 2008). The cytokines produced by T\textsubscript{H}2 cells include IL-4, IL-5, IL-9 and IL-10, IL-13 and IL-25/IL-17E and amphiregulin (Figure 2) (Zhu and Paul, 2008). IL-4 is the positive feedback cytokine for T\textsubscript{H}2 cell differentiation and plays a critical role in the humoral immune response (IgE class switch) against parasitic infections and is of importance in allergic reactions (Zhu and Paul, 2008). IL-5 plays an important role in recruiting eosinophils (Salahuddin, 1989). IL-9 is involved in the proliferation of both T\textsubscript{C} cells and mast cells but also induces mucin production in epithelial cell allergic reactions such as asthma (Longphre et al., 1999). IL-25/IL-17E has shown to play a role in enhancing the production of other cytokines including IL-4, IL-5 and IL-13 (Fallon et al., 2006). This in turn creates a positive feedback cycle for the proliferation of T\textsubscript{H}2 cells and allows for the recruitment of further eosinophils (Zhu and Paul, 2008). Furthermore, IL-25 is also produced by lung epithelial cells in response to allergens
Thus IL-25 from epithelial cells can enhance the effects of the Th2 response.

1.3.1.1.3. Helper 17 T cell

Th17 cells mediate the host response against extracellular bacteria and fungi (Weaver et al., 2006) and have also been implicated as a mediator in autoimmune diseases (Afzali et al., 2007; Zambrano-Zaragoza et al., 2014). The cytokines produced by Th17 cells include IL-17a, IL-17f, IL-21, and IL-22 (Figure 2) (Zhu and Paul, 2008).

IL-17a stimulates the production of various inflammatory cytokines including IL-6 and chemokines such as IL-8 (Yao et al., 1995). IL-17a and IL-17f both recruit and activate neutrophils during immune responses against extracellular fungi and bacteria (Zhu and Paul, 2008). IL-21 is a stimulatory factor for Th17 cells and acts as a positive feedback amplifier (Korn et al., 2007). IL-21 also acts as a modulator of a variety of immune cells including augmenting the proliferation and function of Tc cells and regulating the activity of NK cells (Parrish-Novak et al., 2000). IL-21 also influences B-cells and is a crucial regulator of antibody production and drives the differentiation of B-cells into memory cells (Leonard and Spolski, 2005; Ozaki et al., 2002).
1.3.1.1.4. Regulatory T cells

T\textsubscript{reg} cells play a critical role in maintaining self-tolerance and regulating immune responses. An increase in the number of T\textsubscript{reg} cells can cause an increase in their suppressive function which may be beneficial in auto-immune diseases where self/non-self recognition fails. T\textsubscript{reg} suppressive function is mediated by both cell-to-cell contact and cytokines, including TGF-\(\beta\), IL-10 and IL-35 (Figure 2) (Zhu and Paul, 2008).

IL-10 is an important cytokine in T\textsubscript{reg}-mediated suppression. T\textsubscript{reg}-mediated suppression by IL-10 production has shown to inhibit intestinal inflammation (Asseman et al., 1999), while deletion of the IL-10 gene in mice results in spontaneous colitis and enhanced lung inflammation (Rubtsov et al., 2008).

1.3.1.1.2. Cytotoxic T cells

T\textsubscript{C} cells or otherwise known as CD8\(^+\) T cells are lymphocytes that express the CD8 receptor that recognises antigens that are presented on target cells by the major histocompatibility complex class I (MHC I), which is expressed on virtually all nucleated cells (Bonilla and Oettgen, 2010). The antigens that are presented on MHC I are generally produced from proteins that have been translated within the cell (endogenous antigens) encoded by the host genome or by infecting viruses or pathogens that have been replicating intracellularly (Bonilla and Oettgen, 2010).

T\textsubscript{C} cells are one of the effector cells of the adaptive immune system and kill target host cells in a contact-dependent mechanism. T\textsubscript{C} cells must become activated before they can mediate cell death. While interactions between the TCR and MHC I provide a
partial signal for activation, further interactions between the co-stimulatory molecule CD28 found on the T cell and CD80 or CD86 found on the APCs provides full activation (Chaplin, 2006).

### 1.3.2. Antigen Presenting Cells

APCs are the initiators and modulators of the immune response and are present in large numbers in the skin and mucosal sites, where pathogen detection is most likely. The major cell types acting as APCs are DCs, macrophages and B cells. DCs are primarily located in the skin and the mucosal sites, acting as mobile sentinels. Macrophages are widely distributed throughout the lymphoid organs and connective tissue (Marieb and Hoehn, 2010). Macrophages are phagocytic cells that engulf microbes and particles that have been marked for clearance by Ig, complements or both (Chaplin, 2010).

APCs internalise extracellular proteins and pathogens and process them into antigenic peptides (Chaplin, 2010). These peptides are presented on MHC II molecules where interactions with T
_H
 cells can occur, leading to the initiation of T
_H
 cell immune responses (Banchereau et al., 2000). It should be also noted that DCs can also process these antigenic peptides onto MHC I and present them to and potentially activate T
_C
 cells (Ackerman and Cresswell, 2003).

B and T cells are mediators of the adaptive immune response, but their function is controlled by the DCs. These cells are located in the periphery of the body and concentrated in the secondary lymphoid tissues (Chaplin, 2010). T cells are directly stimulated by DCs; however B cells are indirectly stimulated through activation of T
_H
 cells. While B cells can indirectly recognise an antigen through their B cell receptors (BCRs) with assistance for T
_H
 cells, T cells require the antigen to be processed and
presented to them by an APC (Banchereau et al., 2000; Ahmed et al., 2009). This occurs through an interaction between the TCR and the MHC complex found on the APC. These complexes are found in two types, MHC I and MHC II.

1.3.2.1. B cells

Adaptive humoral immunity is mediated by immunoglobulins (Ig, also known as antibodies) produced by plasma cells which develop from B cells under the influence of signals received from TH cells and other cells, such as DCs (Bonilla and Oettgen, 2010). Constituting 15% of peripheral blood leukocytes, B cells are derived from hematopoietic stem cells in the bone marrow (Bonilla and Oettgen, 2010).

B cells, in contrast to T cells, express an antigen unique BCR on the cell surface. Circulating B cells that have not been exposed to an antigen are termed naive B cells. They become activated when their BCR engages their cognate antigen. B cells are active as APCs and are able to internalise and digest the antigens and present the peptides onto a MHC II molecule to T cells (Ahmed et al., 2009; Bonilla and Oettgen, 2010).

1.3.2.2. Dendritic Cells

Dendritic cells play an important role in the interface between the innate and adaptive immune responses by inducing, coordinating and regulating the responses (Banchereau et al., 2000). DCs not only express MHC I and II, but also TLRs that detect PAMPs such as LPS (TLR 4) and viral RNA (TLR 3 and 7) (Turvey and Broide, 2010). DCs act as APCs which allows DCs to activate T cells by presenting the antigen, co-stimulatory proteins and cytokines; the naive T cell then differentiates into TH cell subsets – TH1, TH2, TH17 and Treg (Banchereau and Steinman, 1998).
DCs produce a range of cytokines and chemokines. Studies have shown that the ligation of CD40, a co-stimulatory protein found on APCs required for its activation, causes a significant increase in IL-12 (Cella et al., 1996; Koch et al., 1996). IL-12 is responsible for the development of T\(_H\)1 responses (Hsieh et al., 1993). Upon antigen presentation to T\(_H\) cells, IL-12 production will cause the differentiation of IFN-\(\gamma\) T\(_H\)1 cells (Hsieh et al., 1993). IFN-\(\gamma\) is important in stimulating macrophages to increase their microbicidal activity and causes the up-regulation of IL-12, which in turns generates more T\(_H\)1 cells (Hsieh et al., 1993; Schroder et al., 2004).

In the presence of IL-4, however, DCs will induce T cells to differentiate into T\(_H\)2 cells which then produce IL-4 and IL-5. These cytokines activate eosinophils and assist B cells into producing antigen specific antibodies (Banchereau and Steinman, 1998). The production of IL-33 by DCs has been shown to also induce the production of cytokines associated with activated T\(_H\)2 cells (Figure 1.3) (Mirchandani, Salmond, and Liew, 2012; Schmitz et al., 2005).
Figure 1.3. **Dendritic cells influence the differentiation of helper T cells.** Production of cytokines from dendritic cells influence the differentiation of naive T\(_H\) cells into Helper 1 T (T\(_H1\)), T\(_H2\), T\(_H17\) and regulatory T cells (Adapted from Kojetin and Burris, 2014).
In summary, the immune system helps protect and eliminate toxic or allergenic substances that are able to breach the anatomical and physiological barriers. Once a pathogen, toxin or allergen has been detected by an APC, the immune response is initiated in an attempt to protect the host and eliminate the toxic or allergic substance (Figure 1.4) (Chaplin, 2010).

**Figure 1.4. Process of immune response to pathogens** Upon detection of pathogens by APCs, the immune response is initiated with production of cytokines and chemokines from APCs activating cells from both the innate (e.g. neutrophils) and adaptive (e.g. T and B cells) immune systems (Adapted from Tokyo, 2010)
1.4. Nervous System

1.4.1. Overview of the Nervous System

The nervous system is divided into two sections: the peripheral nervous system (PNS), with the autonomic (involuntary), somatic (voluntary) and sensory sections, and the central nervous system (CNS). The autonomic PNS is divided into two major sections, the sympathetic (SNS) and the parasympathetic nervous system (PSNS) (Koopman et al., 2011; Marieb and Hoehn, 2010). The SNS and PSNS generally serve the same organs but cause opposite effects from each other. The dual innervation allows for the body to counter balance each other’s activities to allow for optimal performance of the body systems (Marieb and Hoehn, 2010).

Generally, the stimulation of the SNS brings the body into a state of readiness and raised activity called the ‘fight or flight’ response (Marieb and Hoehn, 2010). Physiologically, there is an increase in heart rate and blood pressure, liver glycogenolysis is initiated and peristalsis of the gastrointestinal tract is inhibited (Marieb and Hoehn, 2010; Koopman et al., 2011). In contrast, stimulation of the PSNS enables the ‘rest and digest’ response. This response enables the body to restore the body functions back to normal following SNS stimulation. These responses include the reduction of heart rate and blood pressure, stimulation of gastrointestinal peristalsis and initiation of liver glycogenesis (Marieb and Hoehn, 2010; Koopman et al., 2011).
1.4.2. Sympathetic Neurotransmitters: Adrenaline and Noradrenaline

Adrenaline (AD) and noradrenaline (NAD) are endogenous catecholamines that together with dopamine represent the main catecholamines in humans (Marino and Cosentino, 2013). NAD is primarily produced and released by the post-ganglionic SNS nerve fibres and AD is produced by chromaffin cells in the adrenal glands (Marino and Cosentino, 2013). NAD and to a lesser extent AD act as neurotransmitters in the SNS (Marino and Cosentino, 2013).

When the SNS is mobilised, NAD and AD are produced. This is most pronounced when the body undergoes the ‘flight or fight’ response which is mediated by a stress response (Marieb and Hoehn, 2010).

The peripheral actions of AD and NAD vary and they include: regulation of smooth muscles (in particular the contraction of blood and lymphatic vessels and relaxation of other smooth muscles (e.g. bronchi), cardiac function (increasing the heart rate and the force of the contraction) and endocrine actions (modulation of the secretion of insulin and glucagon) (Piascik and Perez, 2001; Michelotti, Price, and Schwinn, 2000; Itoh and Gerich, 1982).
1.4.3. Sympathetic Nervous System Implication in Inflammation

There is evidence to suggest that the activation of the PNS can generate the cardinal signs of acute inflammation (Basbaum and Levine, 1991). These properties of acute inflammation include vasodilatation (heat and redness), plasma extravasation (swelling), and sensitisation of nociceptors to allow for a lower threshold of pain (Lembeck, Donnerer, and Bartho, 1982). Studies conducted using electrical stimulation of the peripheral nerves (Lembeck and Holzer, 1979) or injection of the neurotransmitter Substance P, which stimulates primary afferent sensory C-nerve fibres (Saria, 1984), showed that these signs of acute inflammation could be induced.

The SNS has also been shown to be implicated in regulating the innate immune response. For example, NAD has been shown to have a range of effects on the innate immune response including changes in cytokine levels after exposure to the bacterial endotoxin LPS (Elenkov et al., 2008). It should also be noted that in chronic inflammatory diseases such as rheumatoid arthritis, increases in SNS activity correlate with heightened disease progression (Capellino and Straub, 2008).
1.4.4. Adrenergic Receptors

Adrenergic receptors (ARs) are a member of the G protein-coupled receptor (GPCR) superfamily that mediate the actions of the endogenous catecholamines, AD and NAD in the SNS (Piascik and Perez, 2001).

1.4.4.1. Alpha and Beta Adrenergic Receptors

The ARs are divided into two subtypes: the $\alpha$-ARs and $\beta$-ARs. By the late 1980s and early 1990s, at least nine AR subtypes ($\alpha_{1a}$, $\alpha_{1b}$, $\alpha_{1d}$, $\alpha_{2a}$, $\alpha_{2b}$, $\alpha_{2c}$, $\beta_{1}$, $\beta_{2}$ and $\beta_{3}$) had been discovered, cloned and found to be expressed stably in murine cells (Hein and Kobilka, 1997).
1.4.4.1.1.  Signalling and Activation

The $\alpha$-AR and $\beta$-AR families both exhibit different ways of signalling through their G-coupled protein receptors. Catecholamines bind to $\alpha_1$-ARs to activate $G_\text{q}$-protein, whose $\alpha$-subunit then activates phospholipase C (PLC) which leads to the production of the secondary messengers inositol triphosphate (IP$_3$) and diacyl glycerol (DAG) and mobilisation of intracellular calcium and activation of protein kinase C (PKC) (Table 1.1) (Hein and Kobilka, 1997).

$\alpha_2$-ARs are primarily coupled to and activate $G_\text{i}$-protein, whose $\alpha$-subunit causes a decrease in adenylyl cyclase (AC) activity. AC is an enzyme which catalyses the conversion of ATP to cyclic adenosine monophosphate (cAMP), a secondary messenger used in signal transduction pathways, primarily signalling through $G_\text{s}$-proteins (Hardin, Bertoni, and Kleinsmith, 2012). However $\alpha_2$-ARs have also been shown to couple with $G_\text{s}$-protein and subsequently cause an increase in AC activity. It has been suggested that at low agonist concentrations, the activation of $\alpha_2$-ARs causes the decrease in cellular cAMP levels, while at higher agonist concentrations, the activation of $\alpha_2$-ARs causes an increase in cellular cAMP level (Table 1.1) (Gyires et al., 2009; Eason et al., 1992; Jones, Halenda, and Bylund, 1991).

$\beta$-ARs are primarily coupled to and activate $G_\text{s}$-protein. However, similar to the $\alpha$-ARs, the $\beta$-ARs family also show dual signalling pathways. Studies have shown that the $\beta_2$-ARs and $\beta_3$-ARs also exhibit $G_\text{i}$-protein coupling (Table 1.2) (Chen-Izu et al., 2000; Soeder et al., 1999; Xiao, 2001).
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Signalling mechanism</th>
<th>Tissue distribution</th>
<th>Physiological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{1A}$</td>
<td>$G_q$ (Phospholipase C stimulation, $Ca^{2+}$ channel)</td>
<td>Human heart, liver, cerebellum, cerebral cortex</td>
<td>Constriction of urethral smooth muscle</td>
</tr>
<tr>
<td></td>
<td>Phospholipase D stimulation</td>
<td>Human prostate and urethra</td>
<td>Contraction of skeletal muscle resistance arteries</td>
</tr>
<tr>
<td></td>
<td>Activation of protein kinase C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_{1B}$</td>
<td>Mitogen activated protein kinases</td>
<td>Human spleen and kidney, somatic arteries and veins</td>
<td>Contraction of arteries and veins</td>
</tr>
<tr>
<td>$\alpha_{1D}$</td>
<td>$G_i/G_o$ (adenylyl cyclase inhibition, $K^+$ channel, $Ca^{2+}$ channel)</td>
<td>Human aorta, blood vessels of human prostate, human bladder</td>
<td>Constriction of arteries</td>
</tr>
<tr>
<td></td>
<td>Phospholipase A2 stimulation</td>
<td></td>
<td>Urethral contractions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Contraction of corpus cavernosum</td>
</tr>
<tr>
<td>$\alpha_{2A}$</td>
<td></td>
<td>Human Brain &gt; spleen &gt; kidney &gt; aorta = lung = skeletal muscle &gt; heart = liver</td>
<td>Presynaptic inhibition of noradrenaline release, hypotension, sedation, analgesia, hypothermia</td>
</tr>
<tr>
<td>$\alpha_{2B}$</td>
<td>$G_i/G_o$ (adenylyl cyclase stimulation)</td>
<td>Human kidney &gt;&gt; liver &gt;&gt; brain = lung = heart = skeletal muscle</td>
<td>Vasoconstriction</td>
</tr>
<tr>
<td></td>
<td>Phospholipase A2 stimulation</td>
<td>Also found in aorta and spleen</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{2C}$</td>
<td>$G_s$ (adenylyl cyclase stimulation)</td>
<td>Human brain ≥ kidney</td>
<td>Presynaptic inhibition of noradrenaline release</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Also found in spleen, aorta, heart, liver, lung, skeletal muscle</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2. Classification of $\beta$-ARs (Adapted from Marino and Cosentino, 2013)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Signalling mechanism</th>
<th>Tissue distribution</th>
<th>Physiological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_1$</td>
<td>$G_s$ (adenyl cyclase stimulation)</td>
<td>Pineal gland, skeletal muscle, liver, superior cervical ganglion, heart, lung, adrenal cortex, cardiac myocytes, brain</td>
<td>Increase of cardiac output (heart rate, contractility, automaticity, conduction)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Renin release from juxtaglomerular cells</td>
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<td></td>
<td></td>
<td></td>
<td>Lipolysis in adipose tissue</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>$G_{i/o}$ (guanylyl cyclase stimulation)</td>
<td>Lung, lymphocytes, skin, liver, heart</td>
<td>Smooth muscle relaxation</td>
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<td></td>
<td></td>
<td></td>
<td>Striated muscle tremor and glycogenolysis</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Increase of cardiac output</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glycogenolysis and gluconeogenesis in liver</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Insulin secretion</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>$G_{i/o}$ (guanylyl cyclase stimulation)</td>
<td>Adipose, gall bladder $&gt;$ small intestine $&gt;$ stomach, prostate $&gt;$ left atrium $&gt;$ bladder</td>
<td>Lipolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thermogenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Relaxation of miometrium and colonic smooth muscle cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vasodilatation of coronary arteries</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Negative cardiac inotropic effect</td>
</tr>
</tbody>
</table>
1.4.4.1.2. Distribution and Function

ARs are distributed across the whole body with expression of ARs in the CNS and virtually all peripheral tissue (Tables 1 and 2) (Marino and Cosentino, 2013; Michelotti, Price, and Schwinn, 2000). ARs are involved in the control of blood pressure, myocardial contractility, airway reactivity as well as other metabolic and CNS functions (Marino and Cosentino, 2013).

1.4.4.1.2.1. Respiratory System

Using radioligand-binding studies, it has been shown that $\beta_2$-ARs are expressed in the respiratory tract, specifically on the airway smooth muscle cells (30-40 000 per cell), with the density of the $\beta_2$-ARs increasing in the lower respiratory tract and high levels in the central airways and alveolar region (Chung, 2008). CT scanning has confirmed that the distribution of $\beta_2$-ARs is greater for small airways rather than the large airways (Hoffman, Chiplunkar, and Casale, 1997; Ueki et al., 1993). Further studies have shown that expression of $\beta_2$-ARs is not limited to the airway smooth muscle cells, but also on lung epithelial and endothelial cells, alveolar type II cells and mast cells (Johnson, 2001). However the expression of $\beta_2$-ARs on these cells is much lower compared to expression of the airway smooth muscle cells.

The primary effect upon activation of $\beta_2$-ARs in airway smooth muscle is to induce bronchodilation. While not fully understood, it is believed that cAMP produced from activation of $\beta_2$-ARs inhibits the release of intracellular Ca$^{2+}$ stores, reduces membrane
Ca\textsuperscript{2+} entry and sequesters intracellular Ca\textsuperscript{2+}, leading to relaxation of the airway smooth muscle (Johnson, 2006).

There has been little research into the expression of α-ARs in the respiratory system. There is evidence to suggest α-AR expression in present in the human airways (Mukherjee and Wasserman, 1986; Beil and de Kock, 1978) with these studies showing that α-ARs have an influence is the constriction of the airways.

1.4.4.1.2.2. Vascular System

Previous studies have shown that all α\textsubscript{1}-AR mRNAs are expressed on peripheral arteries from rats (Piascik et al., 1995; Hrometz et al., 1999) and humans (Rudner et al., 1999). In rats, α\textsubscript{1}-AR subtypes were detected in the medial layer of the aorta, caudal, femoral, iliac, renal, superior mesenteric and mesenteric resistance arteries (Hrometz et al., 1999).

α\textsubscript{1A}-AR has been shown to mediate the contractile function of caudal arteries in rats (Hrometz et al., 1999). α\textsubscript{1D}-AR has been shown to mediate the contractile function of the aorta, femora, iliac and the superior mesenteric artery (Hrometz et al., 1999).

Studies have also used transgenic mice to assist in the explanation of the function of α\textsubscript{1}-ARs in smooth muscle. The deletion of the α\textsubscript{1B}-AR gene resulted in a decrease of mean arterial blood pressure in response to an α\textsubscript{1} agonist (45%) compared to wild type mice (Cavalli et al., 1997). This indicated that α\textsubscript{1B}-AR is a mediator of the blood pressure and contractile responses (Cavalli et al., 1997). However, these results are contradicted
by another study, whereby overexpression of $\alpha_{1B}$-AR gene did not cause a change in blood pressure (Zuscik et al., 2001).

1.4.4.1.2.3. Cellular Signalling Pathways

$\alpha_1$-AR is known to regulate hypertrophic growth responses with these receptors being linked to mitogen activated protein kinases (MAPKs), also known as extracellular signal-regulated kinase (ERK). MAPKs are protein kinases that are involved in signalling transduction pathways that control intracellular responses to a variety of events including acute responses to hormones and major developmental changes in organisms (Pearson et al., 2001). MAPKs regulate cell functions including proliferation, gene expression, differentiation, mitosis and apoptosis (Pearson et al., 2001). The activation of MAPK is also important in the pathway for the activation of immune cells. Many proteins including transcription factors that regulate the production of cytokines are targeted by MAPKs (Guha and Mackman, 2001; Kavelaars, 2002).

There is evidence to suggest that $\alpha_{1A}$-AR is involved in mediating hypertrophic responses in cardiac myocytes (Varma and Deng, 2000; Knowlton et al., 1993). The role of the other $\alpha_1$-AR in regulating growth promoting responses is less well studied. $\alpha_{1D}$-AR has been shown to promote MAPK activity and mediates the hypertrophic growth of rat aortic smooth muscle cells (Xin et al., 1997).

$\alpha_{1B}$-AR has also been shown as a potential mediator of hypertrophic growth. Transgenic mice constitutively expressing $\alpha_{1B}$-AR in the cardiac tissue demonstrated cardiac hypertrophy (Milano et al., 1994). Similar studies showed that mice over expressing $\alpha_{1B}$-AR transgenic mice displayed signs of cardiac hypertrophy that was
characterised by an increased thickness in interventricular septum and posterior wall and an increased isovolumetric relaxation time (Zuscik et al., 2001).

1.5. **Adrenergic Signalling and the Immune System**

1.5.1. **Interactions Between the SNS and Immune System**

It has been generally accepted that the immune system is regulated by at least two different neurohormonal systems: (1) hypothalamic-pituitary-adrenal axis, and (2) the SNS (Bellinger and Lorton, 2014). The primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid organs are innervated primarily by the SNS efferent nerves, which provide a major pathway for the brain to regulate immune function and tissue repair (Bellinger et al., 2006).

1.5.1.1. **Bone Marrow**

The regulation of haematopoiesis and lymphopoiesis is mediated by the nerves within the bone marrow (Bellinger et al., 2006). SNS nerves are in close proximity with hematopoietic and stromal cells in the bone marrow (Tabarowski, Gibson-Berry, and Felten, 1996; Felten et al., 1985). A study of bone marrow using electron microscopy discovered that efferent nerve terminals are distributed between the periarterial adventitial (PAA) cells, a type of stromal cell that is an important source of growth factors (Yamazaki and Allen, 1990). It was also discovered that these efferent nerves also appear in sinus PAA cells, which form a gap junction for the bone marrow stromal cell and the efferent nerve terminals (Yamazaki and Allen, 1990). The close spatial
relationship between the nerves and the haematopoietic and stromal cells in the bone
marrow suggests a role of the SNS in haematopoiesis (Bellinger et al., 2006).

There is also evidence to suggest that a suppressive effect is exerted by the SNS on
specific bone marrow subpopulations during acute stress by β-AR stimulation (Dresch
et al., 1981; Beckman, Mirand, and Fisher, 1980). Likewise for α-ARs, there is
evidence to suggest that stimulation of α₁-ARs on bone marrow cells can suppress
myelopoiesis and enhance lymphopoiesis (Reviewed in Maestroni, 1995).

1.5.1.2. Thymus

The thymic sympathetic nerves arise from the stellate ganglia and other small ganglia of
the thoracic SNS chain (Bulloch and Pomerantz, 1984). The SNS nerves enter the
thymus as dense nerve plexuses with blood vessels and are primarily found in the
medulla. The SNS nerves exit the plexuses to the parenchymal regions of the cortex
where the nerves are situated with the stromal cells and thymocytes (Tollefson and
Bulloch, 1990; Bulloch and Pomerantz, 1984; Bellinger et al., 2006).

Radioligand studies and Northern blot analysis suggests that β₂-AR are expressed on
thymic cell membranes (Marchetti et al., 1994). Radioligand studies and RT-PCR
studies have also shown that thymic epithelial cells, which are important in the process
of negative and positive selection in the thymus, also express β₁- and β₂-ARs (Kurz et
al., 1997).

In general there are limited studies on the expression of α-AR on thymocytes.
Immunohistochemistry and flow cytometry studies provide evidence of expression of
α₁-AR expression on thymic cells (Pesic et al., 2009). Pharmacological studies using
$\alpha_2$-AR agonists indicate that $\alpha_2$-AR expression is also present in thymic cells (Cupic et al., 2003; Colic et al., 2000).

1.5.2. Adrenergic Signalling on Immune Cells

1.5.2.1. Lymphocytes

1.5.2.1.1. Beta-ARs

The expression of $\beta$-ARs on lymphocytes has been well studied. It has been well established that T cells express less $\beta$-AR than B cells (Bidart et al., 1983). Studies have shown that B cells express greatest number of $\beta$-ARs, followed by $T_C$ cells and $T_H$ cells (Karaszewski et al., 1990). It was then reported that CD8$^+$ suppressor T cells expressed more $\beta$-AR than $T_C$ cells (Karaszewski et al., 1991).

Stimulation of $\beta$-AR on lymphocytes may also result in the stimulation of immunity. Noradrenaline may promote DC production of IL-12 that mediates differentiation of naive $T_H$ cells into $T_{H1}$ cells and increase the amount of IFN-$\gamma$ produced by $T_{H1}$ cells (Swanson, Lee, and Sanders, 2001). In B cells, the production of IgE and IgG1 increased when NAD was added either post exposure to antigen (TNP) or within 24 hours of culture with $T_{H2}$ cells (Kasprowicz et al., 2000).

1.5.2.1.2. Alpha-ARs

Little data exists regarding the expression of $\alpha$-ARs on lymphocytes. Ligand binding studies have suggested that there is expression of $\alpha_2$-ARs in $T_H$ and $T_C$ cells, as well as their up-regulation upon exposure to AD (Jetschmann et al., 1997). However, it can be concluded that T cells do express both $\alpha_1$-ARs and $\alpha_2$-ARs. mRNA studies have
shown that T cells expressed both \( \alpha_1 \)-ARs and \( \alpha_2 \)-ARs mRNA. It was also shown that the expression of the mRNA was significantly higher upon exposure to Con-A, a T cell stimulator, than compared to baseline expression (Bao et al., 2006). Results from the same study also demonstrated that the stimulation of \( \alpha_1 \)-ARs did not significantly affect T cell proliferation and IFN-\( \gamma \) and IL-4 production. However, stimulation of \( \alpha_2 \)-ARs did suppress T cell proliferation as well as pro- and anti-inflammatory cytokine production.

There has been limited studies about the expression of \( \alpha_1 \)-ARs in B cells and there are no current studies to suggest that there is specific expression of \( \alpha_1 \)-ARs on B cells (Reviewed in Grisanti, Perez, and Porter, 2011).

### 1.5.2.2. Monocytes/Macrophages

In studies of \( \beta \)-ARs expression on monocytes, it was shown that the \( \beta \)-AR density on monocytes was about 2400 binding sites/cell, and this further increased to 3220 binding sites/cell after physical exercise (Ratge et al., 1988). There is some evidence to suggest that the density of \( \beta \)-ARs on monocytes changes during different stages of maturation. Indeed, during the maturation of monocytes to macrophages in vitro, there is a loss in \( \beta \)-AR responsiveness and function to \( \beta \)-AR agonists (Baker and Fuller, 1995). This could also explain why \( \beta \)-AR agonists lack significant anti-inflammatory effects on alveolar macrophages or in clinical asthma (Ezeamuzie, Shihab, and Al-Radwan, 2011).

Activation of \( \beta \)-ARs on monocytes usually generates anti-inflammatory effects including: the inhibition of oxygen radical production (Schopf and Lemmel, 1983), up
regulation of tumour necrosis factor (TNF) receptors and a reduction in TNF production
and the inhibition of LPS induced macrophage inflammation protein-1α (MIP-1α),
which has an important role in the development of inflammatory responses during
infections by regulating leukocyte trafficking and function (Li et al., 2003). It is
interesting to note that upon activation of β2-AR, the addition of LPS or IL-1 causes
the stimulated monocytes to increase the production of the inflammatory cytokine IL-8. It
also however enhanced the production of the anti-inflammatory cytokine IL-10
(Kavelaars et al., 1997).

RT-PCR studies have shown that the human monocytic cell line, THP-1, expresses
mRNA for α1B- and α1D-AR subtypes (Heijnen et al., 2002). The expression of α1A-
AR was only detectable when the pro-inflammatory cytokines TNF-α and IL-1β were
added to the cell culturing media. It is interesting to note that other reports using the
THP-1 cell line showed that none of the α1-AR subtypes are expressed; however,
following exposure to dexamethasone or the β2-AR agonist terbutaline, α1B- and α1D-
AR subtypes could be detected by RT-PCR (Rouppe van der Voort et al., 1999). This
suggests that α1-AR expression levels on monocytes change according to conditions
which may account for the variability in reporting.

In regards to the functional relevance in α1-AR, a study has shown that in LPS
stimulated human monocytes, the selective α1-AR agonist phenylephrine can induce the
pro-inflammatory cytokine IL-1β production. The production is inhibited when a
selective α1-AR antagonist or inhibitors of PKC are applied (Grisanti et al., 2011).
Similar results were also seen when the non-selective β1-AR agonist isoproterenol was
added to LPS stimulated human monocytes causing an increase in IL-1β. The
application of a selective $\beta_1$-AR antagonist inhibited the production of IL-1$\beta$ (Grisanti et al., 2010).

### 1.5.2.3. Dendritic Cells

There is evidence to suggest that there is expression of ARs on DCs. Immature murine DCs express $\alpha_{1B}$-AR mRNA that is lost upon DC maturation in the lymph nodes (Maestroni, 2000). It is suggested that $\alpha_{1B}$-AR controls DC migration due to the release of NE from lymph nodes influencing the migration of the immature DCs.

In Langerhans cells, DCs that are located in the skin and mucosa express $\alpha_{1A}$-, $\beta_1$-, and $\beta_2$-AR mRNA (Seiffert et al., 2002). It was discovered in the study that EP or NE inhibited the antigen presenting ability of the Langerhans cells. This inhibition was however blocked by a $\beta_2$-AR antagonist, but not by an $\alpha$-AR antagonist.

While many studies have been performed on murine DCs, very little work has been carried out on humans. One study examined the activation of $\beta_2$-ARs by $\beta_2$-AR agonists on human DCs that had been stimulated by either LPS or CD40-CD40L interactions. It was discovered that IL-12 production is inhibited via increased cAMP levels, therefore inhibiting the development of T$_{H}1$ cells while promoting T$_{H}2$ cell differentiation (Panina-Bordignon et al., 1997).

There seems to be extensive evidence to suggest that ARs on murine DCs influence the DC-T cell interactions and thus contributes to the modulation of the adaptive immune
1.6. Adrenergic Signalling in Inflammatory Diseases

1.6.1. Asthma

Asthma is characterised by recurrent symptoms of airway obstruction, bronchial hyperresponsiveness and with evidence of chronic inflammation, which can be reversed spontaneously or after the use of medication. It is the most common paediatric chronic inflammatory airway disease worldwide (Raedler et al., 2015). While there is a variety of asthma phenotypes, clinical asthma is classed broadly into two categories, allergic asthma (AA) and non-allergic asthma (NA), with the former being the most prevalent especially in children (Agache et al., 2012; Romanet-Manent et al., 2002).

AA is characterised by eosinophilic airway inflammation that has specific sensitisation to various antigens and increased levels of IgE and blood eosinophil count. T_{H}2 cytokines are the major drivers of AA with increases in IL-5 and IL-4/IL-13 (Agache et al., 2012; Raedler et al., 2015; Romanet-Manent et al., 2002). The increased numbers of eosinophils can be attributed to the increase in IL-5 secretion, with the increased proliferation and activation of eosinophils mediated by IL-5. IL-4 and IL-13 share the same activity in class switching B cells to IgE production and activating monocytes and macrophages and increasing the production of airway mucus through goblet cell hyperplasia (Robinson, 2010; Wills-Karp, 2004). The increase in production of mucus in respiratory epithelial cells is also thought to be a consequence of increased allergen-induced IL-9 secretion (Longphre et al., 1999).
The cytokines secreted by the airway epithelium, including IL-25, IL-33 and thymic stromal lymphopoitin, might also be an important driver of AA (He and Geha, 2010; Robinson, 2010).

There is evidence to suggest that the expression of $\alpha_1$-AR maybe essential in the pathogenesis of asthma (Davis, Paget, and Turi, 1985). Studies have shown that $\alpha_1$-AR hypersensitivity is a common feature in asthmatic patients (Davis, Paget, and Turi, 1985; Herman et al., 1990). However, this remains controversial (Islami et al., 2014).

$\beta$-AR expression in asthma has been more well studied compared to $\alpha$-ARs. There is evidence to suggest that the expression of $\beta$-AR is increased in asthmatic airway smooth muscle but these receptors are uncoupled, leading to functional hyporesponsiveness (Reviewed in Bai, 1992). Defects in the $\beta_2$-AR gene while not the primary cause of asthma may play an accessory role in the pathogenesis of asthma (Reihsaus et al., 1993). It has been shown that a glutamine 27 $\beta_2$-AR polymorphism is associated with increased levels of IgE in asthma (Dewar et al., 1997).

### 1.6.2. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterised by persistent synovitis, systemic inflammation, auto-antibodies (such as the rheumatoid factor), citrullinated peptides, immune cell infiltration and cartilage destruction (Scott, Wolfe, and Huizinga, 2010; Uysal et al., 2009).

The synovial and cartilage cells are the cells predominantly affected by RA (Scott, Wolfe, and Huizinga, 2010). The synovial cells can be divided into two groups, the fibroblast-like and the macrophage-like synoviocytes. It is suggested that the over
production of pro-inflammatory cytokines is predominantly mediated by the macrophage-like synoviocytes. The fibroblast-like synoviocytes also display abnormal behaviour (Scott, Wolfe, and Huizinga, 2010; Feldmann, Brennan, and Maini, 1996a). Studies suggest that the fibroblast-like synoviocytes infiltrate the cartilage and this abnormal behaviour correlates with the joint destruction associated with RA (Müller-Ladner et al., 1996; Tolboom et al., 2005).

The over production and over expression of TNF-α, IL-1 and IL-6 appear to mediate RA (Feldmann, Brennan, and Maini, 1996b). The interaction between T and B cells, synovial-like fibroblasts and macrophages leads to the production of such pro-inflammatory cytokines (Scott, Wolfe, and Huizinga, 2010; Feldmann, Brennan, and Maini, 1996b).

Studies conducted applying anti-TNF-α antibodies reduced the production of other inflammatory cytokines including IL-1, GM-CSF, IL-6 and the chemokine IL-8 (Brennan et al., 1989). The blocking of IL-1 using a IL-1 receptor antagonist reduced the production of IL-6 and IL-8 but not TNF-α (Feldmann, Brennan, and Maini, 1996a). Therefore it can be concluded that TNF-α appears to be an important mediator in RA and that pro-inflammatory cytokines are linked in a network or cascade (Feldmann, Brennan, and Maini, 1996b).

There have been suggestions that neutrophils may also impact on the chronic inflammatory nature of RA. Arginine deaminase 4 (AD4), an enzyme whose activity is dependent on the levels of extra-cellular Ca^{2+}, is found in neutrophils and could be released when the neutrophils die or become activated. It was found that AD4 regulates and creates citrulline in proteins (Andrade et al., 2010; Cooper, Palmer, and Chapple,
It is interesting to note that the presence of citrullinated proteins is associated with RA (Uysal et al., 2009).

The role of ARs in RA in general is not very clear with more research into β-AR than compared to α-ARs. With the majority of α-ARs expression on innate immune cells (Bellinger et al., 2008), it is interesting to note that RA patients with high disease activity demonstrated α₁-AR expression on their lymphocytes with catecholamines mediating their effect through α₁-ARs. It was also noted that there was a decrease in β₂-AR density (Wahle et al., 1999).

Similar observations were found in juvenile rheumatoid arthritis (JRA), also known as juvenile idiopathic arthritis. Functional α₁-ARs appeared to be upregulated with increased expression observed on the leukocytes of JRA patients when compared to healthy subjects who displayed no expression of α₁-ARs on their leukocytes (Heijnen et al., 1996). Stimulation of these α₁-ARs resulted in increased levels of the pro-inflammatory mediator IL-6 (Roupe van der Voort et al., 2000).

β₂-ARs may play an important role in RA. β₂-ARs have been shown to have a role in the modulation of the immune response of the SNS (Levine et al., 1988; Coderre et al., 1990; Lubahn et al., 2004) and are found to be expressed on both innate and adaptive immune cells (Bellinger et al., 2008).

It was observed that the density of β₂-ARs on peripheral blood mononuclear cells (PBMC), especially T cell cells and B cells, was much lower compared to healthy individuals (Wahle et al., 2001; Baerwald et al., 1997). Therefore it can be concluded that RA PBMCs are also less responsive to NE when compared to healthy subjects. A study showed that B cells from RA patients had impaired responsiveness and signalling to β₂-AR agonists (Wahle et al., 2001). This is of importance because it has been
shown that changes in $\beta_2$-AR density plays a role in determining the cytokines produced by PBMCs (Wahle et al., 2005). With decreased levels of IFN-$\gamma$ and IL-6 produced, a T$_{H}2$ response (humoral immune response) is favoured.

In studies using an adjuvant-induced arthritis model in rats, the application of $\beta_2$-AR agonist prior or at the onset resulted in the worsening of the disease (Coderre et al., 1990; Lubahn et al., 2004). In contrast, the application of $\beta_2$-AR antagonists seemed to decrease the severity of the disease (Coderre et al., 1990). Interestingly, the application of an $\alpha$-AR antagonist or $\beta_2$-AR agonist at the onset of arthritis drastically reduced the severity of the disease indicating that ARs may have a role in the pathogenesis of RA (Lubahn et al., 2004).

1.7. Summary

The immune system is divided into two major sections, the innate and adaptive immune system, each with their own benefits and limitations (Chaplin, 2010). Inflammation is a complex and important mechanism the immune system can utilise (Medzhitov, 2008). It is mediated by a variety of immune cells including APCs and T$_{H}$ cells (Medzhitov, 2010). Adrenergic receptors are member of the GPCR superfamily where they have a wide range of functions in virtually all tissues (Marino and Cosentino, 2013). Adrenergic receptors mediate the action of the endogenous catecholamines EP and NE transmitted by the SNS (Marino and Cosentino, 2013). Adrenergic receptors have been found to be expressed on immune cells with evidence to suggest that the SNS may be mediating the actions of the immune response and furthermore, inflammation (Marino and Cosentino, 2013). Targeting ARs with agonists and antagonists can potentially
arrest some symptoms associated with inflammatory diseases such as bronchoconstriction associated with asthma and severity of disease of RA.

1.8. Hypothesis and Aims

While the expression of β-ARs on immune cells during inflammation has been well studied, α₁-ARs on the contrary has not (Reviewed in Marino and Cosentino, 2013; Reviewed in Grisanti, Perez, and Porter, 2011).

The hypothesis of this project is inflammatory stimuli will differentially upregulate α₁-AR expression on mouse lymphoid immune cells.

The aims of this project will be to:

1. Investigate the baseline α₁-AR expression on immune cells in normal mouse lymphoid tissue.
2. Determine whether α₁-AR expression changes on immune cells after exposure to inflammatory stimuli.
2. Material and Methods

Animal cadavers acquired for this project were approved by the Murdoch University Animal Ethics Committee (Animal ethics – cadaver notice). All reagents used were prepared according to manufacturers specifications, unless indicated otherwise. Additional details (manufacturer and catalogue number) of the reagents are listed in Appendix A.

2.1. Mice

Pathogen-free female BALB/c mice within the age range of 9 to 14 weeks were obtained from the Animal Resource Centre (ARC), Murdoch, WA. Mice were culled by the ARC immediately prior to tissue collection.

2.1.1. Tissue Collection

Spleens from two mice for each experiment were collected aseptically and pooled to minimise biological variability between individual mice. Mice abdomens were sprayed with 70% ethanol (EtOH) before dissection with EtOH-sterile scissors and tweezers. Collected spleens were stored in 5% foetal bovine serum (FBS) and Dulbecco’s phosphate-buffered saline (DPBS) (5% FBS in DPBS) and kept on ice until use for cell extraction.

2.2. Preparation of Splenocyte Suspension

Splenocytes were extracted from collected spleens as a cell suspension. Each spleen was injected at opposite ends with 5% FBS in DPBS using a 5 mL syringe and a 25 gauge needle. Cells dislocated by the injected buffer were gently teased out through the holes made during injection. Clumps of cells were disaggregated by syringing with the same needle and filtered through a cotton wool column. The cell suspension was
initially spun at 1200 RPM for 7 minutes before the supernatant was removed by aspiration. Red blood cells in the pellet were lysed in 5 mL of 1X Flow Cytometry Mouse Lyse Buffer for 10 minutes at room temperature. 5% FBS in DPBS was added in excess to stop the lysis reaction and lysates were removed by aspiration of the supernatant following centrifugation (same conditions as previous centrifugation).

The resultant pellet was resuspended in 3mL of 5% FBS in DPBS before filtering through a nylon membrane yielding a single cell suspension of splenocytes. The number of viable splenocytes present in the suspension was counted manually with an improved Neubauer haemocytometer, at a 1 in 10 dilution of the cell suspension with 0.4% Trypan Blue staining solution. After counting $\geq 100$ viable cells in the middle square, the final concentration of viable cells in cells/mL was determined using the following equation:

$$\text{Cell Concentration (Cells/mL)} = \frac{\text{number of cells counted}}{\text{number of rows counted}} \times \frac{5 \text{ rows}}{\text{rows counted}} \times \text{dilution factor} \times 10^4$$

2.2.1. Storage

Cell suspensions were stored in 1 mL of 5% FBS in DPBS at 4°C for up to two days for further use if required.
2.3. Cell Culturing

2.3.1. Splenocytes

Spleens were taken from 9 – 14 week old female BALB/c mice (ARC, Murdoch, WA) splenocytes were extracted according to the method from section 2.2 (Preparation of Splenocyte Suspension) and resuspended in Roswell Park Memorial Institute (RPMI) media (Gibco) supplemented with 10% FBS. The cell suspension made up to 1×10^6 cells/mL at 6 mL and plated in 96 well round-bottom plates (200μL/well) (Falcon, US). Some of the wells also had 1μg/mL LPS (1mg/mL, Enzo) supplemented into the media. The cells were incubated at 37°C in 5% CO₂.

Separate splenocyte cultures were phenotyped after 24 hours using antibodies which included the lymphocyte and myeloid panel and BODIPY FL-prazosin (BPr) (100μM, Life Technologies).

2.3.2. Cell lines

2.3.2.1. CHO Cells

Chinese hamster ovary (CHO) cells (from Chelsea Back and Linda Wijaya) are derived from the ovary of the Chinese hamster. Two variants of the CHO cells were used with one variant being transfected with α₁-AR (α₁-CHO) and the other variant being non-transfected (NT-CHO). Both variants of CHO cells were cultured on DMEM/F12 media (Gibco, supplemented with 10% FBS, 2mM glutamine. α₁-CHO cells were selected using G418, gentamycin (400μg/mL, Calbiochem). All cells were incubated at 37°C in 5% CO₂.
2.3.2.2. Subculturing/Passaging

CHO cells were subcultured when the desired confluency (80%) was reached. The DMEM/F12 growth media was removed and the monolayer of cells was washed with DPBS (Sigma). 500μL of trypsin (diluted down to a 1x solution, Gibco) was added to the monolayer. The flask was then incubated with the trypsin to disrupt the monolayer. Once the monolayer was disrupted, the cells were resuspended in DMEM/F12 growth media and aspirated. The aspirated cell suspension was then aliquotted into new flasks. Subculturing was used routinely to maintain the cell line with a maximum of 12 passages performed before the cells were discarded.

2.4. Flow Cytometric Analysis of Labelled Cells

Splenocytes collected from spleens were labelled with several fluorescent markers and analysed by flow cytometry.

2.4.1. Fluorochrome-conjugated Monoclonal Antibodies in Staining Panels

Splenocytes collected were split into two groups, which were then individually labelled with either a lymphocyte or myeloid staining panel, of which consisted of several markers (Table 2.1 and Table 2.2).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Antibody</th>
<th>Fluorophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3e</td>
<td>APC</td>
<td>B220</td>
<td>APC-Cy7</td>
</tr>
<tr>
<td>CD4</td>
<td>APC-Cy7</td>
<td>CD11b</td>
<td>APC</td>
</tr>
<tr>
<td>CD8a</td>
<td>PerCP-eFluor710</td>
<td>CD11c</td>
<td>PE-Cy7</td>
</tr>
<tr>
<td>CD19</td>
<td>BV510</td>
<td>F4/80</td>
<td>PerCP-Cy5.5</td>
</tr>
<tr>
<td>CD25</td>
<td>BV421</td>
<td>Ly6GC</td>
<td>BV510</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MHC II</td>
<td>BV421</td>
</tr>
</tbody>
</table>
Table 2.2. Fluorescent reagents used in each staining panel and the respective emission filters used for detection.

<table>
<thead>
<tr>
<th>Panel</th>
<th>Fluorescent reagent</th>
<th>Clone</th>
<th>Emission Filter</th>
<th>Laser Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte</td>
<td>APC-CD3e</td>
<td>145-2C11</td>
<td>660/20 BP</td>
<td>663nM</td>
</tr>
<tr>
<td></td>
<td>APC-Cy7-Cy7</td>
<td>GK1.5</td>
<td>780/60 BP</td>
<td>663nM</td>
</tr>
<tr>
<td></td>
<td>PerCP-eFluro710-Cy7a</td>
<td>53.67</td>
<td>670 LP</td>
<td>488nM</td>
</tr>
<tr>
<td></td>
<td>BV510-Cy7</td>
<td>1D3</td>
<td>450/50 BP</td>
<td>405nM</td>
</tr>
<tr>
<td></td>
<td>BV421-Cy7</td>
<td>PC61</td>
<td>510/50 BP</td>
<td>405nM</td>
</tr>
<tr>
<td>Myeloid</td>
<td>APC-Cy7-B220</td>
<td>RA3-6B2</td>
<td>780/60 BP</td>
<td>663nM</td>
</tr>
<tr>
<td></td>
<td>APC-CD11b</td>
<td>M1/70</td>
<td>660/20 BP</td>
<td>633nM</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7-Cy7</td>
<td>HL3</td>
<td>780/60 BP</td>
<td>488nM</td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5-F4/80</td>
<td>BM8</td>
<td>670 LP</td>
<td>488nM</td>
</tr>
<tr>
<td></td>
<td>BV510-Ly6GC</td>
<td>RB6-8C5</td>
<td>450/50 BP</td>
<td>405nM</td>
</tr>
<tr>
<td></td>
<td>BV421-MHC II</td>
<td>M5/114.15.2</td>
<td>540/50 BP</td>
<td>405nM</td>
</tr>
</tbody>
</table>

2.4.2. Staining of Splenocytes Using the Lymphocyte and Myeloid Panels

Cells were kept on ice (≤ 4°C) at all times during staining and flow analysis to avoid capping (redistribution and internalisation of surface molecules), which would interfere with fluorescence intensity measurements (Loor, Forni, and Pernis, 1972). Stained samples were stored protected from light to prevent photobleaching.

2.4.2.1. Optimisation of Antibody Staining by Titration

To determine optimal staining concentrations, all antibodies were titrated on splenocytes as described in results. Antibodies from the lymphocyte and myeloid panels were titrated in a series of serial dilutions (1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600). CD8a and MHC II were titrated up to 1:3200.

The serial dilutions were prepared in FACS buffer. Cells (1×10^6 cells) were incubated in 100μL of each dilution for 30 minutes on ice. Labelled cells were then washed with
2mL FACS buffer to remove excess antibodies. Unless otherwise mentioned, all cells were resuspended in 600μL FACS buffer for flow analysis.

**2.4.2.2. Antibody Panel Staining**

Non-specific binding between fluorochrome-conjugated mAbs and low affinity Fc receptors expressed on surface of cells was blocked to prevent increased background staining. 2μL Mouse BD Fc Block (purified rat anti-mouse CD16/32) was added to 1×10^6 cells in 100μL FACS buffer and incubated for five mins. Cells were then immediately used for staining.

A 100μL master mix containing the antibodies from either the lymphocyte or myeloid staining panel was prepared and was introduced and incubated to the cell suspension for 30 minutes. Cells were then washed and resuspended in 700μL of FACS buffer.

**2.4.3. Determination of Viability/Dye Exclusion Test**

Due to fluorochromes used, no viability dye was compatible in any of the staining panels. In an attempt to rectify this issue, Propidium Iodide (PI) was used in a separate unstained sample to test for cell viability and give an accurate reading. Trypan blue cells counts (as described in 2.2 Preparation of Splenocyte Suspension) were performed on the cell suspensions on the day of FACS staining and flow cytometric analysis to determine the viability of the cell population.

**2.4.4. Instrumental and Analysis Software**

Cells were analysed within a period of one day after a period of one day after labelling with the BD FACSCanto II flow cytometer (BD Biosciences, USA) at Murdoch University.
Data on fluorescence intensity and forward and side scatter of 300,000 - 500,000 cells was collected with BD FACSDiva software (BD Biosciences, USA; PC version 6.1.2). The data collected was analysed using FlowJo software (Tree Star Inc., USA; PC version 10.0.8).

### 2.4.5. Controls and Compensations

Unstained controls, single stained controls and fluorescence minus one (FMO) controls relevant to each panel were analysed together with their respective panels. Unstained controls contained cells without any fluorochrome markers. Single stained controls contained cells with that had been stained with only one fluorochrome marker from the panels. These cells were analysed to adjust for spillover (spectral overlap) between detection channels. After gating for the positive and negative cell populations for each single stain, compensation matrices were generated using the compensation tool in BD FACSDiva and onto the FMOs and experimental samples.

FMO controls, each comprised of every fluorochrome marker in a panel minus the one being controlled for, were prepared and analysed to help with the determination of gating boundaries. Six FMO controls were analysed for the lymphocyte panel and seven FMO controls were analysed for the myeloid panel.

### 2.5. Optimisation of Prazosin Blocking and BODIPY FL-Prazosin staining by Titration

To determine the optimal staining concentrations of the BODIPY FL-prazosin (BPr) and blocking concentrations of prazosin (1.19mM, Life Technologies), the staining
reagent was titrated on both transfected $\alpha_1$-adrenergic receptors ($\alpha_1$-AR) and non-transfected CHO cells. Prazosin was titrated using transfected $\alpha_1$-CHO cells in combination with BPr to determine the optimal blocking concentration. CHO cells were kept at on ice ($\leq 4^\circ$C) at all times during staining and flow analysis and protected from light.

2.6. **BODIPY-Prazosin**

Transfected $\alpha_1$-AR CHO and non-transfected CHO cells were used to titrate both the BODIPY-prazosin (BPr) staining and the competitive blocking of terazosin. Serial dilutions resulted in BPr concentrations of 400nM, 200M, 100nM, 50nM and 25nM. Cells ($1 \times 10^6$) were incubated in 100 $\mu$L of each concentration for 30 minutes. All cells were washed once as described in section 2.4.2.1 to remove excess BPr. The cells were then resuspended in 1 mL of FACS buffer after wash for flow analysis.

2.7. **Terazosin Competitive Blocking**

Transfected $\alpha_1$-AR CHO cells were used to titrate the competitive blocking of terazosin. Serial dilution of terazosin resulting in concentrations of 5 $\mu$M, 2.5 $\mu$M, 1 $\mu$M and 500nM. Cells ($1 \times 10^6$) were stained in two separate 20 minute incubation steps, the first with dilutions of terazosin and the second with pre-determined dilutions of BPr.

2.8. **Statistic Analysis and Graphing Software**

Numerical data for the averages of triplicates are reported as mean $\pm$ standard error of mean (SEM). Two sample T-tests was used to test for significant changes after cell stimulation with LPS. P-values of $<0.05$ were considered statistically significant.
Statistical analysis and graphing were performed using SigmaPlot (Systat Software Inc., USA; PC version 13).
3. Results

3.1. Optimising Lymphoid and Myeloid Antibody Panels on Murine Spleen Cells

Before the staining reagents were used in combination for lymphocyte and myeloid panel staining, the optimal concentration for each conjugated monoclonal antibody was determined by titration on normal spleen cells and then analysis by FACS. This was to ensure that each staining reagent provided a strong signal without significant non-specific background staining, which can be caused by both cellular auto-fluorescence and non-specific binding.

Two criteria were used to compare the flow data collected from each antibody titration to determine the optimal staining concentration: firstly, the optimal titre had to demonstrate minimal or no background staining, which would be evident when the fluorescence intensity of the negative population of each titration was compared to the unstained cell population from the unstained control. Second, the optimal titre had to provide the best distinction between the negative and positive populations, at the lowest concentration possible.

Overlaying histograms, which displayed fluorescence intensity (or otherwise termed geometric intensity) on the x-axis and cell count on the y-axis, were used to compare the fluorescence profiles between dilutions and optimal staining titres based on the criteria described above.
3.2. Monoclonal Antibody Titrations

3.2.1. Optimisation of Lymphocyte Panel

APC-CD3e mAb was used to identify cells expressing CD3e, a pan-T cell marker, on their surface (Chetty and Gatter, 1994). The optimal staining titre of 2µg/mL (1:100 dilution) for APC-CD3e was determined from a single overlaying histogram and an antibody titration curve (Figure 3.1a, b).

APC-Cy7 CD4 mAb was used to identify cells expressing CD4, a primarily helper T cell (CD4⁺) marker, on their surface (Dialynas et al., 1983). The optimal staining titre of 1µg/mL (1:200 dilution) for APC-Cy7-CD4 was determined from comparing a single overlaying histogram (Figure 3.2a, b).

PerCP-eFluor710-CD8a mAb was used to identify cells expressing CD8a, a primarily cytotoxic T cell (CD8⁺) marker, on their surface (Ledbetter et al., 1980). The optimal staining titre of 0.5µg/mL (1:400 dilution) for PerCP-eFluor710-CD8a was determined from a single overlaying histogram (Figure 3.3a, b).

BV510-CD19 mAb was used to identify cells expressed CD19, a pan-B cell marker, on their surface (Kozmik et al., 1992). The optimal staining titre of 2µg/mL (1:100 dilution) for BV510-CD19 was determined from a single overlaying histogram (Figure 3.4a, b).

BV421-CD25 mAb was used to identify cells expressing CD25, a marker primarily found on activated lymphocytes, on their surface (Lowenthal et al., 1985). The optimal staining titre of 2µg/mL (1:100 dilution) was determined from a single overlaying histogram (Figure 3.5a, b).
Figure 3.1. APC-CD3e mAb titration. (A) Titration curve for APC-CD3e at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen optimal titre was 1:100 dilution (indicated by the asterisk (*)). Data are presented as mean ± SEM, n = 4.

(B) A representative overlay histogram of cells staining positively and negatively with 1:100 dilution of APC-CD3e. Clear separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.
Figure 3.2. APC-Cy7-CD4 mAb titration. (A) Titration curve for APC-Cy7-CD4 at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen titre was 1:400 dilution (indicated by the asterisk (*). Data are presented as mean ± SEM, n = 3.

(B) A representative overlay of cells staining positively and negatively with 1:400 dilution of APC-Cy7-CD4. Clear separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.

Figure 3.3. PerCP-eFluor710-CD8a mAb titration. (A) Titration curve for PerCP-eFluor710-CD8a at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen titre was 1:400 dilution (indicted by the asterisk (*). Data are presented as mean ± SEM, n = 3.

(B) A representative overlay of cells staining positively and negatively with 1:400 dilution of PerCP-eFluor710-CD8a. Clear separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.
Figure 3.4. **BV510-CD19 mAb titration.** (A) Titration curve for BV510-CD19 at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen titre was 1:100 dilution (indicated by the asterisk (*)). Data are presented as mean ± SEM, n = 4.

(B) A representative overlay of cells staining positively and negatively with 1:100 dilution of BV510-CD19. Clear separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.
Figure 3.5. BV421-CD25. (A) Titration curve for BV421-CD25 at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen titre was 1:100 dilution (indicated by the asterisk (*)). Data are presented as mean ± SEM, n = 4.

(B) A representative overlay of cells staining positively and negatively with 1:100 dilution of BV421-CD25. Indistinct separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peaks of the dilution, demonstrating low levels of background staining.
3.2.2. Optimisation of Myeloid Panel

APC-Cy7-B220 mAb was used to identify cells expressing B220, primarily a B lymphocyte marker but also useful for identifying subsets of myeloid cells e.g. plasmacytoid dendritic cells (Bleesing et al., 2001). The optimal staining titre of 1\(\mu\)g/mL (1:200 dilution) was determined from a set of three overlapping histograms from three titrations (Figure 3.6a, b).

APC-CD11b mAb was used to identify cells expressing CD11b, a granulocyte, monocytes and dendritic cell (DC) marker (Lagasse and Weissman, 1996; Gao et al., 2003). The optimal staining titre of 1\(\mu\)g/mL (1:200 dilution) was determined from a

![Figure 3.6. APC-Cy7-B220 mAb titration. (A). Titration curve for APC-Cy7-B220 at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen optimal titre was 1:200 (indicated by the asterisk (*)). Data are presented as mean ± SEM, n = 3.](image)

(B) Overlay histogram of cells staining positively and negatively with 1:200 dilution of APC-Cy7-B220. Clear separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.
single overlaying histogram (Figure 3.7a, b).

PE-Cy7-CD11c mAb was used to identify cells expressing CD11c, a primarily DC marker but also expressed by macrophage populations, on their surface (Gao et al., 2003). The optimal staining titre of 1μg/mL (1:200 dilution) was determined from a single overlaying histogram (Figure 3.8a, b).

PerCP-Cy5.5-F4/80 mAb was used to identify cells expressing F4/80, a primarily macrophage marker, on their surface (Geutskens et al., 2005). The optimal staining titre of 0.5μg/mL (1:400 dilution) was determined from a single overlaying histogram (Figure 3.9a, b).

Figure 3.7. APC-CD11b mAb titration. (A) Titration curve for APC-CD11c at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen optimal titre was 1:200 (indicated by the asterisk (*)). Data are presented as mean ± SEM, n = 4.

(B) Overlay histogram of cells staining positively and negatively with 1:200 dilution of APC-CD11b. Indistinct separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.
BV510-Ly6GC mAb was used to identify cells expressing both Ly-6G and Ly-6C, primarily a granulocyte marker, on their surface (Lagasse and Weissman, 1996). The optimal staining titre of 1μg/mL (1:200 dilution) was determined from a single overlaying histogram (Figure 3.10a, b).

BV421-MHC II mAb was used to identify cells expressing MHC II, a pan-antigen presenting cell (APC) and B cell marker, on their surface (Ernst et al., 1988; Viville et al., 1993). The optimal staining titre of 0.25μg/mL (1:800 dilution) was determined from a single overlaying histogram (Figure 3.11a, b).

**Figure 3.8. PE-Cy7-CD11c mAb titration.** (A). Titration curve for PE-Cy7-CD11c at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen optimal titre was 1:200 (indicated by the asterisk (*)). Data are presented as mean ± SEM, n = 3.

(B) Overlay histogram of cells staining positively and negatively with 1:200 dilution of PE-Cy7-CD11b. Indistinct separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.
Figure 3.9. PerCP-Cy5.5-F4/80 mAb titration. (A). Titration curve for PerCP-Cy5.5-F4/80 at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen optimal titre was 1:400 (indicated by the asterisk (*)). Data are presented as mean ± SEM, n = 5.

(B) Overlay histogram of cells staining positively and negatively with 1:400 dilution of PerCP-Cy5.5-F4/80. Indistinct separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.
**Figure 3.10. BV510-Ly6GC mAb titration.** (A). Titration curve for PE-Cy7-CD11c at serial dilutions of 1:25 to 1:1600 was used to determine the optimal titre. The chosen optimal titre was 1:200 (indicated by the asterisk (*)). Data are presented as mean ± SEM, n = 3.

(B) Overlay histogram of cells staining positively and negatively with 1:200 dilution of PE-Cy7-CD11b. Indistinct separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.
Figure 3.11. BV421-MHC II mAb titration. (A) Titration curve for BV421-MHC II at serial dilutions of 1:25 to 1:3200 was used to determine the optimal titre. The chosen optimal titre was 1:800 (indicated by the asterisk (*)). Data are presented as mean ± SEM, n = 3.

(B) Overlay histogram of cells staining positively and negatively with 1:800 dilution of BV421-MHC II. Clear separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.
3.3. Optimisation of BPr Labelling

Due to the unavailability of a suitable monoclonal antibody specific for mouse $\alpha_1$-adrenergic receptor ($\alpha_1$-AR), the reagent BPr (otherwise known as BODIPY FL-prazosin), a high affinity $\alpha_1$-adrenergic receptor ($\alpha_1$-AR) specific fluorescent antagonist, was used to detect $\alpha_1$-AR (Sugawara et al., 2002). Originally the BPr titrated on the spleen cells, however, staining levels were very low making accurate titrations difficult (Figure 3.12).

![Figure 3.12. BPr titration on spleen cells. Overlay histogram of cells staining positively and negatively with 50nM of BODIPY-Prazosin. Indistinct separation between the positively (higher intensity) and negatively (lower intensity) stained populations was shown for the dilution. The unstained control (tinted curve) overlaps with the negative peak of the dilution, demonstrating low levels of background staining.](image)
In order to overcome this issue, BPr was titrated on Chinese Hamster Ovary (CHO) cells stably transfected with \( \alpha_1 \)-AR and compared to non-transfected CHO cells as controls (see Materials and Methods). A titration curve for BPr is shown in Figure 3.13a. At high concentrations of BPr (>200nM), non-specific staining was observed for both transfected and non-transfected CHO cells (data not shown). However, titration of BPr resulted in a noticeable drop in non-specific staining of BPr on non-transfected CHO cells while maintaining positive staining on transfected cells. Therefore, the optimal concentration for BPr to detect specific staining was determined to be 50nM (Figure 3.13a, b).
3.4. Determining the specificity of BPr for $\alpha_1$-AR

In order to confirm the specificity of BPr for $\alpha_1$-ARs, unlabelled terazosin, both specific $\alpha_1$-AR antagonist (Brosnan et al., 1985), were used in competitive binding assays with BPr on $\alpha_1$-AR CHO cells ($\alpha_1$-CHO) and analysed by FACS. A positive control of single BPr staining was done to compare with the competitor stained cells to ascertain whether blocking was indeed occurring or not.

A high positive percentage and fluorescence intensity was observed on $\alpha_1$-AR CHO cells when compared to the unstained $\alpha_1$-CHO cells (Figure 3.14a). However, after the addition of the terazosin, a marked decreased in positive percentage and fluorescence was observed in $\alpha_1$-CHO when compared to the unstained sample (Figure 3.14b).
In general when working with competitors, 100x the concentration of ligand of interest is used in competitive binding assays. Increasing the concentration of terazosin continued to reduce the fluorescence intensity of the 50nM BPr (Figure 3.15). The addition of terazosin had a marked effect on the geometric mean of the BPr decreasing. In conclusion, 5μM of terazosin was used due to the reduction seen when compared to the other terazosin concentrations and the reduction seen when compared to the 50nM BPr positive control.

**Figure 3.14. Relative mean fluorescence intensity of BPR before and after terazosin blocking.**

A) Overlay histogram of CHO cells that have been stained with 50nM BPR (blue dotted line) and 50nM BPR ± 5μM of terazosin (orange line). Clear separation between the positively (higher intensity) and negatively (lower intensity) stained population was seen for the 50nM BPR only. When incubated with terazosin, the fluorescence intensity of the BPR overlaps the unstained (tinted curve), demonstrating low levels of background staining and unspecific staining.

B) Bivariate contour plots for a single representation experiment of CHO cells stained with 50nM BPR before and after incubation with 5μM terazosin. CHO cells that were BPR⁺ were located in the right half while BPR⁻ was located in the left half.
The competitive blocking of the $\alpha_1$-CHO cells resulted in a significant decrease ($-64.73 \pm 8.97\%$, n=3) in the geometric mean of the $\alpha_1$-CHO cells after blocking ($P < 0.001$, n=3), averaging $136.5 \pm 1.27$ compared to the non-blocked $\alpha_1$-CHO cells with an average geometric mean of $440 \pm 107.87$.

Confirmation of specific binding was also observed when the geometric mean of the blocked $\alpha_1$-CHO cells was compared to the unstained $\alpha_1$-CHO cells. The geometric mean of the unstained $\alpha_1$-CHO cells averaged $138.33 \pm 1.67$, which indicated that geometric mean of the blocked $\alpha_1$-CHO cells was similar to the unstained ($P>0.05$, n=3). This therefore indicated that there was specific binding from the BPr (Figure 3.14a, b).
3.5. Gating for Cellular Analysis by Flow Cytometry

3.5.1. Initial Gating Strategy

Gates set early in analysis were identical for all experimental conditions and used consistently for all sample analyses. Cells were discriminated from cellular debris by gating broadly on forward scatter (FSC) and side scatter (SSC) characteristics (Figure 3.16a). Aggregates of cells were excluded with a doublet discriminator gate (Figure 3.16b). While PI could not be added to the full phenotyping panels due to compatibility issues with the multi-colour staining panels (see Materials and Methods), PI was added in an unstained population to identify the viability of cells within the initial gate (Figure 3.16c).

Figure 3.16. Initial cell gating strategy. Cellular debris and cell aggregates were excluded from analysis by sequential gating around cells and single cells on bivariate dot plots of A) forward scatter area (FSC-A) versus side scatter area (SSC-A), B) FSC-A versus forward scatter height (FSC-H). C) While not used in the early gating strategy, PI fluorescence intensity versus SSC-A was used to identify the live cell population.
3.5.2. Lymphocytes

3.5.2.1. T cells
Initially, T cells present in the heterogeneous cellular spleen cell suspensions were identified by the expression of the pan T-cell marker CD3e (Chetty and Gatter, 1994). On a contour plot of APC-CD3e (x-axis) versus BV510-CD19 (y-axis), T cells (CD3e⁺ CD19⁻) were located in the lower right quadrant (Figure 3.17a). Gating boundaries of the quadrant gates were determined using CD3e and CD19 FMO controls (data not shown).

3.5.2.1.1. CD4+ Helper and CD8+ Cytotoxic T cells

CD4⁺ helper and CD8⁺ cytotoxic T cells present in the cell suspension were identified by the expression of the co-receptor CD4 (helper T cells) and CD8a (cytotoxic T cells) respectively. Although CD4 and CD8a can also be expressed on non-T cells (Vremec et al., 2000), the analysis of these markers was restricted to CD3e⁺ T cells in the current study (Figure 3.17a).

On a contour plot of APC-Cy7-CD4 (x-axis) and PerCP-eFluor710-CD8a (y-axis), helper T cells (CD4⁺, CD3e⁺, CD8a⁻) and cytotoxic T cells (CD8a⁺, CD3e⁺, CD4⁻) were located respectively in the lower right and upper left quadrants (Figure 3.17B). Gating boundaries of the quadrant gates were determined using CD4 and CD8a FMOs (data not shown).
Figure 3.17 Isolation of T and B cells. (A) Bivariate contour plot for a representative experiment (n=3) of cells stained with APC-CD3e and BV510-CD19. T and B cells were located respectively in the lower right and upper left quadrants. Double negative cells were located in the lower quadrant while CD3e+ CD19+ cells were located in the upper right quadrant.

(B) Bivariate pseudocolour dot plot for a single representative experiment of cells stained with APC-Cy7-CD4 and PerCP-eFluor710-CD8a after T cells gating. CD4+ helper T cells and CD8+ cytotoxic T cells were located respectively in the lower right and upper left quadrants. Double negative cells were located in the lower quadrant while CD4+ CD8a+ cells were located in the upper right quadrant.

Figure 3.18. Phenotyping of CD4+ helper T cells. Contour plots for a representative experiment (n=3) of cells stained with APC-Cy7-CD4, PerCP-eFluor710-CD8a, BV510-CD19, BV421-CD25 and BPR after CD4+ T cell isolation. 

A) Gating of only CD4+ T cells was achieved following the gating of CD8a- CD19- located in the lower left quadrant. 

B) CD4+ T cells show low levels of CD25 expression with CD4+ CD25- and CD4+ CD25+ cells located respectively in the lower right and upper right quadrants. 

C) CD4+ T cells show very low levels of α1-AR expression with CD4+ BPR- and CD4+ BPR+ located respectively in the lower right and upper right quadrants.
3.5.2.1.1.1. Phenotyping of CD4+ helper T cells

Isolated CD4+ T cells underwent phenotyping using the lymphocyte panel to determine what markers were expressed. On a bivariate contour plot of PerCP-eFluor710-CD8a (x-axis) versus BV510-CD19 (y-axis), only CD4+ helper T cells (CD8a+ CD19+) were located in the lower left quadrant (Figure 3.18a). The CD8a+ CD19+ cells were used in further analysis with a bivariate contour plot utilising of APC-Cy7-CD4 (x-axis) versus BV421-CD25 (y-axis), regulatory T cells (CD4+ CD25+) were located in the upper right quadrant (Figure 3.18b). The CD8a+ CD19- cells were also used in further analysis with a bivariate contour plot utilising APC-Cy7-CD4 (x-axis) versus BV421-CD25 (y-axis), BPr+ helper T cells (CD4+ BPr+) were located in the upper right quadrant (Figure 3.18c). Utilising these contour plots, it was determined that these CD4+ helper T cells showed the phenotype of CD4+ CD8a- CD19- CD25low BPr- (Table 3.1). Gating boundaries of the quadrant gates were determined using CD4, CD8a, CD19, CD25 and BPr FMO controls (data not shown).

3.5.2.1.1.2. Phenotyping of CD8+ T cells

Gated CD8a+ T cells underwent phenotyping using the lymphocyte panel to determine which markers were expressed. On a bivariate contour plot of APC-Cy7-CD4 (x-axis) versus BV510-CD19 (y-axis), only CD8+ cytotoxic T cells (CD3e+ CD4- CD19-) were located in the lower left quadrant (Figure 3.19a).

The CD4- CD19- cells were used in further analysis with a bivariate contour plot utilising of PerCP-eFluor710-CD8a (x-axis) versus BV421-CD25 (y-axis), CD25+ cytotoxic T cells (CD8a+ CD25+) and CD25- cytotoxic T cells (CD8a+ CD25-) were located respectively in upper left and lower left quadrants (Figure 3.19b).
The CD4−CD19− cells were also used in further analysis with a bivariate contour plot utilising PerCP-eFluor710 (x-axis) versus BPr (y-axis), BPr+ cytotoxic T cells (CD8a+BPr+) were located in the upper right quadrant (Figure 3.19c). Utilising these contour plots, it was determined that these CD8+ cytotoxic T cells showed the phenotype of CD4−CD8a+CD19−CD25−BPr− (Table 3.1). Gating boundaries of the quadrant gates were determined using CD4, CD8a, CD19, CD25 and BPr FMO controls (data not shown).

**Figure 3.19 Phenotyping of CD8+ T cells.** Bivariate contour plots for a representative experiment (n=3) of cells stained with APC-Cy7-CD4, PerCP-eFluor710-CD8a, BV510-CD19, BV421-CD25 and BPR after CD8+ T cell isolation.  
A) Gating of only CD8+ T cells was achieved following the gating of CD4−CD19− located in the lower left quadrant.  
B) CD8+ T cells show very low levels of CD25 expression with CD8+CD25− and CD8+CD25+ cells located respectively in the lower right and upper right quadrants.  
C) CD8+ T cells show very low levels of α1-AR expression with CD8+BPr− and CD8+BPr+ located respectively in the lower right and upper right quadrants.
3.5.2.2. B cells

B cells (CD3ε–, CD19+) present in the heterogeneous cellular suspensions were identified by the expression of the pan B-cell marker CD19 (Kozmik et al., 1992). On a bivariate pseudocolour dot plot of APC-CD3e (x-axis) versus BV510-CD19 (y-axis), B cells (CD3ε– CD19+) were located in the upper left quadrant (Figure 3.17a). Gating boundaries of the quadrant gates were determined using CD3e and CD19 FMO controls (data not shown).
Table 3.1. Summary of phenotypes of normal spleen lymphocyte subsets

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CD3e</th>
<th>CD4</th>
<th>CD8a</th>
<th>CD19</th>
<th>CD25</th>
<th>BPr</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺ T cell</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+ low</td>
<td>-</td>
</tr>
<tr>
<td>CD8⁺ T cell</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B cell</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+ low</td>
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</tr>
</tbody>
</table>
BPr− B cells (BPr− CD19+) were located respectively in the upper left and lower left quadrants (Figure 3.20c). Utilising these contour plots, it was determined that these B cells showed the phenotype of CD4− CD8a− CD19+ CD25low BPr− (Table 3.1). Gating boundaries of the quadrant gates were determined using CD4, CD8, CD19, CD25, BPr FMO controls (data not shown).

3.5.3. Myeloid Cells

3.5.3.1. Conventional Dendritic Cells

Conventional dendritic cells (cDCs) were initially identified by the expression of MHC II and CD11c with a broad gate (Figure 3.21a). However identifying cDCs as CD11chigh MHC II+ does not solely isolate cDCs as there is the possibility of plasmacytoid DCs (pDCs) having a similar phenotype in the spleen (Ferrero et al., 2002). To account for this, a bivariate contour plot of APC-Cy7-B220 (x-axis) versus BV510-Ly6GC (y-axis) was utilised with only cDCs (B220− Ly6GC−) located in the lower left quadrant (Figure 3.21b).

3.5.3.1.1. Phenotyping of Conventional Dendritic Cells

Conventional dendritic cells (cDCs) were initially identified by the expression of MHC II and CD11c with a broad gate (Figure 3.21a). However identifying cDCs as CD11chigh MHC II+ does not solely isolate cDCs as there is the possibility of plasmacytoid DCs (pDCs) having a similar phenotype in the spleen (Ferrero et al., 2002). To account for this, a bivariate contour plot of APC-Cy7-B220 (x-axis) versus BV510-Ly6GC (y-axis) was utilised with only cDCs (B220− Ly6GC−) located in the lower left quadrant (Figure 3.21b). Using these contour plots, it was determined that
these cDCs showed the phenotype of $\text{B220}^{-}\text{CD11b}^{-}\text{CD11c}^{+}\text{F4/80}^{\text{low}}\text{Ly6GC}^{-}\text{MHC II}^{\text{high}}\text{BPr}^{-}$ (Table 3.2). Gating boundaries of the quadrant gates were determined using B220, CD11b, CD11c, F4/80, Ly6GC, MHC II and BPr FMO controls (data not shown).

**Figure 3.21. Gating strategy and phenotyping of cDCs.** Bivariate contour plots for a representative experiment ($n=4$) of cells stained with APC-Cy7-B220, APC-CD11b, PE-Cy7-CD11c, PerCP-Cy5.5-F4/80, BV510-Ly6GC, BV421-MHC II and BPR. **A** MHC II$^{+}\text{CD11c}^{+}$ conventional DCs (cDCs) were located in the upper right quadrant. **B** cDCs were further selected for with the gating of B220$^{-}\text{Ly6GC}^{-}$ cDCs located in the lower left quadrant. **C** B220$^{-}\text{CD11c}^{-}\text{Ly6GC}^{-}\text{MHC II}^{+}$ cDCs show very low levels of F4/80 expression and no CD11b expression with F4/80$^{+}\text{CD11b}^{-}$ cDCs and F4/80$^{-}\text{CD11b}^{-}$ cDCs located respectively in the upper left and lower left quadrants. **D** B220$^{-}\text{CD11c}^{-}\text{Ly6GC}^{-}\text{MHC II}^{+}$ cDCs show no $\alpha_1$-AR expression with BPR$^{-}$ cDCs located in the lower right quadrant.
3.5.3.2. **Macrophages**

Macrophages were initially identified by the expression of primary macrophage marker F4/80 and lack of the monocyte marker Ly6C. On a bivariate contour plot of PerCP-Cy5.5-F4/80 (x-axis) and BV510-Ly6GC (y-axis), macrophages (F4/80⁺ Ly6GC⁻) were located in the upper left quadrant (Figure 3.22a).

### 3.5.3.2.1. Phenotyping of Macrophages

Gated macrophages (F4/80⁺ Ly6GC⁻) underwent phenotyping using the myeloid panel to determine which markers were being expressed. On a bivariate contour plot of APC-Cy7-B220 (x-axis) versus APC-CD11b (y-axis), B220⁺ and CD11b⁻ macrophages (B220⁺ CD11b⁻) were located in the lower right quadrant (Figure 3.22b). On another bivariate contour plot with PE-Cy7-CD11c (x-axis) and BV421-MHC II (y-axis), MHC II⁺ macrophages (CD11c⁺ MHC II⁺), CD11c⁺ macrophages (CD11c⁺ MHC II⁻) and MHC II⁺ CD11c⁺ macrophages were located respectively in the upper left, lower right and upper right quadrants (Figure 3.22c). The gated macrophages were analysed for α₁-AR using a bivariate contour plot utilising PerCP-Cy5.5-F4/80 (x-axis) and BPr (y-axis) with BPr⁻ macrophages (F4/80⁺ BPr⁻) located in the lower right quadrant (Figure 3.22d). Utilising these contour plots, it was determined that these macrophages showed the phenotype of B220⁺ CD11b⁻ CD11c⁺ F4/80⁺ Ly6GC⁻ MHC II⁺ BPr⁻ (Table 3.2). Gating boundaries of the quadrant gates were determined using B220, CD11b, CD11c, F4/80, Ly6GC, MHC II and BPr FMO controls (data not shown).
Plasmacytoid Dendritic Cells

Plasmacytoid dendritic cells (pDCs) were identified by the expression of B220 and Ly6GC. As such, these markers became especially important in the differentiation of cDCs (B220⁺ Ly6GC⁻) from pDCs (B220⁺ Ly6GC⁺). On a bivariate contour plot of BV510-Ly6GC (x-axis) versus APC-Cy7-B220, pDCs (Ly6GC⁺ B220⁺) were located in the upper right quadrant (Figure 3.23a).

Phenotyping of Plasmacytoid Dendritic Cells

Gated pDCs (Ly6GC⁺ B220⁺) underwent phenotyping using the myeloid panel to determine which markers were being expressed. On a bivariate contour plot of BV421-MHC II (x-axis) versus PE-Cy7-CD11c (y-axis), MHC II⁺ pDCs (MHC II⁺ CD11c⁻), CD11c⁺ pDCs (MHC II⁺ CD11c⁺) and MHC II⁺ CD11c⁺ pDCs were located respectively...
in the lower left, upper right and upper left quadrants (Figure 3.23b). On another bivariate contour plot with APC-CD11b (x-axis) and PerCP-Cy5.5-F4/80 (y-axis), CD11b+ F4/80+ pDCs were located in the upper right quadrant (Figure 3.23c).

The isolated pDCs were analysed for α1-AR using a bivariate contour plot utilising APC-Cy7-B220 (x-axis) and BPr (y-axis) with BPr+ pDCs (B220+ BPr+) and BPr- pDCs (B220+ BPr-) located respectively in the upper right and lower right quadrants (Figure 3.23d). Utilising these contour plots, it was determined that these pDCs showed the phenotype of B220+ CD11b+ CD11c+ F4/80+ Ly6GC+ MHC II+ BPr+ (Table 3.2). Gating boundaries of the quadrant gates were determined using B220, CD11b, CD11c, F4/80, Ly6GC, MHC II and BPr FMO controls (data not shown).

Figure 3.23. Gating strategy and phenotyping of pDCs. Bivariate contour plots for a representative experiment (n=4) of cells stained with APC-Cy7-B220, APC-CD11b, PE-Cy7-CD11c, PerCP-Cy5.5-F4/80, BV510-Ly6GC, BV421-MHC II and BPR. A) Ly6GC+ B220+ plasmacytoid DCs (pDCs) were located in the upper right quadrant. B) Ly6GC+ B220+ pDCs show high level of MHC II and CD11c expression with MHC II+ pDCs and CD11c+ pDCs located respectively in the lower right and upper left quadrants. Double negative pDCs were located in the lower left quadrant while MHC II+ CD11c+ pDCs were located in the upper right quadrant. C) CD11b+ F4/80+ Ly6GC+ B220+ pDCs are located in the upper right quadrant. D) MHC II+ CD11c+ pDCs show low levels of α1-AR expression with B220+ BPR+ pDCs and B220+ BPR- pDCs located respectively in the upper right and lower right quadrants.
3.5.3.4. Phenotyping of Neutrophils

Neutrophils were identified by the expression of primary neutrophil marker Ly6GC (Gumley, McKenzie, and Sandrin, 1995). On a bivariate contour plot of BV510-Ly6GC (x-axis) and APC-Cy7-B220 (y-axis), Ly6GC$^+$ neutrophils (B220$^-$ Ly6GC$^+$) were located in the bottom right quadrant (Figure 3.24a).

3.5.3.4.1. Phenotyping of Neutrophils

Gated neutrophils (B220$^-$ Ly6GC$^+$) underwent phenotyping using the myeloid panel to determine which markers were being expressed. On a bivariate contour plot of BV421-MHC II (x-axis) versus PE-Cy7-CD11c (y-axis), MHC II$^+$ neutrophils (MHC II$^+$ CD11c$^-$) and double negative neutrophils (MHC II$^-$/CD11c$^-$) were located respectively in the lower right and lower left quadrants (Figure 3.24b). On another bivariate contour plot with APC-CD11b (x-axis) and PerCP-Cy5.5-F4/80 (y-axis), MHC II$^+$ neutrophils (MHC II$^+$ F4/80$^-$) and double negative neutrophils (MHC II$^-$/F4/80$^-$) were located respectively in the lower right and lower left quadrants (Figure 3.24c).
The gated neutrophils were analysed for $\alpha_1$-AR using a bivariate contour plot utilising BV510-Ly6GC (x-axis) and BPr (y-axis) with BPr$^-$ neutrophils (Ly6GC$^+$ BPr$^-$) located in the lower right quadrant (Figure 3.24d). Utilising these contour plots, it was determined that these neutrophils showed the phenotype of B220$^-$ CD11b$^-$ CD11c$^-$ F4/80$^+$ Ly6GC$^+$ MHC II$^+$ $\alpha_1$-AR$^-$ (Table 3.2). Gating boundaries of the quadrant gates were determined using B220, CD11b, CD11c, F4/80, Ly6GC, MHC II and BPr FMO controls (data not shown).

Table 3.2. Summary of phenotypes of normal spleen myeloid cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>B220</th>
<th>CD11b</th>
<th>CD11c</th>
<th>F4/80</th>
<th>Ly6GC</th>
<th>MHC II</th>
<th>BPr</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDCs</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+ very low</td>
<td>-</td>
<td>+ high</td>
<td>-</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+ high</td>
<td>-</td>
</tr>
<tr>
<td>pDCs</td>
<td>+</td>
<td>+ int</td>
<td>+ int</td>
<td>+ low</td>
<td>+ int</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+ high</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
3.6. Expression of $\alpha_1$-AR on lymphocyte and myeloid cells after LPS exposure

Fresh spleen cells were cultured in two different conditions, normal media or media with LPS supplemented. The introduction of LPS was done to initiate an immune response. Generally, comparisons between the non-LPS and LPS are performed. From examining the unstained profiles, it could be seen that LPS had an effect on the immune cells with the cells become larger (higher FSC value) (Figure 3.25). However, upon analysis of the data, it was observed that comparing between the two cultured conditions would not be possible due to insufficient activation of spleen cells. Statistical analysis also revealed that comparisons between non-LPS and LPS cultured cells would have resulted in statistically insignificant data ($p>0.05$, $n=7$) (data not shown). Therefore comparisons between fresh and LPS stimulated were done.

3.6.1. Lymphocytes

Stimulation of CD4 T cells resulted in a significant decrease (-61.501 ± 9.988%, $n=3$) in the expression $\alpha_1$-AR in LPS stimulated $CD4^+$ T cells ($p<0.05$, $n=3$), averaging 17.1 ± 3.504 compared to fresh cells with an average geometric mean of 55.908 ± 15.338 (Figure 3.26).

Stimulation in cytotoxic ($CD8^+$) T cells resulted in a significant decrease (-63.618 ± 10.473%, $n=3$) in the expression of $\alpha_1$-AR in LPS stimulated $CD8^+$ T cells ($p<0.05$, $n=3$), averaging 18.767 ± 5.3 compared to fresh cells with an average geometric mean of 62.175 ± 14.153 (Figure 3.27).
Figure 3.25. Comparison of unstained profiles of splenocytes stimulated and not stimulated by LPS. Unstained profiles of the two different culturing conditions showing cells with different FSC values. LPS stimulated cells show larger cells than cells cultured without LPS.

Figure 3.26 Comparison of geometric MFI of α₁-AR expression on CD4⁺ T cells after *in vitro* culture. The geometric mean fluorescence intensity (MFI) of BODIPY-prazosin (α₁-AR) staining of spleen CD4⁺ T cells analysed either on freshly isolated cells or after *in vitro* culture with or without LPS for 24 hours. A significant decrease in expression of α₁-AR was detected between LPS stimulated and fresh CD4⁺ T cells (p<0.05). No significant differences were detected for other conditions. Data are presented as mean ± SEM (n=3). * indicates p<0.05.
Stimulation in B cells resulted in a significant decrease (-53.189 ± 16.224%, n=3) in the expression of α₁-AR in LPS stimulated B cells (p<0.05, n=3), averaging 18.833 ± 3.172 compared to fresh cells with an average geometric mean of 53.768 ± 13.896 (Figure 3.28).

**Figure 3.27. Comparison of geometric MFI of α₁-AR expression on CD8<sup>+</sup> T cells after in vitro culture.** The geometric mean fluorescence intensity (MFI) of BODIPY-prazosin (α₁-AR) staining of spleen CD8<sup>+</sup> T cells analysed either on freshly isolated cells or after in vitro culture with or without LPS for 24 hours. Significant differences were detected between the fresh CD8<sup>+</sup> T cells and each of the experimental conditions for expression of α₁-AR (p<0.05). No significant differences were detected between the cultured CD8<sup>+</sup> T cells that had been stimulated by LPS and those that had not been stimulated by LPS. Data are presented as mean ± SEM (n=3). * indicates p<0.05.
Figure 3.28. Comparison of geometric MFI of $\alpha_1$-AR expression on B cells after in vitro culture. The geometric mean fluorescence intensity (MFI) of BODIPY-prazosin ($\alpha_1$-AR) staining of spleen B cells analysed either on freshly isolated cells or after in vitro culture with or without LPS for 24 hours. Significant differences were detected between the LPS stimulated and fresh B cells for $\alpha_1$-AR expression (p<0.05). No significant differences were detected for other conditions. Data are presented as mean ± SEM (n=3). * indicates p<0.05 between the LPS stimulated and fresh cells.

3.6.2. Myeloid Cells

Stimulation in cDCs resulted in a significant decrease (-80.582 ± 8.477%, n=4) in the expression of $\alpha_1$-AR in LPS stimulated cDCs (p<0.05, n=4), averaging 7.155 ± 2.508 compared to fresh cells with an average geometric mean of 33.766 ± 10.766 (Figure 3.29).

Stimulation in macrophages resulted in a significant decrease (-58.513 ± 17.773%, n=4) in the expression of $\alpha_1$-AR in LPS stimulated macrophages (p<0.05, n=4), averaging 23.9 ± 4.54 compared to fresh cells with an average geometric mean of 90.025 ± 31.8723 (Figure 3.30).
Stimulation in pDCs resulted in an increase (26.037 ± 54.026%, n=4) in the expression of α₁-AR in LPS stimulated pDCs. However, the data was not significant with p>0.05 (n=4). An immediate explanation could not be provided. In hindsight, the increase in α₁-AR and statistically insignificant data could have been the result of the large variation in the geometric mean of the fresh cells (20.695 ± 10.347, n=4) and LPS stimulated cells (25.163 ± 6.854, n=4), although this warrants further investigation (Figure 3.31).

Stimulation in neutrophils resulted in a decrease (-64.831 ± 8.313%, n=4) in the expression of α₁-AR in LPS stimulated neutrophils (p<0.05, n=4), averaging 9.725 ± 2.270 compared to fresh cells with an average geometric mean of 21.646 ± 10.823 (Figure 3.32).

**Figure 3.29. Comparison of geometric MFI of α₁-AR expression on cDC after in vitro culture.**
The geometric mean fluorescence intensity (MFI) of BODIPY-prazosin (α₁-AR) staining of spleen cDC analysed either on freshly isolated cells or after in vitro culture with or without LPS for 24 hours. Significant differences were detected between the fresh cDCs and each of the experimental conditions for expression of α₁-AR (p<0.05). There were no significant differences between cDC cultured with or without LPS. Data are presented as mean ± SEM (n=4). * indicates p<0.05.
**Figure 3.30. Comparison of geometric MFI of α₂-AR expression on macrophages after in vitro culture.** The geometric mean fluorescence intensity (MFI) of BODIPY-prazosin (α₂-AR) staining of spleen macrophages was analysed either on freshly isolated cells or after in vitro culture with or without LPS for 24 hours. Significant differences were detected between the fresh macrophages and each of the experimental conditions for expression of α₂-AR (p<0.05). No significant differences were detected between the cultured macrophages that had been stimulated by LPS and those that had not been stimulated by LPS (p>0.05). Data are presented as mean ± SEM (n=4). * indicates p<0.05 between the LPS stimulated or non-LPS stimulated and fresh cells. * indicates p<0.05.

**Figure 3.31 Comparison of geometric MFI of α₂-AR expression on pDC after in vitro culture.** The geometric mean fluorescence intensity (MFI) of BODIPY-prazosin (α₂-AR) staining of spleen pDC analysed either on freshly isolated cells or after in vitro culture with or without LPS for 24 hours. No significant differences were detected between any of the experiment conditions for expression of α₂-AR on pDCs. Data are presented as mean ± SEM (n=4).
Figure 3.32 Comparison of geometric MFI of $\alpha_1$-AR expression on neutrophils after in vitro culture. The geometric mean fluorescence intensity (MFI) of BODIPY-prazosin ($\alpha_1$-AR) staining of spleen neutrophils analysed either on freshly isolated cells or after in vitro culture with or without LPS for 24 hours. Significant differences were detected between LPS-stimulated and non-stimulated cultured neutrophils and fresh neutrophils for $\alpha_1$-AR expression ($p<0.05$). There were no significant differences between neutrophils cultured with or without LPS. Data are presented as mean ± SEM (n=4). Data are presented as mean ± SEM. * indicates $p<0.05$. 
4. Discussion

The purpose of this study was to determine whether inflammation would upregulate the expression of $\alpha_1$-ARs in populations of immune cells. The $\alpha_1$-ARs have known functions in various tissues including the regulation of contractility in smooth muscle, growth promoting responses and involvement in cellular signalling pathways (Piascik and Perez, 2001). Recent studies on immune cells have shown that $\alpha_1$-ARs may have a role in the modulation of immunity, but this is still poorly understood (Reviewed in Grisanti, Perez, and Porter, 2011). While numerous studies have examined the mRNA level of expression, few studies have examined the protein expression during inflammation. The overall aim of this study was to examine the expression of $\alpha_1$-ARs of murine splenocytes upon stimulation by LPS. The projects aim was to determine the baseline protein expression of $\alpha_1$-ARs on fresh immune cells in the spleen and compare the expression of $\alpha_1$-ARs on the immune cells after exposure to LPS. It was discovered that the protein expression of $\alpha_1$-ARs was lower in LPS stimulated immune cells than in fresh immune cells. This decrease was seen throughout the lymphoid and myeloid cell subsets examined.
4.1. Optimisation of Lymphocyte and Myeloid Staining Panels

The primary technique used in this project was flow cytometry, which allows quantitative analysis of surface protein expression on living cells using fluorescently labelled monoclonal antibodies (mAbs) and other fluorescent markers. To achieve optimal results, all mAbs had to be titrated first in order to determine optimal staining profile that would reduce non-specific staining while giving clear positive signals and using the least amount of antibodies. A range of mAbs was used to identify lymphoid and myeloid cell subsets in mouse spleen, along with BODIPY-prazosin (BPr) to specifically identify $\alpha_1$-ARs. Antibodies that were targeted towards highly expressed surface markers on lymphocytes, such as CD3e, CD4, CD8a, CD19, B220 and MHC II, generally gave good titration profiles with clear separation of positive and negative peaks, and optimal dilutions could be easily determined. However, some myeloid markers such as CD11c, primarily a DC marker but also expressed on some other myeloid cells such as neutrophils, were expressed in low levels in the spleen due to the spleen primarily being a site for migration of lymphocytes (Gao et al., 2003; Yang et al., 2013). Other markers that had low expression on splenocytes included CD11b, F4/80 and Ly6GC (Gao et al., 2003; Zwadlo et al., 1985; Lagasse and Weissman, 1996). As such, a proper titration was difficult to do for these markers. The optimal titrations for these markers were determined using the line plots of the geometric mean fluorescent intensity (MFI) for each mAb, as well using the expression profiles by single parameter histogram. In general, the optimal concentration was chosen when the first drop in geometric MFI was seen or when the drop in geometric MFI had become half the highest geometric MFI.
In summary, the titration of the mAbs for the lymphocyte and myeloid panels was successful with an optimal titre determined for all mAbs based on positive fluorescence intensity, low background staining and minimal antibody concentration.

4.2. Optimisation of BODIPY-Prazosin binding and confirmation of specificity

BODIPY-prazosin (BPr) was the chosen fluorescent ligand to use in this study due to its use in previous studies that show that BPr is specific on $\alpha_1$-ARs (Sugawara et al., 2002). BPr a fluorescent analogue of the $\alpha_1$-AR antagonist prazosin, consists of the BODIPY fluorescent dye and prazosin, a very specific $\alpha_1$-AR antagonist. Primarily a staining reagent, studies involving BPr are very diverse from identifying both diffuse and highly mobile clustered $\alpha_1$-AR on various tissues including the endothelial cells of arteries to examination of $\alpha_1$-AR on transfected cell lines (Daly et al., 1998; Sugawara et al., 2002; Daly et al., 2010). However, BPr has also been used in other methods such as efflux studies (Robey et al., 2003; Elliott, Raguz, and Higgins, 2004). The primary methods of analysis of BPr in these studies include flow cytometry and confocal microscopy.

Staining splenocytes in an attempt to find an optimal concentration had already proved to be an issue in this project, as the BPr staining levels were low, and I was unable to determine an optimal concentration (see Results). Similar to mAb titrations, if a high concentration of BPr was used to stain cells, it would have resulted in non-specific staining, resulting in a false positive signal. Consequently, the titration of the BPr on
transfected $\alpha_1$-Chinese Hamster Ovary ($\alpha_1$-CHO) cells was investigated. CHO cells are a commonly used cell line in molecular biology due to their low chromosome number and small size (Puck, Cieciura, and Robinson, 1958). $\alpha_1$-CHO cells are CHO cells which have been transfected to constitutively express $\alpha_1$-ARs.

In order to determine the specificity of BPr, a competitive binding study was performed using terazosin. Terazosin, an $\alpha_1$-AR antagonist, was used as competitive blocker in $\alpha_1$-CHO cells. As the $\alpha_1$-CHO cells express $\alpha_1$-ARs constitutively, terazosin should bind to a majority of the $\alpha_1$-ARs before the addition of BPr. By comparing the MFI of $\alpha_1$-CHO cells to the MFI of the $\alpha_1$-CHO cells stained with BPr and MFI of the unstained $\alpha_1$-CHO cells, it would be possible to determine if BPr was non-specifically binding to the $\alpha_1$-CHO cells or not. A marked decrease in MFI of the terazosin blocked $\alpha_1$-CHO cells when compared to BPr stained only $\alpha_1$-CHO cells would be indicative of specific binding. If the MFI of the terazosin blocked $\alpha_1$-CHO cells had a higher MFI to the unstained $\alpha_1$-CHO cells, then it would be indicative of non-specific binding with the BPr potentially increasing the MFI of the terazosin blocked $\alpha_1$-CHO cells.

The same competitive binding study was performed on non-transfected CHO (NT-CHO) cells to determine if there was any non-specific binding from the BPr because theoretically, there should be no $\alpha_1$-AR expression. Various studies utilising transfected CHO cells have used NT-CHO cells as a negative control; therefore for the purposes of this study, it was also used as the negative control (Shibata et al., 2003; Björk, Vainio, and Scheinin, 2005). The BPr binding experiments on NT-CHO cells resulted in no increase in MFI when compared to the unstained NT-CHO (data not shown).
In conclusion, a significant reduction in the geometric MFI of BPr (p<0.001) for $\alpha_1$-CHO cells blocked with terazosin was observed (Figure 3.14), indicating that BPr was specific for $\alpha_1$-ARs. This conclusion was further endorsed with no non-specific binding on NT-CHO cells.

4.3. Immunophenotyping for better resolution of cell populations and $\alpha_1$-AR expression

Utilising the lymphocyte panel which included the markers CD3e, CD4, CD8a, CD19, CD25, populations could have been identified such as regulatory T cells ($CD4^+ CD25^+$, $CD8^+ CD25^+$) and regulatory B cells ($CD19^+ CD25^+$) (Kessel et al., 2012; Correale and Villa, 2010; de Andrés et al., 2014). However the three populations of lymphocytes of relevance in this study were cytotoxic T cells ($CD4^- CD8^+$), helper T cells ($CD4^+ CD8^-$) and B cells ($CD3e^- CD19^+$). Also of interest were the double positive populations that co-stained when CD4 and CD8 were analysed together ($CD4^{high} CD8^{high}$). While this could be a result of $CD4^+$ and $CD8^+$ T cells sticking together and being analysed at the same time, a study has identified that $CD4^+ CD8^+$ T cells do exist in normal conditions but their numbers increase in immuno-inflammatory conditions (Parel and Chizzolini, 2004). The function of $CD4^+ CD8^+$ T cells is poorly understood but an increase in $CD4^+ CD8^+$ T cells has been observed during viral infections (Nascimbeni et al., 2004). Overall, studies examining $CD4^+ CD8^+$ T cells came to the conclusion that these T cells may take part in the adaptive immune response against infectious pathogens and may have possible immunoregulatory and/or immunosurveillance functions (Zuckermann, 1999; Nascimbeni et al., 2004).
Immunophenotyping of the myeloid populations was more difficult than compared to the lymphocyte populations. The myeloid panel which consisted of B220, CD11b, CD11c, F4/80, Ly6GC and MHC II was used to stain splenocytes. The difficulty arose because myeloid cells make up a low percentage of the total composition of the spleen with lymphocytes being the dominant immune cell type (57.1 – 93.2%) (Yang et al., 2013). A study conducted on C57BL/6J mice determined that myeloid cells composed of 2.9 – 19.5% of the spleen (Yang et al., 2013).

Two DC populations were identified, cDCs (B220⁻ CD11b⁻ CD11c\textsuperscript{high} F4/80\textsuperscript{low} Ly6GC⁻ MHC I\textsuperscript{high}) and pDCs (B220⁺ CD11b⁺ CD11c⁺ F4/80⁺ Ly6GC⁺ MHC II⁺). The two subsets were separated by using B220 as the primary determinant, with B220 primarily expressed on pDCs and not cDCs (Ferrero et al., 2002).

4.4. LPS activation of cell types

Lipopolysaccharide (LPS) was used in cell cultures to attempt to replicate an acute immune response \textit{in vitro}. LPS binds to ‘toll-like receptor 4’ (TLR 4) which is found on numerous immune cell types. TLR4 signalling can be divided into two pathways: MyD88-dependent and MyD88-independent (TRIF-dependent) (Figure 4.1).

In the MyD88-dependent pathway, the stimulation of MyD88 results in the recruitment of IL-1 receptor-associated kinase (IRAK). IRAK is activated by phosphorylation and associates with TRAF6 and activates TAK1. TAK1 then activates IkB kinase and MAPK pathways. These pathways subsequently lead towards the activation of JNK (a MAPK) and transcription factors NF-κB and activation protein 1 (AP-1), which controls the expression of proinflammatory cytokines (Lu, Yeh, and Ohashi, 2008) (Figure 4.2).
Figure 4.1 Overview of LPS/TLR4 signalling. LPS signalling recognition is facilitated by LBP and CD14, and is mediated by the TLR4/MD-2 complex. The LPS/TLR4 signalling can be divided into MyD88 dependent and MyD88 independent pathways, both of which mediate the activation of pro-inflammatory cytokines and type I interferons (Adapted from Lu, Yeh, and Ohashi, 2008).

Figure 4.2. The MyD88 dependent pathway. MyD88 activates IRAKs/TRAF6 complex as well as transcription factors NF-κB, AP-1 and IRF-5. These transcription factors induce the expression of pro-inflammatory cytokines (Adapted from Lu, Yeh, and Ohashi, 2008).
The MyD88 independent signalling pathway consists of TRIF, an important mediator of the MyD88 independent signalling pathway. Studies using TRIF-deficient macrophages have shown that TRIF plays an important role in the activation of transcription factors IRF3, NF-κB and MAPK. The activation of these transcription factors results in the induction of type I interferon production (Lu, Yeh, and Ohashi, 2008) (Figure 4.3).

TLR 4 is part of a family of toll like receptors, a type of pathogen recognition receptor (PRR) that recognises molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs) (Turvey and Broide, 2010). The TLR family consists of 10 TLRs (TLR 1 – TLR 10), each of which recognises a different PAMP (Figure 4.4). For example, TLR 3 recognises dsRNA and TLR 5 recognises flagellin (Takeda and Akira, 2004).

LPS was chosen to be used in this study because it was very commonly used in α1-AR transcription and expression studies where an inflammatory state was induced (Heijnen et al., 2002; Rouppe van der Voort et al., 2000; Guha and Mackman, 2001). In this project, it was unknown if the LPS had any effect on the activation of the splenocytes as cytokine analysis was not performed. However, the unstained negative control that had been cultured with LPS did show a profile with much larger cells (high FSC) than the unstained non-LPS cells, which is indicative of cellular activation (Figure 3.25) (Teague et al., 1999; Sadeghi et al., 2010).
Figure 4.3. The MyD88 independent pathway. TRIF signals the transduction of type I interferons by recruiting TRAF4 and RIP1 to activate transcription factors IRF3, AP-1 and NF-κB (Adapted from Lu, Yeh, and Ohashi, 2008).

Figure 4.4. TLRs and their ligands. Each TLR recognises a different microbial component. TLR 1 – 7 and 9 recognise bacterial components. TLR 9 is the CpG DNA receptor and TLR 3 recognises double stranded RNA.
4.4.1. Lymphocyte Activation via LPS

The introduction of LPS was expected to stimulate naive CD4$^+$ T cells with studies showing that CD4$^+$ cells do express TLR 4 (Gonzalez-Navajas et al., 2010). Upon antigen capture, the APC presents the processed antigen on the MHC II complex to the T cell receptor (TCR) on CD4$^+$ T cells, resulting in the first stage of activation of CD4$^+$ T cells. Full activation of CD4$^+$ T cells requires co-stimulation of the CD28 molecule on CD4$^+$ T cells to the CD80 molecule on APCs (Murphy, 2011). LPS can enhance this process however through signalling TLR4 which activates the APC. Contrary to CD8$^+$ T cells, direct recognition of LPS was not expected to activate naive CD8$^+$ T cells, since TLR 4 is not expressed on murine CD8$^+$ T cells (Komai-Koma, Gilchrist, and Xu, 2009). Therefore, activation of CD8$^+$ T cells occurs through the CD8/MHC I interaction and CD28 on the T cell interacting with either CD80 or CD86 on APCs (Murphy, 2011). However co-stimulation from CD4 T cells through cytokine release was expected, with this causing the activation of CD8$^+$ T cells (Murphy, 2011).

Naive B cells undergo activation through two major pathways: T cell dependent and T cell independent activation. The primary difference between the two pathways is that in T cell independent activation, B cells can undergo activation by the binding of an antigen to the B cell receptor (BCR), which is enough to initiate activation of the B cell. TLR 4 is known to be expressed on B cells and is involved in the activation of naive B cells (Gururajan, Jacob, and Pulendran, 2007; Hua and Hou, 2013).
4.4.2. Myeloid Cell Activation via LPS

The introduction of LPS was used to activate the myeloid cells. It has been shown that cDCs, macrophages, pDCs and neutrophils all express TLR 4, implying that these cells can be directly activated upon exposure to the LPS (Kratky et al., 2011; Richez et al., 2009; Peyssonnaux et al., 2006; Zheng et al., 2012). The activation of these cells by LPS triggers a release of proinflammatory cytokines including TNF-α, IL-6, IL-15 and many others (Feghali and Wright, 1997). The release of cytokines leads to the activation of other immune cell types including lymphocytes.

4.5. Expression of α₁-AR on Lymphoid and Myeloid Cell Subsets

There are three α₁-AR subtypes including α₁A, α₁B, α₁D. While they are found in a variety of tissues including cardiac myocytes, endothelial cells and neurons, they are also found on immune cells (Piascik and Perez, 2001; Marino and Cosentino, 2013).

Results from the analysis of α₁-AR on myeloid cells found that there was a decrease in expression of α₁-ARs on LPS stimulated cells compared to fresh cells. This conclusion was based on data, which showed that the LPS-stimulated myeloid cells had a lower geometric MFI of BPr than fresh myeloid cells (Figure 3.29, 3.30, 3.32). Similar to the myeloid populations, there was an observed decrease in geometric MFI of BPr when the stimulated lymphocytes were compared to fresh lymphocytes (Figure 3.26, 3.27, 3.28). Collectively, these data suggest that in an inflammatory state or at least when the immune cells are stimulated, there is a reduction in α₁-AR protein expression in the
lymphocyte and myeloid cell populations identified. A study using Sprague-Dawley rats that had been exposed to LPS to induce endotoxemia examined the mRNA and protein expression of all $\alpha_1$-AR subtypes (Bucher et al., 2003). It was observed that there was a decrease in all $\alpha_1$-AR subtypes and an increase in the cytokines IL-1$\beta$ and TNF-$\alpha$. It was suggested by the authors that the pro-inflammatory cytokines could have potentially mediated the downregulation in expression of the $\alpha_1$-AR subtypes. It should be noted however that the study was examining expression of $\alpha_1$-AR subtypes in renal mesangial cells and not the immune cells themselves.

Another study examined prazosin treatment and the release of IL-1$\beta$ before and after LPS exposure. It was discovered while prazosin blocking had no effect on basal plasma IL-1$\beta$ levels, pre-treating cell with prazosin before LPS stimulation could block increases in IL-1$\beta$ levels (Dong et al., 2002). Another study examining the release of TNF-$\alpha$ demonstrated that levels of TNF-$\alpha$ were decreased when mice were given prazosin before LPS exposure compared to mice treated with LPS only (Sugino et al., 2009).

**4.5.1. Expression of $\alpha_1$-AR on Myeloid Cells**

One interesting observations is that there was an increase in geometric MFI of BPr in LPS stimulated pDCs when compared to fresh pDCs (Figure 3.31). While the increase wasn’t significant (p<0.05), the increase of geometric MFI in pDCs suggests that the stimulation of other immune cells led to an increase in cytokine production which potentially increased the expression of $\alpha_1$-AR. There is evidence to suggest that cytokine production can increase the expression of $\alpha_1$-AR. For example, in human THP-1 human monocytes, pro-inflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ increased the mRNA levels of $\alpha_{1\alpha}$-AR (Heijnen et al., 2002).
It is interesting to note that a radioligand binding study examining the expression of $\alpha_1$-AR on neutrophils found no specific binding for $\alpha_1$-AR, suggesting that $\alpha_1$-AR expression was not detectable on neutrophils (Casale and Kaliner, 1984). Although no detectable $\alpha_1$-AR was detected in LPS stimulated neutrophils, the fresh cells displayed moderately high geometric MFI for BPr, indicating that there could be expression. In support of this is that there is an observed decrease in the geometric MFI of BPr after LPS exposure, indicating the expression of $\alpha_1$-AR may have decreased.

In this study, it was shown that macrophages had no detectable $\alpha_1$-AR expression in fresh and LPS stimulated cells. On the contrary, a study showed that macrophages do indeed express a functional $\alpha_1$-AR (Muthu et al., 2007). Another study that examined macrophages in the rat thymus utilising immunohistochemistry techniques showed $\alpha_1$-AR expression in monocytes and macrophages (Pesic et al., 2009). While the study was looking at monocytes and macrophages located within the thymus, there is a study to suggest that $\alpha_1$-AR expression is present in both the thymus and spleen (Kavelaars, 2002).

There is evidence to suggest that DCs express $\alpha_1$-ARs (Maestroni, 2000; Yanagawa, Matsumoto, and Togashi, 2010). However, there is little information about the expression of $\alpha_1$-AR on cDCs and pDCs.
4.5.2. Expression of $\alpha_1$-AR on Lymphocytes

Transcriptional studies have indicated that lymphocytes in the mesenteric lymph nodes do express $\alpha_1$-AR (Bao et al., 2006). The same study also showed that stimulation of the lymphocytes with a T cell mitogen, concanavalin A (Con-A), increased the quantity of $\alpha_1$-AR mRNA compared with the resting lymphocytes. Radioligand binding studies using [$^3$H]-prazosin has shown that lymphocytes also do express $\alpha_1$-AR. Another study used flow cytometry to determine that from isolated rat thymus cells, 11.3% express $\alpha_1$-AR (Pesic et al., 2009). The $\alpha_1$-AR expressing population primarily consisted of CD3$^+$ (51.2%) and CD3$^{low}$ (33.2%) T-cells.

B cells on the contrary have had varied reports on whether $\alpha_1$-AR is expressed or not. Studies that have used isolated peripheral blood mononuclear cells (PBMCs), which contained B cells, have suggested that there is no expression of $\alpha_1$-AR on B cells (Casale and Kaliner, 1984; Rouppe van der Voort et al., 2000; Tayebati et al., 2000).

Overall the expression of $\alpha_1$-AR on all lymphocytes was low. The trend however was seen across all lymphocytes with a significant decrease ($p<0.05$) in expression of BPr with LPS stimulated lymphocytes displaying lower expression than compared to naive lymphocytes.

The decrease in $\alpha_1$-AR expression in fresh lymphocytes to LPS-stimulated lymphocytes is perplexing. However, evidence of expression of $\alpha_1$-AR on the LPS-stimulated cells indicates that the $\alpha_1$-AR may have role in the modulation of the immune response. A study has suggested that the presence of noradrenaline could lead to induction of more pro-inflammatory cytokines in activated immune cells (Rouppe van der Voort et al., 2000). Another study has shown that when lymphocytes were stimulated by Con-A, the expression of $\alpha_1$-AR mRNA was significantly higher than the resting lymphocytes (Bao
et al., 2006). This suggests that the activation of T cells increased the expression of $\alpha_1$-ARs.

The impact of the downregulation of $\alpha_1$-AR on immune cells could potentially result in the lessening of immune cell function and potential response. In macrophages, it has been shown that stimulation of $\alpha_1$-AR increases the secretion of TNF-$\alpha$ through a cooperative mechanism with TLR4 (Muthu et al., 2007). The downregulation of $\alpha_1$-AR could potentially decrease the levels of TNF$\alpha$ and reduce the effectiveness of the immune response. Another study examined the impact of blocking $\alpha_1$-AR on the development of thymic cells (Pesic et al., 2009). It was shown that blocking $\alpha_1$-ARs increased the proportion of autoreactive CD4$^+$ CD8$^+$ T cells, therefore affecting possibly the selection stages of T cell development.

An interesting point to consider is that this study has only examined $\alpha_1$-ARs. Other adrenergic receptors such as $\alpha_2$-ARs or $\beta$-ARs could have potentially caused the increase or decrease in $\alpha_1$-AR expression. A study showed culturing human THP-1 monocytes with the $\beta_2$-AR agonist terbutaline resulted in a rapid rise of $\alpha_{1B}$ and $\alpha_{1D}$-ARs mRNA but not $\alpha_{1A}$-AR mRNA (Kavelaars, 2002). However, in this project, the cells were analysed after 24 hours in LPS which could have potentially allowed for the receptors to degrade and be recycled. Studies examining the turnover rate of $\alpha_1$-AR on the muscle cell line BC3H-1 have shown that the turnover rate is ~23 to 25 hours (Hughes and Insel, 1986; Sladeczek et al., 1984).

Another point to consider is that only LPS was used in this study to activate immune cells. There could have been better inflammatory stimuli to use. For instance, a study used phytohaemagglutinin, a T cell mitogen, induced $\alpha_1$-AR expression in stimulated T cells (Rouppe van der Voort et al., 2000).
Overall, the project was limited by various reasons. The use of only one method, flow cytometry, limited how much I was able to analyse the cells. If other methods such as PCR were employed in this study, a more accurate result could have been potentially achieved. Another limitation was only examining one subset of ARs, the $\alpha_1$-ARs. Other receptors such as the $\alpha_2$-ARs or $\beta$-ARs and subsets could have influenced the expression of $\alpha_1$-ARs. The inability to differentiate between the different $\alpha_1$-AR subsets could have been another possible limitation.

4.6. Future Directions

This study has no doubt raised several interesting questions. For one, the observed decrease in $\alpha_1$-AR on all the immune cells examined and how the secretion of cytokines may impact the expression of $\alpha_1$-AR requires immediate attention. However due to the constraints of time available for the project, this was not possible to do. The next question would be how the expression of $\alpha_3$-AR appears in an in vivo model for inflammation. $\alpha_1$-AR investigations of individual cell populations and tissues provide detailed accounts of their expression and possible function. However, the immune system is extremely complex and it is therefore important to know how $\alpha_1$-ARs are affecting the immune system under physiological conditions in vivo. Another question that is raised is what is the expression of the other ARs including the $\alpha_2$-ARs and $\beta$-ARs. The decrease seen in the results could have been a consequence of cytokine production or influence by the other AR subtypes.
4.7. **Conclusion**

In this study, the protein expression of $\alpha_1$-ARs was measured on naive or fresh splenocytes and compared to the protein expression of $\alpha_1$-AR after culturing for 24 hours in the presence of LPS. In addition, a modified protocol for optimising BPr has been established, producing a titration that shows the optimal titre to stain cells with. It could not be concluded if LPS exposure caused the marked reduction in $\alpha_1$-AR protein expression. While stimulation (and therefore induction of inflammation) of immune cells was observed, it could have been potentially caused by the effects of cell culturing rather than exposure to LPS. While further aspects need to be studied including examining the cytokines produced and expression of the other ARs, the results of this project provide preliminary evidence that during the inflammatory state, immune cells show a decrease in $\alpha_1$-AR with potential ramifications on immune cell function and immune cell response.
References


Attach to and Invade Normal Human Cartilage When Engrafted into Scid Mice." 


Sugino, Haruhiko, Takashi Futamura, Yasuhide Mitsumoto, Kenji Maeda, and Yoshinori Marunaka. 2009. "Atypical Antipsychotics Suppress Production of Proinflammatory Cytokines and up-Regulate Interleukin-10 in


### Appendix A: Reagents List

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<tr>
<th>Reagent</th>
<th>Source</th>
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<tr>
<td>APC Hamster Anti-Mouse CD3e</td>
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<td>BD Horizon</td>
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<td>562956</td>
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<td>BD Phar Lyse</td>
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