The role of class IA PI3Kδ in experimental autoimmune encephalomyelitis

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Declaration

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## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine di-phosphate</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukaemia</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP ribosylation factors</td>
</tr>
<tr>
<td>ARNO</td>
<td>ARF nucleotide binding site opener</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>AV</td>
<td>Annexin V</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BD</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2′-Deoxyuridine - Sigma</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CEF</td>
<td>Chicken embryo fibroblast</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescin diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl Pyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenyl</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment, antigen binding</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment, crystalisable</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating proteins</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine di-phosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage – Colony stimulating factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GRP</td>
<td>General receptor for phosphoinositides</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine tri-phosphate</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>IFA</td>
<td>Incomplete Freund’s adjuvant</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>Ins(1,4,5)P_3</td>
<td>Inositol(1,4,5)-trisphosphate</td>
</tr>
<tr>
<td>Itk</td>
<td>Inducible T cell kinase</td>
</tr>
<tr>
<td>KO/KI</td>
<td>Knock-out/knock-in</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin Oligodendrocyte Glycoprotein</td>
</tr>
<tr>
<td>MRCRB</td>
<td>Mouse red cell removal buffer</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PH</td>
<td>Plekstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PIPkins</td>
<td>Proline-rich domain-containing inositol 5-phosphatase kinases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol(4,5)-bisphosphate (PtdIns(4,5)P$_2$)</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol(3,4,5)-trisphosphate (PtdIns(3,4,5)P$_3$)</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospho-lipase C</td>
</tr>
<tr>
<td>PLCγ2</td>
<td>Phospholipase C gamma 2</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid Protein</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonucleocyte</td>
</tr>
<tr>
<td>PP-MS</td>
<td>Primary progressive multiple sclerosis</td>
</tr>
<tr>
<td>PRR</td>
<td>Proline rich region</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PtdIns(4,5)P$_2$</td>
<td>Phosphatidylinositol(4,5)-bisphosphate (PIP$_2$)</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P$_3$</td>
<td>Phosphatidylinositol(3,4,5)-bisphosphate (PIP$_3$)</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog deleted on chromosome ten</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RR-MS</td>
<td>Relapsing-remitting multiple sclerosis</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SHIP</td>
<td>Src homology 2 domain containing inositol polyphosphate phosphatase</td>
</tr>
<tr>
<td>SIP</td>
<td>Standard isotonic Percoll</td>
</tr>
<tr>
<td>SP-MS</td>
<td>Secondary progressive multiple sclerosis</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate-EDTA</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tc</td>
<td>T cytotoxic cell</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells (CD$4^+$/CD$25^+$/FoxP$3^+$)</td>
</tr>
</tbody>
</table>
Publications arising from this work

Manuscripts


Rachel Kohler, Iain Comerford, Scott Townley, Sarah Haylock-Jacobs, Iain Clark-Lewis & Shaun McColl. Antagonism of the chemokine receptors CXCR3 and CXCR4 reduces the pathology of experimental autoimmune encephalomyelitis, Brain Pathology, 2008, 18(4), 504-16.


Conference proceedings

2009: Oral presentation at the Australasian Immunology Retreat (Adelaide, Australia).
*Title:* PI3Kδ is important for Th17 generation and EAE

2009: Poster presentation at the Australasian Society for Medical Research conference (Adelaide, Australia).
*Title:* Activity of the catalytic subunit of PI3Kδ is required for the pathogenesis of experimental autoimmune encephalomyelitis

2008: Poster presentation at the Australasian Society for Immunology Annual Scientific Meeting (Canberra, Australia).
*Title:* Activity of the catalytic subunit of PI3Kδ is required for the pathogenesis of experimental autoimmune encephalomyelitis

2008: Oral presentation at the Australasian Immunology Retreat (Adelaide, Australia).
*Title:* Investigating the role of p110δ in EAE

2008: Poster presentation at the Canadian Society for Immunology conference (Montreal, Canada).
*Title:* The role of chemokine receptor CCR7 in experimental autoimmune encephalomyelitis

2007: Oral presentation at the third Adelaide Immunology Retreat (Adelaide, Australia).
*Title:* Investigating the role of PI3Kδ in experimental autoimmune encephalomyelitis

2006: Oral presentation at the second Adelaide Immunology Retreat (Adelaide, Australia).
*Title:* Investigating the role of p101/PI3Kγ in cell migration

2005: Poster presentation at the Australasian Society for Immunology Scientific Meeting (Melbourne, Australia).
*Title:* The role of chemokine receptor CCR7 in experimental autoimmune encephalomyelitis
Abstract

Through its role in cells of haematopoietic origin, the class IA phosphoinositide 3-kinase delta (PI3Kδ) has a significant impact on both the cell-mediated and innate arms of the immune system. The catalytic protein subunit of PI3Kδ, p110δ, has been implicated in leukocyte activation and survival, Th1 and Th2 differentiation as well as the development of autoimmunity in a model of rheumatoid arthritis. While the impact of p110δ inactivation in vitro is becoming clearer, the precise role that p110δ plays in vivo remains poorly understood, particularly in regard to Th17 differentiation and models of autoimmunity. Here, using mice that express a catalytically inactive form of p110δ (p110δ<sup>D910A/D910A</sup> mice) it is shown that functional p110δ is required for full expression of experimental autoimmune encephalomyelitis (EAE), a Th17-dependent model of the human autoimmune disease multiple sclerosis (MS). In p110δ-inactivated mice, T and B cell activation and function during EAE were markedly reduced, and fewer T and B cells were observed in the central nervous system (CNS) throughout disease. Th17 cell generation was demonstrably more dependent on p110δ than was the Th1 response. The decrease in T cell activation was not due to a defect in dendritic cell (DC) function because p110δ-inactivated DCs migrated, became activated and presented antigen normally. However, there was a significant increase in the proportion of T and B lymphocytes undergoing apoptosis at early stages of EAE. Due to the promising findings observed in the p110δ<sup>D910A/D910A</sup> mice, the ability of the p110δ inhibitor, IC87114, to reduce EAE pathogenesis was investigated. While IC87114 was shown to be a potent inhibitor of Th1 and Th17 activation and differentiation in vitro, administration of this compound failed to reduce EAE disease under the dosing regimen used. Despite this, these findings indicate that p110δ plays an important role in the development of IL-17-dependent inflammation and suggest that small molecule inhibitors for p110δ may be useful therapeutics for the treatment of IL-17-driven pathologies.
CHAPTER 1
Introduction
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1.1 OVERVIEW

The immune system serves to guard against pathogens and cancers whilst maintaining tolerance to symbiotic flora and self-antigens. Efficient function of the immune system involves a milieu of factors that cells require for processes such as growth, metabolism, activation, proliferation, differentiation, adhesion, motility, phagocytosis, effector function and survival. These include both extracellular stimuli and intracellular signalling events. Failures in any of the processes involved in the normal function of the immune system can result in opportunistic infections, chronic inflammation, tumours or autoimmunity.

One autoimmune disease that arises following immune deregulation is multiple sclerosis (MS). Intensive efforts have been devoted to further understanding the mechanisms that govern immune activation and subsequent CNS damage in MS, however there is much that is yet to be elucidated. Significant efforts are also focussing on the development of new therapies for MS patients.

A group of proteins that is a potential target for MS therapy is the phosphoinositide 3-kinase (PI3K) family. These proteins become activated downstream of cognate receptor ligation by peptides such as cytokines, growth factors and chemokines. PI3Ks are also implicated in signalling through antigen receptors. PI3Ks have been directly linked to processes such as insulin signalling and many facets of normal immune system function, and have also been shown to play an important role in a number of different pathologies including allergic inflammatory responses, cancer and autoimmunity (1-10).

In this study, the function of a specific PI3K, PI3Kδ, is investigated using a murine model for MS, experimental autoimmune encephalomyelitis (EAE). This research is conducted with the aim of better understanding the intracellular signalling processes that govern leukocyte activation in autoimmune disease, as well as to investigate whether the PI3Kδ signalling pathway may be a useful therapeutic target in autoimmune diseases.
1.2 FACTORS GOVERNING THE NORMAL IMMUNE RESPONSE

In as much as there are many different types of immune responses that are activated following different immunological challenges, there are also many factors governing these responses. Firstly, proper development of all of the immune cell types is important to ensure availability of the full repertoire of cells involved in immunity. Secondly, normal homeostasis within secondary lymphoid organs and basal surveillance of peripheral tissues for pathogens is necessary. Lastly, efficient innate and cell-mediated/humoral responses, which involve many different cell types, are required for efficient clearance of antigens, tumours and pathogens such as bacteria and viruses. Innate immunity describes the antigen-nonspecific, and often initial, host defences which include physiological barriers as well as inflammatory cells. Cell-mediated/humoral immunity, driven by T and B lymphocytes, is the antigen-specific host immune response. These immune responses are complex and involve a wide variety of cell stimuli and intracellular signalling events. Disruption or dysregulation of any of these events can have profound effects on the overall efficiency of the immune system. There are many different processes and cell types that underlie both the humoral and cell-mediated immune responses, and several that are relevant to this thesis are discussed further here.

1.2.1 Cells of the immune system

A number of different cell types make up the immune system. These include the B and T lymphocytes, neutrophils, macrophages, monocytes, basophils, eosinophils and mast cells. Lymphocytes are required for the generation of antigen-specific cell-mediated/humoral immune responses and allow the development of immunologic memory. Non-specific innate cellular immunity is performed by the other cell types listed above. In addition to this, antigen-presenting cells (APCs) such as DCs are required for efficient activation of the adaptive immune system.

1.2.1.1 T lymphocytes

T lymphocytes arise in the bone marrow and mature in the thymus. Each T cell expresses a unique antigen-binding receptor, the T cell receptor (TCR), which recognises antigenic peptides bound to major histocompatibility complex (MHC)
molecules on the surface of APCs. Three major sub-populations of T cells have been recognised; T helper (Th) cells, T cytotoxic (Tc) cells and T regulatory (Treg) cells (11-15). T helper cells generally express the CD4 membrane glycoprotein and recognise exogenous processed antigen presented by MHC class II, whereas T cytotoxic cells generally express the CD8 membrane glycoprotein and recognise endogenous antigen presented by MHC class I. Regulatory T cells, whose normal function involves maintenance of tolerance, normally express CD4 membrane glycoproteins. These CD4 or CD8 membrane glycoproteins act as co-receptors and enhance TCR signalling. Efficient stimulation of T cells also requires co-stimulation of another membrane-anchored glycoprotein, CD28, by CD80 or CD86 on the surface of APCs (16).

When activated, T helper cells produce a variety of cytokines and chemokines which assist in the activation of other immune cell types and their recruitment to sites of infection. T helper cells, of which there are three well-defined types (Th1, Th2 and Th17), also help drive specific types of immune responses according to the cytokines they produce. Th1 cells produce high levels of the cytokines IL-2, IFN-γ and TNF-α and are responsible for functions such as delayed-type hypersensitivity and activation of CTLs (17-19). Th2 cells secrete high levels of IL-4, IL-5, IL-10 and IL-13 and are responsible for allergic reactions and humoral immunity by providing help to B cells (17, 18, 20-23). Th17 cells produce IL-17 and IL-23 and, while their function is less well-defined than Th1 and Th2 cells, they are thought to be important for immunity against bacteria and fungi (24-28). They are also believed to drive the severe pathology observed in many autoimmune disorders (12, 29-34). T helper cell differentiation is discussed in more detail in section 1.2.2.

Cytotoxic T lymphocytes (CTLs) do not produce as many cytokines as Th cells, but instead function to induce cell-death in altered self-cells such as tumour cells, foreign tissue grafts and virus-infected cells (35, 36).

Regulatory T cells, which produce the cytokine IL-10, are responsible for the maintenance of peripheral tolerance to both self-antigens and symbiotic intestinal
flora and also aid in the regulation of T cell numbers during immune responses (37, 38). Breakdown of Treg function often results in autoimmunity (19). Treg cells are generally defined as CD4+/CD25+ cells that express the forkhead transcription factor FoxP3 (39-43).

T cell activation results in not only proliferation and differentiation of T cells to effector Th or CTLs, but also to central memory T cells (44). Unlike the effector T cells produced in response to infection, these cells survive long-term in the host. If the host is re-exposed to the antigen then central memory T cells undergo rapid expansion and differentiation resulting in the generation of a faster cell-mediated immune response.

1.2.1.2 B lymphocytes

B cells mature in the bone marrow before moving to the peripheral secondary lymphoid organs. Like T cells, B cells also express a unique antigen binding receptor, the B cell receptor (BCR). The BCR is a membrane bound antibody molecule which consists of an Fc (‘fragment, crystalisable’) fragment which is bound by disulphide bonds to an antigen-specific Fab (‘fragment, antigen-binding’) fragment. B cell activation occurs when the BCR comes into contact with cognate antigen. It can be stimulated by T cell-dependent or T cell-independent antigens. T cell-dependent antigen-mediated stimulation of B cells requires direct contact between B and T cells and stimulation of B cells by T cell-produced cytokines and relies on the ability of B cells to present antigen to T cells via MHC Class II (45, 46). T cell-independent activation of B cells occurs when B cells encounter thymus-independent antigens, particularly bacterial proteins such as lipopolysaccharide (LPS) (45, 47).

Upon activation, B cells proliferate and differentiate into B memory cells (which, like T memory cells, are long-lived cells which afford rapid secondary immune responses) and Plasma cells which secrete high levels of antigen-specific antibody. The humoral immune response, which is driven by B cells, is therefore characterised by high levels of antibody in the plasma, lymph fluid and tissues. Antibodies then
bind cognate antigen, resulting in a number of outcomes including complement activation, opsonisation and neutralisation. Different isoforms of antibody are produced depending on the antigen and infection usually results in B cell isotype class-switching from initial IgM responses to the production of isotypes with different spatial characteristics and modes of action (48-50).

1.2.1.3 Dendritic cells
DCs are APCs which can be found in most areas of the body such as in the skin, tissues, blood and secondary lymphoid organs. There are a number of different types of DCs and their identification can be difficult. Most relevant to this study are migratory DCs such as the Langerhans cells. These DCs reside in the epidermis and mucous membranes and, upon exposure to antigen, mature and traffic to the secondary lymphoid organs where they present antigen, most commonly in the context of MHC II, to T helper cells (51, 52). They express MHC class II and up-regulate co-stimulatory molecules upon activation, making them highly efficient APCs. They also express high levels of CD11c. Langerhans cells are the most potent of the ‘professional APCs’ and are crucial for priming the adaptive immune response.

1.2.1.4 Macrophages
Macrophages are mononuclear cells whose primary function is to ingest and degrade (phagocytose) both external (e.g. microorganisms) and internal (e.g. dead cell debris) antigens. Opsonisation of antigens such as bacteria by B cell-produced antibodies enhances this process as the Fc region of antibodies can be bound by Fc receptors on the macrophage. In addition to their phagocytic function, macrophages can also induce antimicrobial and cytotoxic mechanisms through oxygen-dependent and oxygen-independent killing mechanisms as well as through complement (53, 54). They are also one of the ‘professional APCs’ and can present antigen to T helper cells via MHC II on their cell surface. Macrophages typically express high levels of the cell marker F4/80.
1.2.1.5 Neutrophils

Neutrophils are granulocytes that are early mediators of the immune response to pathogens such as bacteria. Due to their multi-lobed nucleus they are often referred to as polymorphonuclear leukocytes (PMNs). Neutrophils, like macrophages, are phagocytic cells that also use oxygen-dependent and -independent mechanisms to perform their antimicrobial function (55-57). These cells typically express high levels of the cell marker Ly6G. Due to the high number of circulating neutrophils in the blood, and the ease of isolating these cell types, neutrophils have been commonly used to investigate processes such as leukocyte trafficking.

1.2.2 T helper cell differentiation

T cell differentiation is complex and requires a milieu of stimulating factors and a number of intrinsic processes. Upon ligation of the TCR, as well as co-stimulation of CD28 (without which T cells generally become anergic (58)), T cells rapidly proliferate and begin producing cytokines (59, 60). Cytokine stimulation of T cell subsets results in up-regulation of disparate transcription factors (depending on the stimulating cytokine) and subsequent production of a cytokine signature for that subset. This is described in more detail below. A schematic diagram of T helper cell differentiation is shown in Figure 1.1.

1.2.2.1 Th1 and Th2 differentiation

Th1 and Th2 cells drive cell-mediated and humoral immune responses respectively. While Th1 and Th2 cells are both activated following stimulation of the TCR by antigen presented on MHC II, different cytokines produced by the APC stimulate the naïve T cell to up-regulate expression of different transcription factors, thus resulting in directed differentiation. In the case of Th1 cells, TCR-induced activation along with stimulation with the cytokine IL-12 ultimately results in upregulation of the transcription factor T-bet (61). Th2 differentiation is regulated by stimulation with the cytokine IL-4 and up-regulation of the transcription factor GATA-3 (20-23). Expression of these transcription factors ultimately results in production of T helper cell type-specific cytokines (i.e. IL-2, IFN-γ and TNF-α by Th1 cells and IL-4, IL-5, IL-10 and IL-13 by Th2 cells) and differential effector function.
1.2.2.2 Th17 and Treg differentiation

Th17 cells typically produce high levels of the pro-inflammatory cytokine IL-17 and require stimulation by the cytokines TGF-β, IL-6, and IL-1β, as well as expression of the transcription factor RORγt, for their differentiation and activation (29, 31-34). Of these cytokines, IL-6 seems to be the most important. Without IL-6, TGF-β-induced differentiation results in the generation of Treg cells. While IL-23 is not required for Th17 differentiation per se, it is necessary for sustained survival and expansion of the Th17 cell type (32, 33).

Treg cells express CD4, CD25 and have high expression of the transcription factor FoxP3 (39-43, 62). They are reliant on TGF-β stimulation and FoxP3 expression for differentiation. However exposure to IL-6 in the presence of TGF-β drives cells away from the Treg-type to the Th17 cell type (63).

Unlike Th1 and Th2 differentiation, which is thought to be relatively finite, it has been demonstrated that there is plasticity in Th17 and Treg cell differentiation (15). For example, Treg cells can be induced to produce cytokines typical of Th17 cells (64-67) and both Treg and Th17 cells can be induced to express cytokines more typical of Th1 and Th2 cells (68-71). This kind of plasticity has not been observed in Th1 and Th2 cell types (15, 65). The consequences and prevalence of these phenomena in vivo are not yet clear.

1.3 INFLAMMATION OF THE CENTRAL NERVOUS SYSTEM

The CNS includes the brain, optic nerves and spinal cord and provides the body with the nervous signals vital for life. It is thought that the CNS may be an immuno-privileged site and that trafficking of immune cells through the healthy CNS is limited and therefore they are rarely exposed to CNS-specific antigens (72-74). Despite this, autoimmunity of the CNS can occasionally occur. In these cases, a CNS-specific, cell-mediated, immune response is initiated, such as that observed in MS patients. The following sections provide an overview of the healthy CNS, the possible causes of MS, the experimental animal models of MS employed in research and the immunopathology and current treatments for the disease.
1.3.1 The healthy central nervous system

The CNS is separated from the periphery by a number of barriers, including the blood brain barrier (BBB) which exists to provide a physical barrier and restrict the passage of cells, pathogens and some chemical compounds to the CNS from the periphery. Within the CNS are neurons, which conduct nervous impulses, and at least four different types of glial cells. Oligodendrocytes are responsible for the production of myelin which is a lipid and protein layer that surrounds neuronal axons and affords rapid nervous conduction. The myelin sheath is made up of several proteins (including myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) and proteolipid protein (PLP)) and is schematically shown in Figure 1.2. Microglia function in a similar way to macrophages and are responsible for immune surveillance within the CNS. Astrocytes are responsible for synaptic maintenance, the integrity of the BBB and the regulation of extracellular pH and K⁺ levels. The functions of the CNS-specific ependymal cells are not yet clear. In a healthy CNS, these cell types all work in concert to protect, regenerate and maintain the CNS environment and allow efficient neuron function.

1.3.2 Multiple Sclerosis

Multiple sclerosis is a debilitating disorder of the CNS that affects approximately 2.5 million people worldwide (National MS Society, USA). The clinical manifestation usually begins in young adulthood (75). The symptoms of the neurological damage that underlies MS vary widely between patients but generally include impairment of motor, sensory and/or cognitive function. Episodes and physical symptoms are unpredictable but often include fatigue, numbness and visual disruption and can progress to more severe paralysis. A slow deterioration of each individual’s capabilities is generally observed. Most cases (~85%) of MS begin with a relapsing-remitting (RR-MS) disease course where patients suffer acute relapses, interspersed with periods of little disease activity, before developing a more steady neurological degeneration later in life (termed secondary progressive MS (SP-MS)) (76, 77). While most patients present with the RR-MS type, 10-20% of patients initially develop the primary progressive (PP-MS) chronic disease course which is normally
more typical of later stages of disease. PP-MS patients display continued and chronic neurological damage and physiological symptoms.

The mechanism of MS pathology is still under investigation. However, it is clear that there are several steps that result in disease. Symptoms arise because an inappropriately activated immune system, particularly (although not exclusively) T and B cells, results in destruction of the myelin sheath which insulates neuron axons and affords rapid nervous conduction (77-79). Once the immune system is inappropriately activated, antigen-specific and non-specific leukocytes infiltrate the CNS in response to chemokines and cytokines and further contribute to the damage (77, 80). The immunopathology of MS, and the cell types involved, are further discussed in section 1.3.4.

While the aetiology of MS is yet to be elucidated, there are clear genetic elements to the disease. MS is prevalent in Caucasians (affecting 0.05-0.15% of the Caucasian population) and is more common in women than men. It is rarely observed in individuals of African or Asian descent, even in areas of high MS prevalence (81, 82). Studies of family members of MS patients have revealed that blood relatives have a higher incidence of the disease, whereas non-blood relatives have the same MS prevalence as the general population, indicating that there is an element of genetic predisposition (83). Several genes, such as \textit{IL7R}, \textit{IL2RA}, \textit{CLEC16A}, \textit{CD226}, \textit{KIF1B} and \textit{TYK2}, have been identified as potentially being involved in MS susceptibility and severity (84-93). However, the MHC class II encoding \textit{DR15} locus is the most consistently associated with MS (84, 94-96), providing strong support not only for the genetic basis of MS, but also for the autoimmune nature of the disease. Future studies are expected to cast further light on the role that genetics plays in MS development.

In addition to the genetic element in MS aetiology, environmental factors are thought to play a role. MS is more prevalent in temperate climates (97) and viral infections have been demonstrated to be associated with MS relapses (98). An ‘MS epidemic’ in the Faroe Islands, where MS was unknown before the 1940s, followed the arrival
of British troops at this time (99), indicating that that there may be some kind of communicable element that contributes to MS (even though MS is not an infectious disease). Viral proteins that are similar to self proteins found exclusively in the CNS may be able to stimulate the immune system to generate a response against the self proteins via a process known as molecular mimicry. Several studies have implicated different pathogens that may contribute to virus-induced, CNS-specific immune cell activation, however as yet there is little firm evidence to implicate any of these in causing MS (100-102). It is known that almost all MS patients have had prior exposure to Epstein-Barr virus (103) and this virus is consistently associated with MS (104-107). However, the mechanism of any link between MS and Epstein-Barr virus remains unclear.

1.3.3 Animal models of multiple sclerosis
While there are established links between genetic and environmental factors in MS, it is still unknown how and why the cells of the immune system become activated and attack the CNS. It is also possible that CNS damage plays a role by exposing CNS-specific proteins to the immune system. While studies are ongoing, research into MS pathology is currently limited as samples of diseased CNS are not easily accessible. Tissues from deceased individuals usually show advanced clinical manifestations and therefore do not give a detailed representation of all phases of the disease. While modern imaging techniques, such as magnetic resonance imaging (MRI), allow visualisation of the CNS, they do not afford differentiation of the cellular make-up of MS lesions. Most cellular research in MS patients has therefore been performed using blood or cerebrospinal fluid (CSF) samples. These studies also only provide a limited ‘snapshot’ of MS pathology. Animal models have been developed to circumvent these issues.

1.3.3.1 Experimental autoimmune encephalomyelitis
The most commonly used model for multiple sclerosis is EAE. Various EAE models share clinical and histopathologic similarities to MS. EAE is induced in susceptible strains of animals (most commonly mice and rats) by immunising with whole spinal cord homogenate or a neuroantigen emulsified in adjuvant. The neuroantigens are
either immunogenic peptides or neuro-proteins that are part of the myelin sheath (see Figure 1.2). The result of this immunisation is an ascending paralysis that displays either a chronic or remitting-relapsing disease course (depending on the strain of animal and immunogen used). There are several benefits of this experimental model. EAE is a robust, inducible, affordable and time-efficient model that allows research into the underlying causes of neuroinflammation. Details of antigenic proteins/peptides used to induce EAE and susceptible animal strains are shown in Table 1.1 and several are described further below.

1.3.3.2 Whole spinal cord homogenate-induced EAE
Initial development of an animal model that had similarities to MS in humans arose following the observation that infection with CNS-infecting viruses (such as smallpox and measles) or treatment with rabies vaccine (that was generated by infecting rabbits before immunising humans with rabbit spinal cord emulsions) often resulted in neurological complications. It was speculated that the exposure of the peripheral immune system to CNS-specific antigens was resulting in the development of a self-reactive immune system and consequent neurological damage (108-112). Subsequent studies, where several strains of animals were injected with CNS homogenate and were observed to develop neuroinflammation, lead to the development of EAE as a model for MS (113-115). The addition of complete Freund’s adjuvant (CFA) to the immunising emulsion enhanced the susceptibility of immunised animals to neurodegeneration (116). This method of inducing EAE has since been demonstrated to result in the activation of the immune system against a variety of myelin sheath antigens (Table 1.1).

1.3.3.3 Induction of EAE using immunogenic peptides of myelin sheath proteins
Several animal strains have been demonstrated to be highly-susceptible to EAE induced by a number of myelin sheath epitopes (Table 1.1). However, the immunogenicity of each epitope varies greatly between animal strains. The myelin proteins that are most commonly utilised to actively induce EAE are MOG, MBP and PLP (117). Numerous peptides of each of these proteins have been demonstrated to be immunogenic in rats and mice (Table 1.1). Immunisation of C57BL/6 mice
with MOG$_{35-55}$ results in an ascending paralysis that follows a chronic disease course which is similar to the later progressive phases of MS (118). Immunisation of SJL/J or Biozzi ABH mice with PLP or MOG antigens induces a remitting-relapsing form of EAE, thus allowing more specific research in to the remission and relapse phases of the disease (119, 120). Different methods of EAE induction can therefore allow research into disparate phases and mechanisms of MS/EAE.

**1.3.3.4 Adoptive transfer**
Adoptive transfer of EAE involves priming donor animals by immunising with myelin antigens, isolating and re-stimulating lymphocytes prior to transferring them to non-immune recipient animals (118-131). Recipient animals typically begin showing signs of ascending paralysis within a week after transfer. This transfer process allows the study of the effector, trafficking, demyelination and relapse phases of the attack on the CNS independently of the priming phase of the immune response.

**1.3.3.5 Transgenic mice**
A limited number of mice have been generated which have TCRs that are specific for myelin epitopes (Table 1.2). These animals can develop EAE spontaneously (incidence range of 20-100% of animals), depending on the strain and genetic manipulation of the animal (117). These animals have proven useful for the study of factors involved in the development of autoimmunity without exogenous manipulation. Humanised transgenic animals carrying human myelin-specific TCRs and multiple sclerosis-associated MHC class II molecules have also afforded research into human immune mechanisms in an *in vivo* context (94, 95, 132).

**1.3.3.6 Cuprizone-mediated demyelination**
Another method of inducing an MS-like pathology is by oral administration of the copper chelator cuprizone (bis-cyclohexanone-oxaldehydrazone). This results in copper deficiency and demyelination, and is used as a model of the demyelination that is observed in the white matter during MS (133). Following administration of cuprizone to C57BL/6 mice, oligodendrocytes are ablated and microglia, along with
peripheral macrophages, phagocytose myelin in the CNS. The BBB remains intact throughout the disease course. Removal of cuprizone from the diet results in remyelination and recovery from the disease. This model therefore allows the study of demyelination independently of antigen-specific immune cell responses and affords investigation into the molecular mechanisms of remyelination in the CNS.

1.3.3.7 Limitations of animal models for MS
Despite the plethora of knowledge that in vivo animal experimentation has generated, as with most disease models there are limitations in how this knowledge can be used to advance treatment of the equivalent human disease (108, 134, 135). Several successful MS therapies have been devised from animal studies. These include Copaxone (galtiramer acetate) (136), Mitoxantrone (137) and Natalzumab (138). Despite this, many have also failed. Interpretation of actively-induced EAE data therefore requires consideration of the differences between animal and human immune systems, particularly when proposing new human therapeutics.

1.3.4 Immunopathology of MS
A particular challenge for researchers studying and medical professionals treating MS is that the disease rarely follows a predictable course. While patients can generally be categorised with respect to whether they have RR-MS or PP-MS, it is impossible to predict the neurological outcomes of their disease. As described above, several models are available for MS research and each allows study into different phases and types of MS-like disease. These models, as well as samples and imaging from MS patients, have afforded a wider understanding of the immunopathology that causes demyelination, prevents remyelination and underlies MS disease.

There are a number of steps required for the development of neuroinflammation. The BBB, which normally protects the CNS from an influx of peripheral leukocytes, must become permeable (139). Whether this occurs before or after the activation of neuroantigen-specific lymphocytes remains unclear. However, once the BBB is more permeable, an influx of antigen-specific and non-specific cells occurs, mediated by the production of pro-inflammatory proteins such as cytokines and chemokines. In
fact, many different cytokines and chemokines have been implicated in EAE disease progression (140-167). This cellular infiltration results in demyelination, oligodendrocyte death and axon loss which are the processes that underlie the neurological manifestations observed in MS.

Many different blood-derived cell types have been implicated in MS/EAE pathogenesis. These include neutrophils (168, 169), macrophages (168-171), mast cells (172-174), natural killer (NK) cells (175-179) and CD8+ T cells (123, 171, 180-185). In addition, activation of microglia in the CNS further exacerbates CNS damage (186). However, the most important cell types in MS/EAE pathology are CD4+ T cells and B cells (29, 33, 115, 127, 150, 151, 155, 156, 158, 159, 162, 164, 187-191). A schematic diagram of MS/EAE immunopathology is shown in Figure 1.3. In addition, the role of T and B cells in MS/EAE pathology is further discussed below.

1.3.4.1 CD4+ T cell-mediated pathology in MS/EAE

CD4+ T cells were first described as the driving force behind EAE pathology after their isolation from MBP-immunised Lewis rats and adoptive transfer resulted in the development of classical EAE in recipient animals (187). It has since been demonstrated that rodents, primates and humans possess potentially autoaggressive T-cell clones, where the TCR is specific for myelin sheath proteins like MBP (192, 193). However, the method of activation of autoreactive T cells is currently unclear. As mentioned, these T cells may be activated due to molecular mimicry by infecting pathogens. Alternatively, damage to the CNS may result in exposure of peripheral autoreactive T cells (which would not normally traffic through the CNS) to neuroantigens, resulting in their subsequent activation. There is a plethora of published research that describes the role of CD4+ T cells in MS/EAE (34, 150, 151, 155, 156, 158, 159, 162, 164, 175). Several specific subsets of CD4+ T cells have been implicated in MS/EAE pathology. These include Th1, Th17 and Treg cells, all of which can be derived from naïve T cells (Figure 1.1 and section 1.2.2).
Interferon gamma (IFN-γ)-producing, IL-12-driven, CD4+ Th1 cells have been widely implicated in MS/EAE pathogenesis. In fact, until fairly recently Th1 cells were the main cell type thought to be responsible for driving the autoimmune response in these diseases. This was primarily due to the fact that high levels of IFN-γ and TNF-α are expressed by encephalitogenic cells and these Th1-type cytokines are also expressed in the CNS of mice with EAE (155, 162, 163). However, despite the initial findings implicating Th1-type cells in EAE, it is now apparent that this cell type is unlikely to be the main cause of the disease. Several studies have demonstrated that knock-out of proteins or receptors thought to be classical determinants of Th1 differentiation and function (such as IL-12, IL-18 and IFN-γ) resulted in enhanced, not reduced, EAE pathogenesis (151, 157, 194-198).

The recent discovery of Th17 cells has resulted in a paradigm shift in regards to the primary cell type that is responsible for the severe pathogenesis observed in MS/EAE. Th17 cells have been implicated in autoimmune conditions including rheumatoid arthritis (RA) (30, 159, 199, 200) and inflammatory bowel disease (201-204) and Th17 cells have also been demonstrated to play an important role in the mediation of autoimmune inflammation of the CNS during EAE (34, 150, 151, 156, 158, 159). Studies have demonstrated that Th17 cells, more than Th1 cells, are necessary for the development of severe EAE pathology. Mice without a functional IL-12 receptor develop EAE, whereas EAE pathogenesis is reduced when mice are administered antibodies that neutralise IL-17 (154). Adoptively transferred Th17 cells are also more efficient at initiating severe EAE pathology in recipient mice than Th1 cells (159), however it has also been demonstrated through adoptive transfer EAE studies that while both Th1 and Th17 cells can drive EAE, neither can establish the same neurological damage without some cooperation from the other cell type (154, 156, 160). The ratio of Th17 to Th1 cells also appears to determine lesion distribution in the CNS; a high Th17:Th1 ratio results in increased brain pathology and severe EAE disease compared with a lower Th17:Th1 ratio (165). These data indicate that, while both Th1 and Th17 cells are required for complete EAE pathogenesis, it is Th17-driven autoimmunity that is responsible for the severe disease phenotype observed in EAE.
While many aspects regarding the role of Th17 cells in MS are yet to be elucidated, it has been demonstrated that MS patients have higher levels of IL-17 in lesions and the CSF (168, 205, 206) and that IL-17-producing memory T cells infiltrate into MS lesions (207). Human Th17 cells have been demonstrated to break down the BBB with a higher efficiency than Th1 cells (207) and human BBB endothelial cells express lower levels of tight junction proteins after culture with IL-17. IL-17 expression has also been shown to stimulate the expression of matrix metalloproteinases in RA (208-210). Taken together, these studies suggest that Th17 cells may be responsible for not only CNS damage in MS but also for increased BBB disruption, thus allowing the influx of other cell types to the CNS. Interfering with Th17 function may prove to have significant therapeutic advantages for MS patients.

The function of Tregs is to control and down-regulate the immune system, particularly to prevent autoimmunity. Deficiencies in Treg function have been demonstrated to be at least partly responsible in several different autoimmune diseases including RA (211-213) and diabetes (214-216). Adoptive transfer of antigen-specific Treg cells has been associated with the prevention or reduction of EAE (62, 217-220), and depletion of Tregs in EAE results in a more severe disease phenotype and prevents remission (221, 222). Patients with MS have lower levels of FoxP3 in their peripheral CD4⁺/CD25^high cells which has been associated with a loss of Treg function (223-225). Therefore, deficiencies in Treg function result in an increased propensity to autoimmune diseases such as MS.

While all of these T cell types have been implicated in MS/EAE pathology, recent evidence describing the plasticity of these lineages, particularly in the case of Th17 and Treg cells, must be taken in to account (15). It has been demonstrated that cells expressing both IFN-γ and IL-17 are present in the CNS during EAE (68) and that these may be Th17 cells that are undergoing differentiation to the Th1-type. While there is clear evidence to suggest that Th17 cells drive the most severe MS/EAE pathology, future research will further define the specific role of each of these T cell subsets.
1.3.4.2 B cells and MS/EAE

While T cells have been critically linked to MS/EAE, it is clear that B cells are also involved. Myelin-specific antibodies are often observed in blood samples from MS patients as well as animals with EAE (226-229). It has since been demonstrated, using a transgenic mouse model with a MOG-specific TCR, that T cell-dependent activation of myelin-specific B cells occurs during EAE (127). In addition to secreting myelin-specific autoantibodies, mature B cells act as APCs and present local autoantigen within the CNS, which exacerbates disease (188, 190). They are also capable of secreting inflammatory cytokines and activating complement, thereby further contributing to the innate immune response (189, 191, 230, 231).

In addition to autoantibody-secreting plasma B cells, regulatory B cells have also been implicated in MS/EAE. Mice deficient in functional B cells have been shown to develop, but not recover from, the MBP-induced remitting EAE disease course (115). Animals treated with antibodies that neutralise CD20 (a B cell-specific marker involved in development) before EAE immunisation display increased disease severity, whereas animals treated with this antibody only from peak disease show declining EAE severity (191). This is attributed to a requirement for regulatory B cells in preventing the early phases of EAE. Therefore, while it is clear that B cells play a role in the pathogenesis of MS/EAE, therapies aimed at B cells for the treatment of autoimmunity must take the function of regulatory B cells into account.

1.3.5 Current therapy for MS

Current therapy for MS patients generally involves treating both the inflammatory and neurodegenerative aspects of the disease. Immunomodulatory drugs such as glatiramer acetate (Copaxone) and β-interferons (Avonex, Betaferon and Rebif) can successfully reduce the relapse rate of the disease (136, 232-235) and a limited number of monoclonal antibodies are in preclinical development or, in the case of Natalizumab, which blocks VLA-4 (α4β1 integrins), are newly approved for MS treatment (138, 236). Intravenous or oral corticosteroids are useful for treating acute exacerbations through their anti-inflammatory and immunosuppressive action. While these methods of treatment have been variously successful, most of them need to be
taken regularly by MS patients. Furthermore, they currently only reduce MS severity and relapses, and long-term neurodegeneration usually still occurs. It is therefore important that a more focused approach to MS treatment/prevention is developed. Several new therapies for relapsing MS, which either block T and B cell activation/function or induce immune tolerance to neuroantigens, are currently in phase 2 clinical trials (237).

1.3.6 Summary

MS/EAE pathology is a complex process that is not yet fully understood. Pharmacologic intervention of any specific immune cell type is not likely to be adequate to completely prevent pathology. However, there are several possibilities for development of targeted intercession of the immune system. These include the targeting of specific cytokines, chemokines and/or concurrent cell types. Intracellular molecules specific for cells of the immune system are also an attractive target. One family of proteins that includes isoforms that are largely immune-cell specific is the PI3K family.

1.4 THE CLASS I PHOSPHOINOSITIDE 3-KINASE FAMILY

The PI3K family consists of 3 classes (I, II and III) defined by their sequence homology (5). Because class II and III PI3Ks have not been implicated in immune signalling they will not be discussed further here. Class I PI3Ks are further classified into class IA and class IB, based on the method of activation. Class IA PI3Ks are activated following receptor tyrosine phosphorylation which occurs after proteins such as growth factors and cytokines, as well as peptides, bind to their cognate receptor. They are also activated downstream of T-cell and B-cell antigen receptors making them fundamental for processes such as cell growth, metabolism, survival, activation and differentiation in many different cell types (238-252). The Class IB PI3K is activated by serpentine transmembrane G protein-coupled receptors (GPCRs), such as chemokine receptors and are primarily involved in cell migration (3, 8, 253). Further details on each of the class I PI3K sub-groups are detailed in sections 1.5 and 1.6.
Once activated, class I PI3Ks phosphorylate membrane-anchored phosphatidylinositol(4,5)-bisphosphate (PtdIns(4,5)P$_2$) at the 3’-OH position of the inositol head group (254, 255). A structural diagram of membrane-anchored inositols in shown in Figure 1.4. Phosphorylation of PtdIns(4,5)P$_2$ (PIP$_2$) results in the generation of PtdIns(3,4,5)P$_3$ (PIP$_3$), which is a docking site for proteins with plekstrin homology (PH) domains such as kinases, phosphatases and lipases that reside in, or are recruited to, the cell membrane. A number of signal transduction pathways resulting from PI3K activation are described in section 1.4.2.

The following sections provide a summary of PI3K structure, expression and regulation, intracellular signal transduction regulated by PI3Ks and the function of specific PI3K isoforms.

1.4.1 Class I PI3Ks – Structure and expression

Class I PI3Ks function primarily as heterodimers that consist of a catalytic p110 subunit coupled with a regulatory subunit. A schematic figure representing all of the catalytic and adaptor subunits is shown in Figure 1.5. There are four different p110 catalytic subunits, p110$\alpha$, p110$\beta$, p110$\delta$ and p110$\gamma$, which are encoded on the PIK3CA, PIK3CB, PIK3CD and PIK3CG genes respectively. Of these catalytic subunits, p110$\alpha$, $\beta$ and $\delta$ are responsible for class IA enzymatic function, and p110$\gamma$ is the sole catalytic subunit in class IB. The p110 isoforms are highly homologous and have several hallmark domains. These include a Ras-binding domain (all p110 subunits bind Ras and it is thought that in many cases Ras is imperative for stimulation of the p110 catalytic subunits) (256-259), a C2 domain (possibly involved in membrane-recruitment), a structural helical backbone and the catalytic domain (which is responsible for adenosine tri-phosphate (ATP) binding and substrate specificity) (259). The expression of class I PI3K catalytic subunits is varied (Table 1.3). Whilst p110$\alpha$ and $\beta$ are expressed ubiquitously, expression of the class IA p110$\delta$ and class IB p110$\gamma$ is largely limited to cells of the immune system (260). This is an important distinction for the rationale of this study.
Class IA PI3K p110 subunits constitutively bind to one of seven regulatory subunits that are derived from three different genes; p85α (PI3KRI), p85β (PI3KR2), and p55γ (PI3KR3). The p110α, β and δ catalytic subunits of class IA can form functional complexes with each of these regulatory subunits (5). The p85 and p55 adaptor proteins are expressed in many cell types (see Table 1.3) and share similar functional domains in that they all contain proline-rich regions and SH2 domains (the region between two of these is responsible for binding to the N terminus of class IA p110 proteins) (261). The p85 proteins also have N-terminal SH3 and RhoGAP regulatory domains thereby allowing participation of other intra- and inter-molecular interactions (262, 263). The regulatory proteins bind to receptor tyrosine kinases (RTKs) via their SH2 domains following receptor dimerisation and tyrosine autophosphorylation events induced by ligand binding. The role of the regulatory subunits in PI3K signalling is thought to be three-fold: to stabilise the p110 catalytic subunits, to inhibit basal activity of the p110 subunits and to recruit them to the phosphorylated residues of RTKs where they are activated (9, 264).

The sole class IB PI3K catalytic subunit, p110γ, binds to one of two regulatory subunits, p101 or p84 (also known as p87PIKAP) (265-267). Aside from demonstrated p110γ and Gβγ binding-domains in p101 (268, 269), both p101 and p84 are still relatively uncharacterised in regards to the presence of other functional domains. As is the case with p110γ, it has been shown that p101 and p84 expression is largely limited to cells of the immune system (266, 267), however both p84 and p110γ have also been shown to be expressed in cardiac tissue (Table 1.3) (267, 270).

The functions of class IA and IB PI3Ks are discussed further in sections 1.5 and 1.6.

1.4.2 Signalling events downstream of PI3Ks
PI3Ks phosphorylate membrane-anchored PtdIns(4,5)P₂ at the 3’-OH position of the inositol head group to form PtdIns(3,4,5)P₃, which results in the generation of a docking site for proteins with PH domains (271). PH domains have been identified in over 100 proteins (255) and consist of a C-terminal α-helix and short β-strands that are connected by highly variable loops (272). Not all of these PH domain-containing
proteins can interact with 3-phosphoinositides. The most commonly studied proteins involved in PIP3-induced activation following RTK or GPCR ligation and PI3K signalling are Akt/PKB, the Tec family tyrosine kinases (e.g. Btk and Itk in B and T lymphocytes) and the small GTPases of the Rho and Arf families. All of these proteins initiate distinct signalling pathways. Signalling events that occur following PI3K activation are complex and are still yet to be fully elucidated. Therefore, only the most well researched elements are discussed briefly here. Figure 1.6 and Figure 1.7 show schematic representations of class IA and IB activation and signalling respectively.

Due to difficulty in directly measuring 3-phosphoinositide levels within cells, one of the most commonly used output readings for PI3K function in mammalian cells is observing levels of phosphorylation of the serine/threonine kinase Akt/PKB (protein kinase B), usually via western blot. Akt/PKB has a PH domain and binds directly to PIP3 following both class IA and IB PI3K activation (255, 273), resulting in Akt/PKB phosphorylation. There are several substrates for Akt/PKB that have been identified and these studies have demonstrated how important Akt/PKB signalling pathways are for processes such as cell survival and function (274-277). Suppression of apoptosis is mediated in part via Akt/PKB phosphorylation and subsequent deactivation of BAD, restricting its ability to bind to and inhibit the anti-apoptosis proteins Bcl-2 and Bcl-XL (thereby indirectly inhibiting apoptosis) (278). Akt/PKB can phosphorylate and inhibit caspase-9, a protease important for later stages of apoptosis, thereby promoting cell survival (276, 278). Akt/PKB can also, in cooperation with protein kinase C (PKC), bind to and activate IκB kinases (IKKs) which are crucial for the regulation of the nuclear factor κB (NFκB) transcription factor (279). This activation of IKKs leads to degradation of IκB which allows NFκB to act as an integral transcription factor for anti-apoptotic proteins. Akt/PKB has also been implicated in the function of forkhead (FH) transcription factors which, upon phosphorylation by Akt/PKB, are retained in the cytosol and prevent expression of proteins such as the Fas ligand, p27kip, Rb2/p130, Bim and TRAIL which are involved in the initiation of apoptosis pathways and control of the cell-cycle (280-284). Akt/PKB is also important for insulin signal transduction through its role in phosphorylating and inhibiting glycogen synthase kinase 3 (GSK3) (285), resulting
in downstream effects on metabolic enzymes involved in augmenting glycogen synthesis.

Another important PH-domain containing protein family that is activated by 3-phosphoinositides are the Tec family of tyrosine kinases. This family of proteins includes Btk (Bruton’s tyrosine kinase) and Itk (inducible T cell kinase) which are vital for B and T cell antigen receptor function respectively (242, 246, 286, 287). Following BCR activation, the main Btk substrate, phospholipase-C-gamma-2 (PLCγ2), hydrolyses PtdIns(1,4,5)P$_3$ to form Ins(1,4,5)P$_3$ and diacylglycerol (DAG), which leads to intracellular Ca$^{2+}$ mobilisation (287-289). This process also influences activation of PKC and thus further impacts on IKK phosphorylation and downstream effects on the control of apoptosis (290, 291). The role of Itk, which is activated downstream of PI3K signalling induced by TCR stimulation, is less clear, however it is thought to be involved in TCR-dependent actin polymerisation (242, 246).

PI3K-induced activation of the small GTPases of the Rho family is fundamental for cell motility. The Rho family proteins (e.g. Rho, Rac and cdc42) are activated by guanine nucleotide exchange factors (GEFs – which convert GDP to GTP) and inactivated by GTPase-activating proteins (GAPs – which dephosphorylate GTP), both of which contain PH domains (292-295). Rho GTPases are involved in adapting actin reorganisation and in cytoskeletal rearrangements; i.e. processes which are required for chemotaxis (294).

As well as the GTPases responsible for Rho family protein activation, GTPases involved in Arf (ADP ribosylation factors) function are also regulated via PH domains (296, 297). These include general receptor for phosphoinositides 1 (GRP1), ARNO (ARF nucleotide binding site opener), centaurin-α1 and cytohesin-1 and 2. These proteins are responsible for integrin-mediated adhesion of leukocytes as well as regulating the leading edge during cell migration.
In summary, the proteins discussed here have been shown to require PI3K signalling for their activation, however it is likely that future research will shed further light on the complexity and significance of PI3Ks signalling.

### 1.4.3 Negative regulation of PI3K activity

Research has yielded significant insight into the negative regulation of PIP3. Once PI3Ks have performed their function and have generated PIP3, negative regulation of the PI3K-generated signal is predominantly performed via de-phosphorylation of PIP3 by the phosphatases PTEN (phosphatase and tensin homolog deleted on chromosome ten) or SHIP (Src homology 2 domain containing inositol polyphosphate phosphatase). These lipid phosphatases de-phosphorylate PIP3 to form PtdIns(4,5)P2 and PtdIns (3,4)P2 respectively (i.e. PTEN de-phosphorylates at the 3’-OH position (298, 299) and SHIP at the 5’-OH position of PtdIns(3,4,5)P3 (300) (as shown in Figure 1.8)). This not only controls basal activity of proteins with PH domains, but also establishes localisation of PI3K signals (PIP3) to specific parts of the cell, for example at the leading-edge following cell activation by chemotactic stimuli. A brief summary of the most important findings of research targeting PTEN and SHIP is discussed below.

PTEN is well-known as a tumour suppressor. PTEN knock-out is embryonic lethal, and animals heterozygous for PTEN show a high incidence of cancer, defective Fas-mediated cell death, lethal polyclonal autoimmunity and lethal lymphoproliferative disease (301-305), mostly due to deregulated Akt/PKB activation (and therefore deregulated apoptosis). PTEN has also been demonstrated to be important for cell chemotaxis (306, 307). Further to its role in chemotaxis and cell survival, PTEN function also impacts on process such as glycogen synthesis (through GSK3 which is initiated by insulin receptor signalling) and can therefore influence the outcome of Type 2 diabetes (308-311). PTEN has also been shown to be important for *in vivo* neutrophil responses to both bacterial infection and during auto-antibody-induced arthritis in mice. A lack of PTEN leads to diminished bacterial containment and clearance and reduced neutrophil-induced arthritic inflammation respectively in these models (307).
There are two closely related SHIP homologues: SHIP1 and SHIP2. SHIP2 proteins have also been strongly implicated in the negative regulation of insulin signalling (312), and genetic knock-out of SHIP2 confers resistance to obesity and diabetes (313). Whilst the mechanism for this has not been delineated, it is likely that deregulated signalling through Akt/PKB in Ship2-/- mice results in increased insulin responsiveness and reduced obesity-related insulin resistance. It has also been shown that SHIP2 can be up-regulated in some breast cancer cell lines (314), but that it does not influence oncogenesis in a myeloma cell line (315) implying that a role for SHIP2 in cancer progression is likely to be cell-type specific. SHIP1 is possibly more relevant in immunology as its expression is limited to cells of haematopoietic lineage (316). Similar to PTEN heterozygous mice, SHIP1-null mice suffer from a lethal myeloproliferative disorder (which bears some similarity to chronic myelogenous leukaemia) where myeloid cells infiltrate the lungs, most likely due to deregulated apoptosis and cell motility (317, 318). Mutations in the SHIP1 gene have been implicated in acute myeloid leukaemia as well as acute lymphoblastic leukaemia (319, 320). SHIP1 has also been implicated in phagocytosis (321, 322), mast cell activation and degranulation (323, 324), histamine-mediated allergic reactions (325-327), macrophage function (328, 329) and T and B lymphocyte survival and function (317, 330-338).

1.4.4 Methods of experimentally disrupting class I PI3K function

There are several methods of investigating PI3K function. These include pan-PI3K inhibitors, isoform-specific small molecule inhibitors and genetic targeting strategies in mice. All of these mechanisms of investigating PI3K function have led to increased understanding of the roles that PI3Ks play.

1.4.4.1 Pan-PI3K inhibitors

Two low-molecular-weight, cell-permeable pan-PI3K inhibitors, Wortmannin and LY294002, have been commercially available for a number of years and have enabled many initial studies into the function of PI3Ks (7, 339-343). These reagents have been important analytical tools for the development of the PI3K field and our current understanding of PI3K signalling. The chemical structures of Wortmannin
and LY294002 are shown in Figures 1.9A and 1.9B respectively. Both compounds potently inhibit class I PI3Ks at low inhibitory concentrations (IC$_{50}$) by binding to the ATP binding-pocket in the catalytic domain of the p110 subunits (344, 345). Wortmannin has a lower IC$_{50}$ whereas LY294002 has a longer half-life and both have been used successfully, independently or in combination. It must be taken in to account that both have off-site effects in that they both also inhibit the mammalian target of rapamycin (mTOR) and DNA-dependent protein kinase (DNA-PK). Wortmannin also inhibits ataxia telangiectasiamutated protein (ATM) and type II Proline-rich domain-containing inositol 5-phosphatase kinases (PIPkins) $\alpha$ and $\beta$, whilst LY294002 can also inhibit casein kinase-2 (CK-2) (255, 346-350). However, if both Wortmannin and LY294002 are used at low concentrations (approximately 20-50nM and 10-100$\mu$M respectively) their specificity is greatly limited to PI3Ks.

In recent years cell-permeable, small-molecular-weight and isoform-specific inhibitors for PI3Ks have been developed. These are discussed below.

1.4.4.2 $p110\alpha$, $p110\beta$ and $p110\gamma$ specific inhibitors

Like the pan-PI3K inhibitors Wortmannin and LY294002, the isoform-specific inhibitors of class I PI3Ks are targeted to the ATP-binding pocket of the catalytic p110 subunits (351). Inhibitors for $p110\alpha$ (352-354), $p110\beta$ (355, 356) and $p110\gamma$ (357, 358) have been developed, with mixed success. Current $p110\alpha$ inhibitors have many off-target effects and inhibit other important kinases such as isoforms of protein kinase C (354), and are therefore not considered to be a useful tool for specifically studying $p110\alpha$ function. Similar issues are likely with $p110\beta$ inhibitors. This is attributed to the ubiquitous expression and function of both $p110\alpha$ and $p110\beta$ and indicates that widespread inhibition of these proteins may not prove to be useful for studying their function or as therapeutics.

Due to their more limited expression, targeting of $p110\delta$ and $p110\gamma$ is a more attractive prospect. The most successful $p110\gamma$-specific inhibitor to date is AS604850 (357). This inhibitor shows high selectivity for $p110\gamma$ with few off-target effects. Inhibitors of $p110\delta$ are discussed below.
1.4.4.3 *P110δ*-specific inhibitors

Several small-molecule *p110δ*-specific inhibitors have been generated. These include IC87048 (359), IC980033 (360) and IC486068 (361). The research using these inhibitors is limited and they will not be further discussed here. The most potent inhibitor with the least off-target effects is IC87114 (Figure 1.9C). IC87114 was first described by Sadhu and colleagues in 2003 (359). The IC$_{50}$ value for PI3Kδ inhibition by IC87114 is 0.5μM, whereas the IC$_{50}$ values for PI3Kα, PI3Kβ and PI3Kγ are 100μM, 75μM and 29μM respectively, indicating that IC87114 is highly-specific for PI3Kδ. In order to avoid inhibition of any of the other PI3K isoforms, and to achieve optimal *p110δ* inhibition, IC87114 is optimally used at concentrations between 1-10μM. Importantly, IC87114 was also shown to have no off-target effects on several other protein kinases including Akt1 (PKBα), PKCα, PKCβII, p38 MAPK, DNA-PK, c-Src, casein kinase I and checkpoint kinase I (359). IC87114 is highly effective at inhibiting PIP$_3$ biosynthesis and significantly reduces PI3Kδ function (238, 240, 359, 360, 362-372).

1.4.4.4 Knock-out and knock-out/knock-in mice

A list of mice with targeted regulatory and catalytic PI3K subunits is shown in Table 1.4. There have been a limited number of different genetic approaches to targeting both PI3K catalytic and regulatory subunits in animal models. The most prevalent is protein knock-out mice. While this is a useful way to measure PI3K function this approach has been proven to have some serious limitations. Several knock-outs have proven unsuccessful as the target protein is probably important for embryonic development and the mice are either embryonic lethal (p110α (373) and p110β (374)) or do not survive for long post-birth (p85α+p55α+p50α (375)), thus highlighting the ubiquitous importance of these PI3K subunits in mammalian biology. Despite this, several successful PI3K subunit knock-out mice have been generated by targeting the p110δ (239, 244), p110γ (376), p85α (305, 377), p55α (378), and p85β (379) proteins. A double knock-out (p110δγ−/−) strain has also been produced (380, 381).

One of the problems with PI3K knock-out mice is that genetic deletion of one family member has been shown to affect expression of others. For instance, up-regulation of
the PI3K p85 regulatory subunit has been observed in p110δ knock-out mice (373). This has led to the development of PI3K ‘knock-out/knock-in’ (KO/KI) mice. Indeed, p110δ (p110δ\textsuperscript{D910A/D910A}) and p110γ (p110γ\textsuperscript{K833R/K833R}) mice have not been reported to show any differences in expression of any of the other PI3K subunits (248, 270). In addition, the p110γ subunit has been confirmed to be expressed in cardiac tissue and a hallmark study comparing cardiac function in p110γ\textsuperscript{−/−} versus p110γ KO/KI mice demonstrated that p110γ is important for scaffolding via catalytic-activity-independent binding with phosphodiesterase 3B (PDE3B) in the heart (270). This demonstrates that catalytically inactive KO/KI mice may be a more relevant method of observing PI3K function without disrupting other, possibly unknown, roles for these proteins.

1.5 P110α, P110β, P110γ AND THE PI3K REGULATORY SUBUNITS – NORMAL FUNCTION AND INVOLVEMENT IN DISEASE

The focus of this thesis is on the functional role of the class IA catalytic subunit, p110δ. However, it must be taken into account that p110δ functions in the cellular environment in close relationship with not only class IA PI3K regulatory subunits, but also often with other class IA and IB PI3K catalytic subunits. A brief summary of class I PI3K regulatory and catalytic p110α, β and γ subunit biology is provided below.

1.5.1 Studies targeting the PI3K regulatory subunits

Attenuation of the function of class I PI3K regulatory subunits has resulted in great advancements in understanding of PI3K function. The most widely studied class I PI3K regulatory subunits are those of the class IA sub-family, p85α, p85β, p55α and p50α, all of which can bind to and mediate activation of the class IA PI3K catalytic subunits (p110α/β/δ) (4). These subunits have been implicated in insulin signalling/diabetes (4, 382-390), autoimmunity (391), cancer (387, 392-395) and immune cell function (303, 305, 387, 396-402). Since genetic knock-out of the p85α gene (PIK3R1; in which p55α and p50α are also encoded) results in embryonic or perinatal lethality (303, 375) these proteins are thought to be important for
development, probably through their role in p110α and p110β function, as knock-out mice for these proteins also die during gestation (373, 374).

While there has been a plethora of research focusing on the class IA PI3K regulatory subunits, there has been less on the class IB regulatory subunits p101 and p84. The expression of both is limited mostly to cells of the immune system, however p84 is also expressed in cardiac tissue and has been demonstrated to be important for kinase-independent p110γ/PDE3B-mediated scaffolding in the heart (267, 270, 403, 404). Both subunits have been implicated in p110γ activation, and p101 overexpression has been demonstrated to enhance survival of T cells (405). However, aside from this, very little is known about the specific function of p101 and p84.

It is undoubtable that the regulatory PI3K subunits all play an important role in controlling catalytic function. However, research efforts have generally focussed on further understanding the functional outcome of activation of the catalytic subunits. This is further discussed below.

1.5.2 Class IA PI3Kα and PI3Kβ
Both p110α and p110β are expressed ubiquitously (4, 255, 406) and genetic deletion of p110α or p110β is embryonic lethal (373, 374). This has restricted the study of p110α and p110β function, however it is evident that p110α is primarily involved in insulin-dependent signalling (407, 408) and p110β is responsible for platelet aggregation and thrombosis and sustained insulin signalling (409-411). P110α has also been implicated in phagocytosis and pinocytosis in macrophages (412), whereas p110β is capable of acting downstream of GPCRs and in some cases is functionally redundant with p110gamma (413). Both p110α and p110β are also involved in the cell cycle and survival (411, 414, 415). Of relevance to this role, p110α has been shown to be up-regulated in many different types of cancer, including ovarian, lung, thyroid, cervical and gastric carcinomas as well as neuroblastoma (392, 394, 416-422). While the involvement of p110β in cancer is not as well documented, it has been implicated in ovarian, prostate and thyroid cancers as well as neuroblastoma (392, 394, 416, 419, 423).
Despite the implication of both p110α and p110β in disease, it is unlikely that specific therapeutic targeting of these proteins would be beneficial due to their widespread expression and involvement in several critical cellular processes. Without cell-specific methods of administration of p110α or p110β therapeutics, targeting of the p110δ and p110γ catalytic PI3K subunits is more realistic.

1.5.3 Class IB PI3Kγ

As mentioned, expression of p110γ, p101 and p84 is largely limited to the immune system (266, 267, 270). Upon binding of a ligand to its cognate GPCR, the PI3Kγ complex (p110γ coupled to either p101 or p84) is activated. GPCRs are so-named due to their coupling with G proteins consisting of α, β and γ subunits. Upon receptor ligation, the Ga subunit is phosphorylated thus allowing dissociation of the Gβγ subunits (which remain as a heterodimer). The Ga subunit then activates the phospholipase C (PLC) pathways responsible for hydrolysis of membrane anchored PtdIns(4,5)P2, ultimately resulting in increased intracellular Ca2+ levels (424, 425). This drives processes such as actin polymerisation and cytoskeletal rearrangements, which are critical processes for cell motility. Following release from Ga, the Gβγ heterodimer directly activates PI3Kγ, resulting in PIP3 production (3, 255, 426).

There are several known isoforms of Gβγ which are all thought to activate the PI3Kγ complex with equal efficiency, at least in vitro (265). As discussed earlier, activation of PI3Kγ results in the generation of PH domains and intracellular processes important for cell growth, survival and activation. However, since PI3Kγ is activated following GPCR ligation with proteins such as chemokines, it is generally accepted that the main function for this complex is in cell migration. In fact, it has been clearly demonstrated that in normal cells PI3Kγ is the predominant PI3K isoform involved in leukocyte motility both in vitro and in vivo, particularly at early time points (3, 8, 253, 307, 368, 369, 376, 427-434). Due to the role of PI3Kγ in cell migration it has been directly implicated in inflammation (376, 429), cancer (394, 423, 433), autoimmune disorders (357, 370, 433, 435-437) and heart disease (438, 439). PI3Kγ has also been implicated in the activation of T cells (440), although its role in this respect is less well-defined. It is clear that, through its role in cell movement and
activation, p110γ could be involved in a wide range of human pathologies such as cancer metastasis, autoimmunity and chronic inflammation.

1.6 P110δ IN IMMUNOLOGY AND DISEASE

Through its role in cells of haematopoietic origin, p110δ has a significant impact on both the innate and cell-mediated arms of the immune system. It has been demonstrated that p110δ is imperative for efficient activation, migration and function of many different leukocyte subsets. As a consequence, p110δ has also been implicated in numerous immune pathologies. Deregulated p110δ-mediated signalling has also been shown to specifically play a role in several different types of cancer. These findings are further discussed below.

As outlined above, p110δ inactivation has been achieved in several different ways (most commonly through genetic means such as p110δ−/− or p110δD910A/D910A mice or with the p110δ inhibitor IC87114 as discussed in section 1.4.4). Therefore, for the purpose of this discussion the term ‘p110δ inactivation’ is used to imply inhibition by any of these methods.

1.6.1 p110δ and leukocyte migration

Current research suggests that the class IB PI3Kγ (which binds to GPCRs like chemokine receptors) is the predominant PI3K responsible for driving directed cell migration (3, 8, 253, 307, 368, 369, 376, 427-434). However there have been several recent reports that also implicate p110δ in trafficking of cells. These studies have primarily highlighted a role for p110δ in the migration of B cells, NK cells and neutrophils. While T cell migration in vivo has also been shown to involve p110δ signalling, this may be indirect.

1.6.1.1 p110δ and migration of T and B cells

Despite a lack of evidence to implicate p110δ in T cell migration in vitro (241, 432, 441), it has been demonstrated that without p110δ, T cells are not capable of efficiently migrating towards antigenic tissue in vivo (441). This reduction in trafficking is TCR-dependent and is supported by findings which show that
chemokine-induced T cell migration, at least in the case of CXCR4-mediated migration, requires cross-talk between TCR- (mediated by ZAP-70) and chemokine-mediated signals (245). The class IA PI3K regulatory subunit p85 has also been implicated in T cell trafficking in the lymph nodes, but this is more likely to be due to disrupted cell-cell interactions as opposed to direct effects on migratory pathways (442). These findings demonstrate that, while p110δ may not play a role in direct migration of T cells, its activation may be indirect, affecting other activities such as T cell activation, adhesion to endothelium and the expression of endothelial adhesion molecules, which are required for efficient extravasation and trafficking of T cells in vivo.

The p110δ protein has however been clearly implicated in B cell trafficking both in vitro (241, 432) and in vivo (241, 244, 248, 432). B cells lacking functional p110δ cannot migrate effectively towards CXCL13 in vitro (432) and a lack of p110δ in transferred B cells affects their ability to home to Peyer’s patches and splenic white pulp in vivo (a process generally governed by the B cell response to CXCL13) (432). Mice lacking functional p110δ are incapable of forming germinal centres, a phenomenon that may be at least in part attributable to defects in B cell homing (241, 244, 248, 432). Aside from this, further effects of p110δ inactivation on B cell homing in vivo (such as to sites of autoimmunity) are yet to be elucidated.

1.6.1.2 p110δ and neutrophil migration

Neutrophils are commonly used to study cell trafficking and p110δ has been implicated in migration of this cell type. It has been reported that p110δ-inactivation in neutrophils can result in reduced cell trafficking in vitro, however this is dependent on the chemotactic stimulus driving neutrophil migration (307, 359, 365). Neutrophil migration in vivo (which is reliant on a range of different chemotactic cues) also requires p110δ (368-370). Expression of functional p110δ in endothelial cells helps govern their adhesive state (369). OT-II mice without functional p110δ express lower levels of ICAM-1 and VCAM-1 in the lung following ovalbumin (OVA) challenge, further supporting a role for p110δ in endothelial adhesion molecule expression (367). In addition to the importance of functional p110δ in the
endothelium, neutrophils with inactive p110δ are not capable of efficiently migrating across leukotriene (LTB)₄- or TNF-α-treated inflamed microvessels in vivo (369, 370) and functional p110δ is required for efficient infiltration of neutrophils to joints of mice with autoantibody-induced arthritis (370). Furthermore, it was shown that p110δ plays a more important role in later stages (4+ hours) of migration whereas the class IB p110γ is more important for initial stages of neutrophil motility in vivo (368).

1.6.1.3 p110δ and migration of natural killer and mast cells

Through its role in SCF-dependent adhesion to fibronectin and transwell chemotaxis in vitro, p110δ has been implicated in the migration of mast cells (362, 363). NK cells also utilise p110δ-dependent pathways for chemotaxis. P110δ-inactivated NK cells cannot migrate efficiently towards CXCL12 or CCL3, however p110γ has also been demonstrated to be important for NK migration towards these chemokines (434). In addition, p110δ is required for NK migration towards CXCL10 and sphingosine-1-phosphate (S1P) whereas p110γ is not (434). Interestingly, even when chemotaxis is dependent on p110δ but not p110γ it is still sensitive to pertussis toxin (434). This indicates that the signalling is occurring through, and p110δ may be directly activated by, GPCRs. NK cells from mice lacking active p110δ cannot efficiently migrate to the peritoneum in response to LPS-induced inflammation, nor to the uterus in response to normal chemotactic signals that occur during pregnancy (434), indicating that the p110δ isoform is necessary for efficient recruitment of NK cells in vivo.

1.6.2 p110δ and cancer

Cells require several ‘hallmarks’ to become cancerous. These include self-sufficiency in growth signals and insensitivity to anti-growth signals, limitless replicative potential, the ability to drive angiogenesis, tissue invasion and metastasis (443). As previously discussed, PI3Ks are involved in regulation of the cell-cycle, apoptosis and cell activation. Mutations in PI3K subunits have been strongly implicated in several different types of cancers (444-446). In addition to this, several studies have indirectly implicated PI3Ks by demonstrating unregulated Akt/PKB
signalling or reduced PTEN-mediated de-phosphorylation of PIP₃ in cancer cells (444-446). As p110δ is highly expressed in leukocytes and is the predominant class IA PI3K in leukocyte signalling it is reasonable to speculate that deregulation of p110δ may be a primary cause of increased survival (by loss of apoptosis) in leukocyte tumours. Analysis of samples from human patients with acute myeloid leukaemia (AML) delineated that p110δ, but not p110α, β or γ, is consistently expressed at high levels in blast cells from AML (371), however this is not due to a mutation in p110δ (447). The p110δ inhibitor, IC87114, can suppress AML proliferation and Akt/PKB activation in blasts to the same extent as the pan-PI3K inhibitor LY294002 (371). Inhibition of p110δ also results in reduced proliferation and increased cell death in acute promyelocytic leukaemia (APL) cells (448) and is capable of reducing the proliferation of the WEHI-231 B cell lymphoma cell line, as well as impairing Akt/PKB phosphorylation in these cells (238).

P110δ deregulation has also been specifically implicated in several different types of non-myeloid cancers. In addition to high p110δ expression in cells of haematopoietic origin, p110δ has also been shown to be expressed in endothelial cells. Mice with Lewis lung carcinoma (a cancer of the endothelial cells of the lung) or GL261 hind limb endothelial tumours display reduced tumour growth when treated with a selective p110δ inhibitor (IC486068) in conjunction with radiation when compared to either treatment alone (361). It has also been demonstrated that, through a role downstream of the epidermal growth factor receptor, breast cancer cells require p110δ for migration (449). P110δ is up-regulated in some neuroblastoma tumours and contributes to survival and uncontrolled growth in these cells (416). Studies observing the role of p110δ in the induction of oncogenic transformation of chicken embryo fibroblasts (CEF) have demonstrated that this isoform can confer Ras-independent oncogenic potential to these cells (423, 450).

These studies reveal that the development of p110δ-specific inhibitors for treatment of cancers of both myeloid and non-myeloid types may provide therapeutic intervention of PI3Ks in a manner that would not disrupt off-target cellular functions (which may occur with pan-PI3K inhibition).
1.6.3 The role of p110δ in Neutrophil function
Neutrophils are critical for the innate immune response, and, as discussed above, p110δ has been demonstrated to be integral for efficient migration of these cells. However, p110δ is not only important for neutrophil migration, but also for their activation and function. Initial reports showed that, without active p110δ, neutrophils are not capable of superoxide generation in response to TNF-α and N-formyl-methionyl-leucyl-phenylalanine (fMLP) \textit{in vitro} (360), and they produce less PIP₃ following fMLP stimulation (369). \textit{In vivo}, p110δ inactivation can reduce LPS-mediated neutrophil accumulation in a model of acute pulmonary inflammation (369) and neutrophil accumulation in the lungs of mice challenged with OVA is reduced when p110δ is inhibited by IC87114 treatment (367). Without functional p110δ, mice do not develop autoantibody-induced arthritis to the same extent as wild-type counterparts (370), a phenomenon which was attributed, at least in part, to reduced neutrophil accumulation in joints of p110δ-deficient animals. Future research may further elucidate the role of p110δ in the specific function of neutrophils, however these findings indicate that therapeutic inactivation of p110δ may prove a useful strategy in disorders where infiltration by neutrophils is an underlying cause, such as asthma and RA (451-457).

1.6.4 The role of p110δ in Mast cell-mediated allergic responses
Mast cells are important for inflammatory responses to invading pathogens and parasites. Importantly, they also contribute to atypical inflammatory responses such as asthma, autoimmunity and other allergic disorders. As discussed in section 1.6.1.3, p110δ is involved in adhesion and migration of mast cells. However, it has also been implicated in mast cell development and function.

P110δ-inactivation has been shown to result in reduced phosphorylation of Akt/PKB in mast cells following SCF- and IL-3-stimulation \textit{in vitro} (362, 363). Signalling through the mast cell FceRI receptor with IgE-antigen is also reduced when p110δ is genetically or pharmacologically inactivated (362, 363). Furthermore, p110δ inactivation results in reduced FceRI-mediated mast cell-dependent cutaneous
anaphylaxis *in vivo* following intradermal administration with dinitrophenyl (DNP)-directed IgE and systemic DNP challenge (362).

These findings are particularly important for the interpretation of a number of studies implicating p110δ in airway hyperresponsiveness (367, 458, 459). Although those studies did not specifically concentrate on mast cells, this cell type has been independently implicated in asthma and airway inflammation (460). It is clear that without efficient p110δ-driven signals mast cells are not capable of participating in the immune response, which could partially explain why reduced airway inflammation was observed in these studies following p110δ inactivation (367, 458, 459). However, mice lacking functional p110δ (p110δ<sup>D910A/D910A</sup> mice) have fewer mast cells in the dermis of the ear as well as the submucosa and muscularis of the stomach (362, 363). Therefore, the impact of p110δ on mast cell development and differentiation must be taken into account when considering the effects that this protein has on mast cell function.

1.6.5 The effect of p110δ on natural killer cell-mediated cytotoxicity

Aside from the role that p110δ plays in NK cell migration (discussed in section 1.6.1.3), this protein is also integral for efficient NK function. NK cells are key mediators of innate immunity, playing a significant role in mediating cytotoxicity towards virus-infected and tumour cells. Without p110δ, NK cells do not efficiently develop and mature (380, 461, 462). NK cells with inactive p110δ fail to efficiently clear transplanted lymphoma cells due to reduced CXCR3-mediated chemotaxis of NK cells *in vivo* (434). NK cells from mice lacking active p110δ were not capable of mediating cytotoxicity against influenza virus infection (461). This indicates that p110δ may be more important for NK mediated antiviral responses than in tumour clearance. In addition to this, inactivation of p110δ results in profound defects in secretion of the cytokines and chemokines IFN-γ, GM-CSF, MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) by NK cells (461, 462). This lack of p110δ-mediated cytokine and chemokine secretion by NK cells is likely to have significant detrimental effects on the immune response.
1.6.6 The role of p110δ in dendritic cells
At this stage, the only published evidence implicating p110δ in DC function showed that bone marrow-derived DCs (BMDCs) from p110δD910A/D910A mice produce less IL-6 compared to BMDCs from wild-type animals following stimulation in vitro with cholera toxin (463). While this was not further investigated in the published study, it is possible that reduced IL-6 production by DCs due to targeted p110δ inactivation may affect the differentiation of T cells upon antigen presentation by these APCs. Because pathogenic Th17 cells require IL-6 for efficient differentiation it is possible that reduced IL-6 production by DCs could affect the activation of this specific T cell subset. However, there is no further published evidence to either support or disprove a role for p110δ in DC function as yet.

1.6.7 The role of p110δ in B cell activation and function
In addition to the requirement for p110δ in B cell trafficking, the involvement of p110δ in B cell activation and function has been widely studied. Inhibition of p110δ function has arguably the most profound negative impact on B cells when compared with any other cell type.

Several studies have elucidated an important role for p110δ in B cell specific processes, particularly those downstream of the BCR, and p110δ has been implicated in both T-dependent and T-independent B cell responses. Without p110δ function downstream of the BCR, B cells have lower levels of phosphorylated Akt/PKB and Btk, reduced PLCγ2-mediated Ca2+ flux following anti-IgM exposure and also show an impaired ability to proliferate in response to B cell stimuli such as anti-IgM, anti-CD40, IL-4, CpG and LPS in vitro (238-241, 243, 244, 248). B cells lacking functional p110δ show reduced activation of cell-cycle regulators such as cyclins D2, A and E, retinoblastoma protein (Rb), p107 and E2F1 after anti-IgM stimulation (238, 398, 401) and fail to successfully progress through the cell-cycle (398, 401). Other downstream effectors of BCR signalling that are not effectively activated following anti-IgM stimulation include the forkhead transcription factor FOXO3a and GSK3α (238). In vivo, p110δ-inactivated B cells generate lower levels of serum immunoglobulin in response to T-dependent and T-independent antigens (239, 241,
and fail to generate germinal centres in the spleen, mesenteric lymph nodes and Peyer’s patches upon adoptive transfer or in T-dependent and T-independent models of inflammatory bowel disease (244, 248, 432). These studies identified the critical importance of p110δ in B cell function and activation.

Without functional p110δ, it has been reported that B cells show uncontrolled class-switching to IgE and IgG1 after anti-CD40/IL-4, LPS/IL-4 and CpG/IL-4 treatment in vitro (240, 372). This occurs despite reductions in the production of other immunoglobulin isoforms and type 2 cytokines (IL-4, IL-6, IL-10 and IL-13) (240, 372, 459). Therefore, if lacking p110δ, B cell signalling through CD40/IL-4 receptors as well as toll-like receptors appears to result in faulty regulation of IgG1 and IgE B cell responses. These findings demonstrate that inactivation of p110δ may have further implications that must be taken into account when considering attenuation of p110δ function in disease.

Defects in B cell development have also been reported and the most significant outcomes of this are a reduction in peritoneal B1 B cell and CD23−CD21+ marginal zone B cell numbers in mice lacking functional p110δ (239, 241, 248). There are also fewer cells in secondary lymphoid organs of mice lacking functional p110δ (244, 248). The down-regulation of RAG gene expression, which is necessary for light chain allelic/isotype exclusion during maturation, is also p110δ-dependent (247). Without this regulation of RAG, unique BCR production does not occur. Therefore p110δ is not only important for the development and differentiation of B cells but also for the development of the BCR repertoire.

In response to IL-4-stimulation, p110δ−/− B cells do not display increased levels of phosphorylated Akt/PKB thereby rendering them incapable of preventing apoptosis through downstream phosphorylation of proteins such as IκBα and Bcl-XL (238, 239). Despite this, B cell survival has not yet been addressed in an in vivo setting; without proper p110δ-mediated survival signals, efficient B cell generation and immune responses are unlikely to occur.
In summary, p110δ has been implicated in the control of B cell development, antibody production, BCR specificity and survival. As p110δ has been shown to play an important role in B cell function both \textit{in vitro} and in disease models \textit{in vivo}, it is likely that autoimmune diseases which have a B cell component (such as MS/EAE and RA/collagen-induced arthritis (CIA)) would benefit from inhibition of p110δ function.

1.6.8 The role of p110δ in T cell activation and function

T cells are integral for the cell-mediated immune response and inactivation of p110δ in T cells results in profound defects in TCR signalling (248). T cells lacking functional p110δ are not capable of effectively undergoing processes such as phosphorylation of Akt/PKB, Ca$^{2+}$ flux and proliferation following TCR stimulation with anti-CD3 antibodies (248, 249).

T cells from p110δ KO/KI mice that have been crossed with OT-II TCR transgenic mice (resulting in p110δ-inactivated T cells with a TCR that is specific for OVA) show a reduced capacity to proliferate in response to OVA antigen (249) and are incapable of sustained production of IFN-$\gamma$ or IL-4 under Th1- or Th2-skewing culture conditions respectively (249), indicating that both Th1- and Th2-type immune responses are reduced without functional p110δ. \textit{In vivo}, these T cells fail to undergo clonal expansion or to produce IFN-$\gamma$ in response to immunisation with OVA/LPS (249). As discussed in section 1.6.7, T-dependent B cell responses are also attenuated in mice lacking functional p110δ (239, 244, 248) which may be due to defects in T cell function, B cell function or, most likely, both.

While T cell development appears to be normal in mice without functional p110δ, there are significantly fewer T cells in the secondary lymphoid organs of these animals (248, 381). Despite the smaller secondary lymphoid organs, normal ratios CD4$^+$ and CD8$^+$ are observed (248, 381).

In addition to the well-documented role for p110δ in Th1 and Th2 cell differentiation and function, it has also been reported that p110δ$_{D910A/D910A}$ mice have impaired
development and function of CD4⁺/CD25⁺/FoxP3⁺ Treg cells (464). Development of Tregs is impaired as evidenced by fewer regulatory T cells in the periphery when compared with wild-type mice (464). The ability of these Tregs to suppress proliferation of CD4⁺/CD25⁺ cells in response to anti-CD3/CD28 (with exogenous IL-2) is reduced, as are the levels of the cytokine IL-10 that are produced by p110δ-inactivated Tregs (464). Adoptively transferred Tregs without functional p110δ cannot protect recipient mice from disease in a model of experimental colitis (464). These findings further support the importance of p110δ in TCR-mediated activation of T cells.

Unlike that observed in p110δ-inactivated B cells, no link has been made between p110δ inactivation and increases in T cell apoptosis (249) (although mice lacking both p110δ and p110γ show increased thymocyte apoptosis - see section 1.6.9). Despite the clear influence that p110δ exerts on activation and differentiation of Tregs and Th1- and Th2-type T cells, the influence of p110δ on the differentiation of IL-17-producing Th17 cells is as yet undetermined. It is also unclear as to whether the reduction in T cell differentiation is purely a result of defective T cell function, or whether a lack of p110δ signalling also affects the presentation of antigen to T cells by APCs such as DCs.

1.6.9 Attenuating both p110δ and p110γ function

As discussed in previous sections, the expression of both p110δ and p110γ is largely limited to cells of the immune system and they perform different yet often complementary functions within leukocytes. There is also some evidence that they can function through receptors normally not thought to be involved in activation of each of these specific enzymes, such as p110δ activation by GPCRs and p110γ activation through RTKs (252, 434), however the prevalence and implications of these findings are not yet clear. It is rarely shown that inactivation of either p110δ or p110γ results in a complete inhibition of the output reading, such as chemotaxis or cell activation, indicating that there may be some redundancy between these systems. It is however important to note that when p110δ (and maybe p110γ) is inactivated that p110α/β may also be able to execute some of the functions normally performed
by these proteins. Despite this, it is an attractive option to cooperatively target both the p110δ and p110γ isoforms to further reduce cell function (465). To address this, two approaches have been used. Firstly, mice in which both p110δ and p110γ have been genetically inactivated have been generated (p110γ/δ−/− mice (380, 381) and p110γ−/δD910A/D910A mice (428)). Secondly, a combination of genetically-altered mice and small molecule p110 subunit inhibitors is commonly used to investigate the effects of inhibition of both of these PI3K catalytic subunits (366, 368, 370). However these approaches have not always shown that concurrent inhibition results in any functional differences when compared to inhibition of one of the subunits alone (364, 365).

Several studies have focussed on the effect of attenuating p110δ/γ function in different cell types, the most widely studied being neutrophils. In vivo, the migration of neutrophils to TNF-α is additionally reduced when compared with only p110γ-inactivation (368). This has been attributed to differential roles for p110γ and p110δ in early and late phases of the neutrophil emigration response respectively. Furthermore, mice lacking both p110δ and p110γ function display significantly reduced mean ankle thickness, altered histology, bone and cartilage erosion, and neutrophil infiltration in a model of autoantibody-induced arthritis (370). The attenuation of both p110δ and p110γ in this model showed further reductions in all of these outputs when compared to the inhibition of only one catalytic subunit alone. Therefore both p110γ and p110δ are important for movement of neutrophils into inflamed joint tissue.

While no major defects in T cell development have been described in p110δ-inactivated animals, p110γ inactivation has been shown to lead to reduced thymus size, cellularity and defects in thymocyte selection (433). Mice lacking both p110δ and p110γ function have further defects in thymocyte differentiation as well as increased apoptosis in double-positive thymocytes (251, 381). Extrathymic T cells from these animals are less capable of mobilising intracellular Ca2+ in response to TCR cross-linking than was observed in both p110δ−/− and p110γ−/− mice, indicating
that both of these proteins work in concert to mediate TCR signalling in mature T cells (251).

Mice with deleted p110\(\gamma\) and inactive p110\(\delta\) (p110\(\gamma^{-/-}\delta^{D910A/D910A}\) mice) have reduced B and T cell numbers and lower levels of serum immunoglobulin compared with wild-type animals (428). In addition, function of Th1 and Th2 cells from these animals is altered; lower levels of IFN-\(\gamma\) and TNF-\(\alpha\), but higher levels of IL-5 and IL-4 are observed following anti-CD3 or anti-CD3/anti-CD28 stimulation \textit{in vitro}. Perhaps the most striking phenotype of these mice however is that they spontaneously develop eosinophil- and T cell-dominated inflammation in the stomach and salivary glands which may be attributable to reduced Treg function and increased Th2 development (428).

As demonstrated by the aforementioned studies which co-ordinately inactivated both p110\(\delta\) and p110\(\gamma\) function, there may be benefits to reducing the function of these proteins concurrently. However serious side-effects, in particular the high incidence of spontaneous inflammation in multiple organs, must be taken into account when considering this approach.

1.7 HYPOTHESES AND AIMS OF THE STUDY

As discussed above, the p110\(\delta\) protein has been shown to be integral for many immunological processes, including those required for migration, activation, differentiation and/or function in neutrophils, mast cells, NK cells, B and T cells. All of these cell types have been implicated in either MS or EAE, and it is postulated that reduction in their function may result in reduced disease. In particular, CD4\(^+\) T cells have been demonstrated to be highly pathogenic and are generally accepted to be the major initiating cell type in both EAE and MS. Research has shown that p110\(\delta\)-inactivation reduces the capacity for efficient T cell differentiation to the Th1-, Th2- and Treg-types. However, to date, little is known about the role that p110\(\delta\) plays in Th17-cell differentiation, nor has p110\(\delta\) been specifically implicated in the generation of the autoimmune response in MS or EAE. The published findings, and speculation that arises from them, have led to the following hypotheses:
1) That inactivation of p110δ results in reduced differentiation of naïve CD4+ T cells to the IL-17-producing Th17-type.

2) That genetically-inactivating p110δ function influences EAE disease pathogenesis through reducing immune cell activation and function.

3) That treatment of murine cells with the p110δ inhibitor IC87114 reduces their activation and differentiation.

To address the above hypotheses, the experimental aims were as follows:

1) To further characterise the KO/KI p110δ<sup>D910A/D910A</sup> (kinase-dead) mice and assess their ability to generate an autoimmune response in the model for multiple sclerosis, EAE.

2) To characterise the autoimmune response in p110δ<sup>D910A/D910A</sup> mice with particular regard to the leukocyte infiltration to the CNS, DC activation and B and T cell activation and survival.

3) To assess the role of p110δ in the generation of IL-17-producing Th17 cells.

4) To investigate the ability of the p110δ inhibitor, IC87114, to reduce differentiation of T cells and influence EAE outcome.

The results of the experimental investigation of the aforementioned hypotheses are presented and discussed in the subsequent chapters of this thesis.
Figure 1.1: T cell differentiation. Following antigen-specific activation, naïve T cells may undergo differentiation to several different types depending on the stimuli received. When activated in the presence of TGF-β alone they up-regulate the FoxP3 transcription factor and differentiate into regulatory T cells. When activated in the presence of TGF-β and IL-6 they up-regulate the RORγt transcription factor and become IL-17-producing Th17 cells. Th1 cells are driven by IL-12 and expression of the transcription factor T-bet. Th2 cells differentiate following IL-4 stimulation and GATA3 up-regulation. IFN-γ and IL-4, produced by Th1 and Th2 cells respectively, negatively regulate Th17 differentiation. IFN-γ also suppresses Th2 differentiation, and IL-4 suppresses Th1 differentiation. (Adapted from (150))
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Figure 1.2: Proteins of the myelin sheath. The myelin sheath, which surrounds and protects axons and affords rapid nervous conduction, is comprised of lipids (80%) and protein (20%) and is formed by oligodendrocytes. Proteins that make up the myelin sheath include PLP (proteolipid protein), MOG (myelin oligodendrocyte protein) and MBP (myelin basic protein). (Adapted from (466))
Figure 1.3: Immunopathology of multiple sclerosis. There are many facets to CNS inflammation. Upon presentation of neuro-antigen to naïve T cells by APCs and exposure to cytokines, naïve CD4+ T cells differentiate to the Th1- and Th17-types, before entering the CNS where they are restimulated by CNS resident cells and secrete cytokines such as IL-17, IFN-γ and TNF-α. These cytokines also further disrupt the BBB, exacerbating the influx of antigen-specific and non-specific cells. CD8+ T cells and B cells also become activated in an antigen-specific manner. Mature cytotoxic T cells and plasma cells then cause further CNS damage, including oligodendrocyte cell death and local antibody-production and complement induction respectively. CNS-resident microglia are also activated. Cytokines and chemokines produced within the CNS by T cells, plasma cells and microglia further exacerbate disease by recruiting more of these cell types, as well as macrophages, neutrophils and NK cells to the CNS. The ultimate result of this immune response is myelin destruction, neuron death and inefficient nervous conduction. (Adapted from (230))
NOTE:
This figure is included on page 49 of the print copy of the thesis held in the University of Adelaide Library.
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**Figure 1.4: Chemical structure of membrane-anchored phosphatidylinositol.**
The inositol head group is attached to the membrane-anchored diacylglycerol fatty acid tails by a phosphodiester link. (Adapted from (6, 255))
**Figure 1.5: Class I PI3K catalytic and regulatory subunits.** The class IA PI3Ks consist of three p110 catalytic subunits (α, β and δ) that couple to p85 or p55 regulatory subunits. The class IB catalytic subunit p110γ binds to either p101 or p84 regulatory subunits to efficiently function. All p110 subunits have a C2, Ras binding and catalytic domains as well as a helical backbone. The class IA regulatory subunits all have p110α/β/δ binding and SH2 domains as well as proline rich regions (PRR). The p85 proteins also have GAP and SH3 domains. The class IB regulatory subunit p101 binds p110γ at the N terminus and Gβγ at the C terminus. The p84 protein is currently uncharacterised. (Adapted from (1))
CHAPTER 1: Introduction

Class IB PI3K

Class IA PI3K
Figure 1.6: Class IA PI3K signalling. Following ligand binding to RTKs, receptor homodimerisation and autophosphorylation, class IA PI3Ks are activated. This activation results in phosphorylation of PIP$_2$ to PIP$_3$ which provides a docking site for proteins with PH domains. Activation of class IA PI3Ks contributes to diverse cell processes such as activation, migration, metabolism, differentiation, survival, growth and phagocytosis. (Adapted from (3))
CHAPTER 1: Introduction

Growth factor/cytokine etc.

Class IA PI3K

Ras

p110α/β/δ

p85/p55

p50

PI3K

RTK

Metabolism

Growth

Differentiation

Phagocytosis

Cell migration

Activation

Survival

PI3P

Cytosol

PH domain-containing protein
Figure 1.7: Class IB PI3K signalling. The binding of GPCR agonists (such as chemokines) to their cognate GPCR results in activation of G proteins and subsequent class IB PI3K activation. PI3Kγ then phosphorylates PIP$_2$ to PIP$_3$ which generates a docking site for proteins with PH domains. Activation of class IB PI3K contributes to cell processes such as migration and survival. (Adapted from (3))
CHAPTER 1: Introduction

Cell migration
Survival

Chemokine

GPCR (e.g. chemokine receptor)

Gα/β/γ
Gβγ
PI3Kγ
p110γ
Ras
p101

PI3K

P110γ

PI3Kγ

PH domain-containing protein

Cytosol

PIP3

containing PH domain-protein

6α/β/γ

Cell migration
Survival
CHAPTER 1: Introduction

Figure 1.8: Negative regulation of PIP$_3$. Following PtdIns(3,4,5)P$_3$ generation by class I PI3Ks, PTEN and SHIP de-phosphorylate PIP$_3$ at the 3’ or 5’ position of the inositol head group to form PtdIns(4,5)P$_2$ and PtdIns(3,4)P$_2$ respectively. (Adapted from (255)).

NOTE:
This figure is included on page 58 of the print copy of the thesis held in the University of Adelaide Library.
Figure 1.9: Chemical structure of the PI3K inhibitors Wortmannin, LY 294002 and IC87114. The pan-PI3K inhibitors (A) Wortmannin and (B) LY 294002 (7). (C) The p110δ-specific small molecule inhibitor IC87114 (359).

NOTE:
This figure is included on page 59 of the print copy of the thesis held in the University of Adelaide Library.
Table 1.1: Commonly used immunising antigens in EAE. The CNS antigens most commonly used to immunise animals to develop EAE are listed. Shown are the antigenic peptide, animal strain that is commonly used and the induction protocol used to generate EAE disease (Induced = actively immunised. Transferred = adoptively transferred encephalitogenic cells). (Adapted from (117))

*Legend*
M = Mice, R = Rat, P = Primate.
* Most commonly used models of EAE.
<table>
<thead>
<tr>
<th><strong>Immunising antigen</strong></th>
<th><strong>Autoantigen</strong></th>
<th><strong>Animal strain</strong></th>
<th><strong>Induction</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myelin basic protein (MBP)</strong></td>
<td>MBP</td>
<td>SJL/J (M)</td>
<td>Induced</td>
<td>(467)</td>
</tr>
<tr>
<td></td>
<td>MBP</td>
<td>Lewis (R)</td>
<td>Induced</td>
<td>(125, 129)</td>
</tr>
<tr>
<td></td>
<td>MBP</td>
<td>Rhesus monkey (P)</td>
<td>Induced</td>
<td>(468)</td>
</tr>
<tr>
<td></td>
<td>MBP Ac1-11</td>
<td>B10.PL and PL/J (M)</td>
<td>Induced</td>
<td>(121, 130)</td>
</tr>
<tr>
<td></td>
<td>MBP 21-35</td>
<td>Lewis (R)</td>
<td>Induced</td>
<td>(469)</td>
</tr>
<tr>
<td></td>
<td>MBP 29-84</td>
<td>Lewis (R)</td>
<td>Induced</td>
<td>(125, 129)</td>
</tr>
<tr>
<td></td>
<td>MBP 61-82</td>
<td>C3H/HeJ Lewis (R)</td>
<td>Transferred</td>
<td>(123)</td>
</tr>
<tr>
<td></td>
<td>MBP 79-87</td>
<td>SJL/J (M)</td>
<td>Induced</td>
<td>(125, 129)</td>
</tr>
<tr>
<td></td>
<td>MBP 80-105</td>
<td>Lewis (R)</td>
<td>Induced</td>
<td>(469)</td>
</tr>
<tr>
<td></td>
<td>MBP 89-101</td>
<td></td>
<td></td>
<td>(125, 129)</td>
</tr>
<tr>
<td></td>
<td>MBP 170-186</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Myelin oligodendrocyte glycoprotein (MOG)</strong></td>
<td>MOG</td>
<td>SJL/J (M)</td>
<td>Induced/Transferred</td>
<td>(119, 127)</td>
</tr>
<tr>
<td></td>
<td>MOG</td>
<td>Biozzi ABH (M)</td>
<td>Induced</td>
<td>(119)</td>
</tr>
<tr>
<td></td>
<td>MOG</td>
<td>DA, BN (R)</td>
<td>Induced</td>
<td>(470)</td>
</tr>
<tr>
<td></td>
<td>MOG</td>
<td>Common Marmoset (P)</td>
<td>Induced</td>
<td>(471)</td>
</tr>
<tr>
<td></td>
<td>MOG1-22</td>
<td>Biozzi ABH (M)</td>
<td>Induced</td>
<td>(119)</td>
</tr>
<tr>
<td></td>
<td>MOG14-36</td>
<td>Common Marmoset (P)</td>
<td>Induced</td>
<td>(471)</td>
</tr>
<tr>
<td></td>
<td>MOG34-56</td>
<td>Rhesus Monkey (P)</td>
<td>Induced</td>
<td>(119)</td>
</tr>
<tr>
<td></td>
<td>MOG35-55</td>
<td>C57BL/6 (M)</td>
<td>Induced</td>
<td>(468)</td>
</tr>
<tr>
<td></td>
<td>MOG35-55</td>
<td>C57BL/6 (M)</td>
<td>Induced</td>
<td>(118)</td>
</tr>
<tr>
<td></td>
<td>MOG43-57</td>
<td>B10.PL and PL/J (M)</td>
<td>Transferred</td>
<td>(118)</td>
</tr>
<tr>
<td></td>
<td>MOG74-90</td>
<td>Biozzi ABH (M)</td>
<td>Induced</td>
<td>(124)</td>
</tr>
<tr>
<td></td>
<td>MOG92-106</td>
<td>DA, BN (R)</td>
<td>Transferred</td>
<td>(119)</td>
</tr>
<tr>
<td></td>
<td>MOG93-107</td>
<td>SJL/J (M)</td>
<td>Induced/Transferred</td>
<td>(128)</td>
</tr>
<tr>
<td></td>
<td>MOG134-148</td>
<td>DA, BN</td>
<td>Induced</td>
<td>(119)</td>
</tr>
<tr>
<td></td>
<td>MOG</td>
<td>Biozzi ABH (M)</td>
<td>Induced</td>
<td>(119)</td>
</tr>
<tr>
<td><strong>Proteolipid Protein (PLP)</strong></td>
<td>PLP</td>
<td>C3H/HeJ (M)</td>
<td>Induced</td>
<td>(122)</td>
</tr>
<tr>
<td></td>
<td>PLP 36-70</td>
<td>Biozzi ABH (M)</td>
<td>Induced</td>
<td>(472)</td>
</tr>
<tr>
<td></td>
<td>PLP 104-117</td>
<td>SJL/J (M)</td>
<td>Induced</td>
<td>(473)</td>
</tr>
<tr>
<td></td>
<td>PLP 139-151</td>
<td>SJL/J (M)</td>
<td>Induced</td>
<td>(120)</td>
</tr>
<tr>
<td></td>
<td>PLP 178-191</td>
<td>SJL/J (M)</td>
<td>Induced/Transferred</td>
<td>(122)</td>
</tr>
<tr>
<td></td>
<td>PLP 215-232</td>
<td>C3H/HeJ (M)</td>
<td>Induced</td>
<td>(122)</td>
</tr>
<tr>
<td><strong>Oligodendrocyte-specific Glycoprotein (OSP)</strong></td>
<td>OSP</td>
<td>C57BL/6 (M)</td>
<td>Induced</td>
<td>(474)</td>
</tr>
<tr>
<td></td>
<td>OSP</td>
<td>SJL/J (M)</td>
<td>Induced</td>
<td>(126, 131)</td>
</tr>
<tr>
<td></td>
<td>OSP 57-72</td>
<td>SJL/J (M)</td>
<td>Induced</td>
<td>(126)</td>
</tr>
<tr>
<td></td>
<td>OSP 179-207</td>
<td>C57BL/6 (M)</td>
<td>Induced</td>
<td>(474)</td>
</tr>
<tr>
<td><strong>Nogo-A</strong></td>
<td>rNogo-66</td>
<td>C57BL/6 / SJL/J (M)</td>
<td>Induced</td>
<td>(475)</td>
</tr>
</tbody>
</table>
Table 1.2: Spontaneous models of EAE. These models of EAE involve the use of transgenic mice to study the mechanisms of autoimmunity independently of exogenous manipulation (e.g. immunisation with CNS antigenic epitopes). (Adapted from (117))

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Epitope</th>
<th>Model</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>MOG&lt;sub&gt;35,55&lt;/sub&gt;</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; TCR Tg</td>
<td>Paralytic EAE and optic neuritis.</td>
<td>(476)</td>
</tr>
<tr>
<td></td>
<td>MOG&lt;sub&gt;35,55&lt;/sub&gt; and MOG</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; TCR Tg x BCR Tg</td>
<td>Paralytic EAE and optic neuritis.</td>
<td>(190)</td>
</tr>
<tr>
<td></td>
<td>Neo-self antigen OVA</td>
<td>ODC-OVA Tg x OT01 (CD8&lt;sup&gt;+&lt;/sup&gt;) TCR Tg</td>
<td>Paralytic EAE and locomotor defects.</td>
<td>(477)</td>
</tr>
<tr>
<td>SJL/J</td>
<td>PLP&lt;sub&gt;139-151&lt;/sub&gt;</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; TCR Tg</td>
<td>Paralytic EAE.</td>
<td>(478)</td>
</tr>
<tr>
<td></td>
<td>MOG&lt;sub&gt;92-106&lt;/sub&gt;</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; TCR Tg</td>
<td>Paralytic and ataxic EAE, relapsing-remitting.</td>
<td>(127)</td>
</tr>
<tr>
<td>B10.PL</td>
<td>MBP Ac1-9</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; TCR Tg</td>
<td>Paralytic EAE.</td>
<td>(479)</td>
</tr>
<tr>
<td></td>
<td>MBP Ac1-11</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; TCR Tg</td>
<td>Paralytic EAE.</td>
<td>(480)</td>
</tr>
</tbody>
</table>
Table 1.3: Tissue distribution of the mammalian class I PI3K protein subunits.
(Adapted from (387))

<table>
<thead>
<tr>
<th>PI3K class</th>
<th>Subunit</th>
<th>Expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class IA (catalytic)</td>
<td>p110α</td>
<td>Ubiquitous.</td>
<td>(260, 373, 481)</td>
</tr>
<tr>
<td></td>
<td>p110β</td>
<td>Ubiquitous.</td>
<td>(260, 374, 481, 482)</td>
</tr>
<tr>
<td></td>
<td>p110δ</td>
<td>Highly expressed in leukocytes. Moderate expression in neurons and cancer cell lines from various origin (melanoma, breast, colon). Moderate expression in endothelium.</td>
<td>(260, 369, 449, 481, 483, 484)</td>
</tr>
<tr>
<td>Class IA (regulatory)</td>
<td>p85α</td>
<td>Ubiquitous. Lowest in skeletal muscle.</td>
<td>(481, 485, 486)</td>
</tr>
<tr>
<td></td>
<td>p55α</td>
<td>Brain and muscle. Undetectable in other tissues.</td>
<td>(485, 487)</td>
</tr>
<tr>
<td></td>
<td>p50α</td>
<td>High in liver, low in kidney and brain.</td>
<td>(485, 488)</td>
</tr>
<tr>
<td></td>
<td>p85β</td>
<td>Ubiquitous. Lowest in skeletal muscle.</td>
<td>(481, 485, 486)</td>
</tr>
<tr>
<td></td>
<td>p55γ</td>
<td>Low protein expression in liver, muscle, fat, spleen. High mRNA in brain and testis.</td>
<td>(263, 481, 485)</td>
</tr>
<tr>
<td>Class IB (catalytic)</td>
<td>p110γ</td>
<td>Highly expressed in leukocytes. Low expression in most other tissues.</td>
<td>(270, 376, 489-491)</td>
</tr>
<tr>
<td>Class IB (regulatory)</td>
<td>p101</td>
<td>Highly expressed in leukocytes. Highly expressed in leukocytes and heart.</td>
<td>(266, 267)</td>
</tr>
<tr>
<td></td>
<td>p84</td>
<td>Highly expressed in leukocytes. Highly expressed in leukocytes and heart.</td>
<td>(266, 267)</td>
</tr>
</tbody>
</table>
Table 1.4: Genetic manipulation of PI3K subunits. (Adapted from (4))

<table>
<thead>
<tr>
<th>PI3K class</th>
<th>Targeted subunit</th>
<th>Viability</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class IA (catalytic)</td>
<td>p110α p110β p110δ KO p110δ KO/KI</td>
<td>Embryonic lethal</td>
<td>(373) (374) (239, 244, 369) (248, 362)</td>
</tr>
<tr>
<td>Class IA (regulatory)</td>
<td>p85α + p55α + p50α (PIK3RI) p85α p55α + p50α p85β p55γ</td>
<td>Perinatal lethality (homozygous) Viable (heterozygous) Viable Viable Viable</td>
<td>(303, 375, 492-494) (305, 377) (378) (379) (379)</td>
</tr>
<tr>
<td>Class IB (catalytic)</td>
<td>p110γ KO p110γ KO/KI</td>
<td>Viable</td>
<td>(376, 429, 430, 433, 437, 495) (270)</td>
</tr>
</tbody>
</table>
CHAPTER 2

MATERIALS AND METHODS
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CHAPTER 2: Materials & Methods

2.1 REAGENTS

2.1.1 General Solutions

2.1.1.1 Phosphate buffered saline (PBS)
PBS was obtained from the Central Services Unit at the Department of Molecular and Biomedical Sciences (The University of Adelaide). Endotoxin free PBS was obtained from the Media Production Unit at the Institute of Medical and Veterinary Sciences (IMVS) (Adelaide, SA).

2.1.1.2 PBS/Tween
Polyoxyethylene-sorbitan monolaurate (Tween 20) (Sigma Australia, Castle Hill, NSW, Australia) was added to PBS to a final concentration of 0.05 % (w/v) and the solution mixed thoroughly.

2.1.1.3 ELISA coating buffer
NaCHO₃ was dissolved in Milli-Q water to a concentration of 0.1M, the pH was adjusted to 9.6 and the solution filter sterilised.

2.1.1.4 Mouse Red Cell Removal Buffer (MRCRB)
MRCRB was prepared by mixing 9 parts Solution A (8.3g NH₄Cl made up to 1L with Milli-Q water) with 1 part Solution B (20.594g TRIS base made up to 1L with Milli-Q water and adjusted to pH 7.65 with HCl). MRCRB was adjusted to pH 7.2, filter sterilised and stored at 4°C.

2.1.1.5 Hank’s Balanced Salt Solution (HBSS)
The following reagents were dissolved in Milli-Q water to generate 10X stocks:= and sterilised by autoclaving: 80g/L NaCl, 4g/L KCl, 0.32g/L NaHPO₄ and 10g/L D-glucose. When required for use, the solution was diluted to 1X in Milli-Q water, and HEPES buffer (pH 7.4) and CaCl₂ were added to a final concentration of 0.01M and 1.6mM respectively.
2.1.1.6 Standard isotonic Percoll (SIP)
SIP was prepared by mixing one part 1X HBSS with 9 parts Percoll (Amersham Pharmacia Biotech Australia, Castle Hill, NSW).

2.1.1.7 Tail tip lysis buffer
Tail tip lysis buffer was made by adding the following ingredients to Milli-Q water: 100mM Tris-HCl pH 8.5, 5mM EDTA, 0.2% SDS and 200mM sodium chloride.

2.1.1.8 Tris Acetate-EDTA Buffer (TAE)
1 x TAE consisted of 0.04M Tris-acetate, 0.02M Na acetate and 1mM EDTA (pH 7.4).

2.1.1.9 DNA loading buffer
1ml of DNA loading buffer consisted of 50μl 1M Tris, 10μl 0.5M EDTA, 100μl Bromophenol Blue 0.15%, 100μl Xylene cynole 0.5% and 750μl 80% Glycerol. DNA loading buffer was stored at 4°C.

2.1.1.10 PBS/BSA/Azide for flow cytometry
PBS was mixed with 1% bovine serum albumin (BSA, Sigma) and 0.04% NaN₃ (both w/v) and stored at 4°C.

2.1.1.11 PBS/Azide for flow cytometry
PBS was mixed with 0.04% NaN₃ (w/v) and stored at 4°C.

2.1.1.12 1-4% Paraformaldehyde (PFA)
PFA (w/v) was prepared by dissolving paraformaldehyde in PBS pH 7.4 at 55°C with stirring for at least 30 minutes. PFA was stored at 4°C for up to one month or at -20°C until required.

2.1.1.13 DNase solution for 5-Bromo-2'-Deoxyuridine (BrdU) labelling
Stock solutions of DNase were prepared by dissolving DNaseI (Sigma) to 5,000 U/ml in a solution of 4.2mM MgCl₂ + 0.15mM NaCl in Milli-Q water (pH 5.0).
Aliquots of the stock solution were stored at -20°C. When required, an aliquot of stock solution was thawed and diluted to 50 U/ml in 4.2mM MgCl₂ + 0.15mM NaCl.

2.1.1.14 Annexin V staining buffer
Annexin V staining buffer was made up of Milli-Q water with the following additives: 10mM HEPES (pH 7.4), 140mM NaCl and 5mM CaCl₂.

2.1.1.15 1% acid alcohol
10ml of concentrated hydrochloric acid was added to 300ml of distilled water. Seven hundred ml of commercial grade ethanol was added to the acid/water solution and mixed thoroughly.

2.1.1.16 Scott’s tapwater substitute
The following reagents were dissolved in Milli-Q water: 2g sodium bicarbonate and 20g magnesium sulphate, then made up to 1L with additional Milli-Q water. The solution was filter-sterilised and stored at room temperature.

2.1.1.17 Diethyl Pyrocarbonate (DEPC)-treated water
DEPC was diluted to 0.1% (v/v) in Milli-Q water, incubated overnight at room temperature and then autoclaved.

2.1.1.18 Gill’s haematoxylin
250ml ethylene glycol, 2g Haematoxylin 75290, 0.2g sodium periodate (NaIO₄), 17.6g aluminium sulphate (Al₂(SO₄)₃.18H₂O) and 20ml glacial acetic acid were added in that order to 730ml distilled H₂O while stirring. The solution was stirred for 1 hour at room temperature until dissolved.

2.1.1.19 Chemotaxis buffer
Chemotaxis buffer was made up by dissolving 0.25g of BSA in 50ml of RPMI-1640 media with 1.25ml of 1M HEPES (IMVS, South Australia).
2.1.2 Reagents used in vitro

2.1.2.1 Antibodies
Antibodies used for flow cytometry, ELISA, immunohistochemistry (IHC) and cell culture are listed in Table 2.1.

2.1.2.2 ELISA reagents
ELISA peroxidase reactions were developed using o-Phenylenediamine dihydrochloride (OPD) (Sigma, St Louis, MO, USA).

2.1.2.3 Chemokines
A list of chemokines used in chemotaxis experiments in this study is provided in Table 2.2. The chemokines used in this study were provided by Professor Ian Clark-Lewis (UBC, Vancouver). All chemokines were functionally tested in this laboratory using either chemotaxis and/or calcium mobilisation assays.

2.1.2.4 Cytokines
A list of the commercial cytokines used in this study is provided in Table 2.3.

2.1.3 Antigens and Adjuvants used in vivo

2.1.3.1 Myelin Oligodendrocyte Glycoprotein (MOG) peptide 35-55
MOG\textsubscript{35-55} is an encephalitogenic peptide of myelin oligodendrocyte glycoprotein. This was obtained as a kind gift from Professor Iain Clark-Lewis (Biomedical Research Centre, University of British Colombia, Vancouver, Canada) and was also purchased from the Biomolecular Research Facility, John Curtin School of Medical Research, Australian National University, Canberra, Australia as well as GL Biochem (Shanghai) Ltd. (Shanghai, China). The sequence (N→C terminus) is as follows: MEVGWYRSPFSRVVHLRYNGK. MOG\textsubscript{35-55} was received in desiccated form and stored at 4°C. For use, 16mg of MOG\textsubscript{35-55} was dissolved in 1ml of mQ water before 9ml of sterile endotoxin-free PBS was added. Suspended MOG\textsubscript{35-55} was stored at -20°C.
2.1.3.2 Ovalbumin (OVA) 323-339

OVA323-339 is an antigenic peptide which is recognised by the transgenic TCR of OT-II (H-2b) mice (section 2.2.1). OVA323-339 was obtained from the Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University, Australia. The sequence (N→C terminus) is as follows: ISQAVHAAHAINEAGR. OVA323-33 was received in desiccated form and stored at 4°C.

2.1.3.3 Incomplete Freund’s Adjuvant (IFA)

IFA was made by combining mineral oil (85%; Sigma) and mannide manoooleate (15%; Sigma) and stored at 4°C.

2.1.3.4 Complete Freund’s Adjuvant (CFA)

CFA was prepared by supplementing IFA with 8.33mg/ml *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA) and stored at 4°C. The bacteria were ground using a mortar and pestle in IFA.

2.1.3.5 Pertussis Toxin

Lyophilised, salt free toxin from *Bordatella pertussis* (List Biological Laboratories) was reconstituted to 50μg/ml in endotoxin-free PBS. Reconstituted Pertussis toxin was stored at 4°C and diluted to the required concentration in endotoxin-free PBS immediately before use.

2.1.4 Inhibitors

All inhibitors are listed in Table 2.4 and are described in further detail below.

2.1.4.1 LY294002

LY294002 was purchased from Calbiochem (Catalogue number 440202) and was re-suspended in Dimethyl sulfoxide (DMSO) (Sigma Australia # D8418) for use.
2.1.4.2 IC87114
The small molecule p110δ inhibitor, IC87114, was synthesised and purified as described (Patent reference number WO0181346 (359)) and provided for this study by Kamal Puri at Calistoga Pharmaceuticals (Seattle, USA). IC87114 is a highly selective inhibitor of p110δ. Its IC50 values for p110α, p110β and p110γ are at least 200-, 150- and 60-fold higher than that for p110δ (359). IC87114 has no inhibitory activity in vitro toward a number of other kinases, including Akt1, PKCa, PKCβII, c-Src, p38 MAPK, casein kinase I, checkpoint kinase 1 and DNA-PK (359). For in vitro studies, IC87114 was re-suspended in DMSO (Sigma Australia # D8418). For in vivo studies IC87114 was re-suspended in the vehicle described below (section 2.1.4.3).

2.1.4.3 Vehicle for IC87114 use in vivo
IC87114 was administered in vivo in a solution containing 0.5% (w/v) methylcellulose (supplied by Calistoga Pharmaceuticals)/0.1% (v/v) Tween 80 (Sigma) (Kamal Puri, personal communication). The solution was prepared in the following manner: 100ml of Milli-Q water was heated to 70-90°C on a heated stir plate. Methylcellulose (0.5g per 100ml) and Tween 80 (0.1ml per 100ml) was added to the water and left on the heated stir plate for 15 minutes. The solution was then covered with aluminium foil and placed in the dark to stir overnight on a stir plate at 4°C. The vehicle solution was stored at 4°C in the dark for up to 30 days.

IC87114 was suspended in vehicle at a concentration of 7.5mg/ml and thoroughly vortexed. Mice were administered approximately 30mg/kg of this suspension (i.e. approximately 80μl per 20g mouse). To stop IC87114 from settling out of the viscous vehicle the solution was shaken vigorously throughout the dosing procedure.

2.1.5 Cell culture media

2.1.5.1 Foetal calf serum
Foetal calf serum (FCS) was obtained from JRH Biosciences (Lenexa, KS, USA) and was heat-inactivated by incubation at 55°C for one hour.
2.1.5.2 Incomplete media
RPMI 1640 medium (Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 0.1% heat-inactivated FCS and 1% penicillin/gentamycin stock solution (IMVS, South Australia).

2.1.5.3 Complete media
RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 1% penicillin/gentamycin stock solution.

2.1.5.4 BMDC media
RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1% penicillin/gentamycin stock solution, 2mM L-glutamine (IMVS, South Australia), 55μM 2 beta Mercaptoethanol (IMVS, South Australia).

2.2 ANIMAL MODELS

2.2.1 Mouse strains and conditions
All animals were housed at the University of Adelaide Animal House (Adelaide, SA, Australia), under standard light and temperature conditions, with food and water ad libitum. All mice were aged 6-10 weeks at the initiation of experiments. Severely paralysed mice were afforded easier access to food and water. The p110\(^{δD910A/D910A}\) mice (on the C57Bl6 background) (248) used in this study were obtained from Dr. Margaret Hibbs, Ludwig Institute for Cancer Research, Melbourne, Australia and bred at the University of Adelaide Animal House. The OT-II mice used in this study were obtained from Dr. Christian Engwerda, Queensland Institute for Medical Research, Queensland, Australia. Age and sex-matched C57BL/6 mice were purchased from The University of Adelaide Animal House, South Australia or the Australian Research Council and housed at the University of Adelaide Animal House. All animals were housed in specific pathogen-free (SPF) barrier rooms. Animal experiments were performed as approved by The University of Adelaide Animal Ethics Committee.
2.2.2 Genotyping p110δ^{D910A/D910A} mice

Approximately 5mm of the tail tips of p110δ^{D910A/D910A} were taken at the time of weaning (section 2.3.1.1). Tail tips were digested overnight at 55°C in 100μl of tail tip lysis buffer (section 2.1.1.7) containing 0.1mg/ml of Proteinase K (Roche # 1-373-196). The following morning, tubes were placed at 95°C for 5 minutes before 400μl of Milli-Q water was added. Tails were then centrifuged for 15 minutes at 13,000 RPM and the supernatant used as template for polymerase chain reactions (PCR). All genotyping PCR was carried out in 0.2ml thin walled PCR tubes (Scientific Specialities Inc., Lodi, CA, USA) in a Bio-Rad MyCycler thermocytometer. Primers used for PCR amplification are listed in Table 2.5. All primers were purchased from GeneWorks (Adelaide, SA) and were of PCR purity. Primers were received in a lyophilised form, diluted in sterile Milli-Q water and stored at -20°C. Reactions were carried out with the Immolase enzyme (Bioline # Bio 21047) with supplied PCR buffer supplemented with MgCl₂ at a final concentration of 2.5μM and dNTPs (Sigma) at a final concentration of 1μM/base per reaction. Enzyme was added at 2.5U/20μl reaction and 0.5μl of tail tip DNA was added to each tube. Cycling conditions were as follows: Initial denaturation at 95°C for 7 minutes, then 35 cycles of 94°C for 20 seconds, 69°C for 20 seconds and 72°C for 30 seconds. Reactions were incubated for an additional 10 minutes at 72°C to ensure efficient A-tailing, then maintained at 4°C. Genotypes were determined by running PCR products on a 1.5% agarose gel before being stained with GelRed (Jomar # 41003-S) and measured with a Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA) using Quantity One™ software. Lanes with bands at 500bp and 300bp were considered to be heterozygous (p110δ^{D910A/WT}) mice and lanes with bands only at 500bp were considered to be homozygous p110δ^{D910A/D910A} mice.
CHAPTER 2: Materials & Methods

2.2.3 EAE Model

2.2.3.1 Active induction of EAE with MOG\textsubscript{35-55}

Female C57BL/6 mice received an intravenous dose of 300ng of pertussis toxin (Sapphire Biosciences # 181) in endotoxin-free PBS on the day of induction. One hour later mice were immunised with 100μg MOG\textsubscript{35-55} (Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University and GL Biochem) in CFA (section 2.1.3.4). The emulsion contained equal volumes of MOG\textsubscript{35-55} (1.6mg/ml in endotoxin-free PBS) and CFA. Fifty microlitres was injected subcutaneously (s.c.) into each hind flank and 20μl was injected s.c. into the scruff of the neck. Two days later mice were again injected intravenously with 300ng of pertussis toxin.

2.2.3.2 Clinical assessment of EAE

Mice were observed daily until day 30 for clinical signs of EAE. Disease severity was scored on a scale from 0 (asymptomatic) to 5 (moribund). Clinical scores were assigned as follows: 0 = no clinically detectable signs of EAE were observed; 1 = partial tail and/or hind limb paralysis; 2 = complete tail paralysis; 2.5 fully flaccid tail and some visible hind limb paralysis; 3 = hind limb and tail paralysis; 4 = hind limb and forelimb paralysis; 5 = moribund. Animals that reached a score of 4 were euthanased after 24 hours if no partial recovery was made.

2.2.4 CFA immunisation

CFA emulsions were prepared by thoroughly mixing one part CFA (section 2.1.3.4) with one part endotoxin-free PBS (IMVS, South Australia). Fifty microlitres of CFA emulsion was injected subcutaneously in both of the hind flanks of mice and 20μl was injected subcutaneously into the scruff of the neck.

2.2.5 In vivo administration of IC87114

Mice were administered IC87114 via oral gavage. A 20 gauge needle (Popper # 7902) was used to inject 30mg/kg of IC87114 in vehicle (sections 2.1.4.2 and
2.1.4.3) twice daily (at approximately 9am and 5pm). Mice used were between 16-22 grams each and were between 6-11 weeks of age.

2.2.6 FITC paint assay
The abdomen of naïve mice was shaved and painted with 150μl of a 1:1 solution of acetone and di-butylphthalate containing 1% FITC powder. Forty-eight hours later single cell suspensions were generated from the inguinal and brachial lymph nodes as described in section 2.3.1.4. Cells were then stained with anti-CD11c antibody (Table 2.1) and were analysed by flow cytometry (sections 2.3.2.2 and 2.3.2.7).

2.3 ANALYTICAL AND FUNCTIONAL ASSAYS

2.3.1 Collection of tissues

2.3.1.1 Collection of tail tips for genotyping
Mice were weaned from their mothers at three weeks of age by staff members of the University of Adelaide Animal House. During the weaning process, mice were anaesthetised with Isoflourane (VCA, NSW, Australia) and approximately 0.5cm of the tip of their tails was removed with scissors and stored in an eppendorf tube. Tail tips were frozen at -20ºC until use.

2.3.1.2 Collection of mouse serum
Mice were sacrificed by carbon dioxide asphyxiation. The rib cage was then immediately reflected back to expose the heart, which was perforated at the left ventricle with surgical scissors. Blood was collected from the chest cavity into eppendorf tubes and allowed to clot overnight at 4ºC. Tubes were then centrifuged at 1,000 x g for 10 minutes at 4ºC. Serum was collected and stored at -20ºC.

2.3.1.3 Collection of mouse plasma
Mice were sacrificed by carbon dioxide asphyxiation. The rib cage was then reflected back to expose the heart, which was perforated at the left ventricle with surgical scissors. Blood was collected from the chest cavity and immediately placed in
Lithium Heparin coated tubes (IMVS, South Australia) and centrifuged at 2,500 x g for 15 minutes at 4ºC. Plasma was collected and stored at -80ºC. Shipments of plasma were sent on dry ice.

2.3.1.4 Preparation of single cell suspensions from lymphoid organs
Mice were sacrificed by carbon dioxide asphyxiation and lymph nodes and/or spleens were removed by blunt dissection. Organs were individually gently homogenised using two microscope slides with frosted ends (VWR Scientific # 48312-002) in 5ml of complete media (section 2.1.5.3). When lymph node cells were being stained with anti-DC markers they were also treated for 30 minutes with collagenase (Sigma # C2674) at 37ºC and with 5% CO2 before being homogenised. The resulting suspensions were filtered through cell strainers (BD Biosciences Labware) and suspended in a further 5ml of complete medium. Following 5 minutes of centrifugation, lymph node cells were counted and re-suspended in the appropriate solution for the subsequent experiment. Before cell counting, splenocytes were suspended in 7ml MRCRB (section 2.1.1.4) and incubated at 37ºC for 5 minutes. Following centrifugation splenocytes were re-suspended appropriately for subsequent experiments.

2.3.1.5 Collection of spinal cords for flow cytometry
To remove spinal cords, mice were sacrificed by carbon dioxide asphyxiation and perfused through the left ventricle with PBS to remove circulating leukocytes. The spinal cord was extracted from the spinal column using scissors to cut the vertebrae and a scalpel to remove the cord. Spinal cords were then gently crushed through a cell strainer. The resultant cell suspension was collected in 30ml of RPMI 1640 plus 10% FCS, and 20ml of 90% Percoll (Amersham Pharmacia Biotech, Little Chalfont, UK) was added, before centrifuging at 1000 x g for 25 minutes at 4ºC, with no brake. Following centrifugation, the myelin cake was discarded, the cells were washed twice then re-suspended in PBS/BSA/Azide for antibody labeling (section 2.1.1.10).
2.3.1.6 Collection and storage of spinal cords for immunohistochemistry

To remove spinal cords, mice were sacrificed by carbon dioxide asphyxiation and perfused through the left ventricle with PBS to remove any circulating lymphocytes. The spinal cord was extracted from the spinal column using scissors to cut the vertebrae and a scalpel to remove the cord. Spinal cords were embedded in Tissue-Tek® OCT embedding medium (Sakura Finetek, Torrance, CA, USA) and frozen using the Gentle Jane snap-freezing system (Instrumedics Inc. Hackensack, NJ, USA). Blocks were stored at -80°C until sectioning for IHC (section 2.3.3).

2.3.1.7 Collection of bone marrow-derived dendritic cells

Mice were euthanased and femurs were removed, rinsed with 70% ethanol and placed into warm PBS. Bone marrow was flushed out by cutting either end of the bone with dissecting scissors into a 10cm² tissue culture dish (Falcon) with a 1mL syringe and 27G needle using warm BMDC medium (section 2.1.5.4). The cells were then centrifuged at 300 x g for 5 minutes at room temperature before being suspended in 6mL of MRCRB (section 2.1.1.4) and incubated for 5 minutes at 37ºC. Cells were then centrifuged and washed with 10ml of PBS, re-centrifuged and re-suspended in PBS while cell counts were performed.

2.3.2 Flow cytometry

2.3.2.1 Labelling cells with Carboxyfluorescin diacetate succinimidyl ester (CFSE)

Single cell suspensions were generated as described in section 2.3.1.4 and cells were suspended in incomplete media (section 2.1.5.2) at a concentration of 2 x 10⁷/ml to be labelled with CFSE (Molecular Probes, Eugene, OR, USA # C1157). CFSE, which was stored at a stock concentration of 5mM in DMSO, was diluted 1:40 in incomplete media. Two microlitres of diluted CFSE was added per 100μl of cells and immediately shaken vigorously to ensure even staining of CFSE. After incubation at 37°C for 10 minutes, the reaction was quenched by the addition of 15ml of complete media and incubated at room temperature for 5 minutes, followed by two washes in the same medium. Cells were then re-suspended at the required concentration for subsequent assays.
2.3.2.2 Standard surface staining protocol
Cells were isolated as described (section 2.3.1.4 and 2.3.1.5) before being resuspended to 4 x 10^6 viable cells/ml in PBS. Fc receptors were blocked by incubating for 20 minutes at room temperature with 50μg of murine gamma-globulin (Rockland D609-0100) per million cells, then 50μl of cells was aliquoted into wells of 96 well round-bottom plates (BD Falcon™ #353077). For experiments where an un-conjugated primary antibody was used, cells were incubated for 30 minutes at room temperature with 10μl of antibody at concentrations indicated in Table 2.1. Following this incubation, cells were washed with 180μl of PBS/BSA/Azide (section 2.1.1.10), centrifuged at 400 x g for 1 minute, after which the supernatant was flicked off. Fifty microlitres of fluorophore-conjugated secondary antibody was then added to relevant wells at concentrations indicated in Table 2.1. Following a 30 minute incubation at 4°C in the dark, cells were once again washed with 180μl of PBS/BSA/Azide. If conjugated secondary antibodies were then being used, any potential cross reactivity of the anti-rat secondary for rat and mouse immunoglobulins was prevented by pre-blocking the secondary antibody with 10μl of 2mg/ml rat gamma-globulin (Rockland D611-0050) for 20 minutes at 4°C. Fluorochrome-conjugated antibodies were then added and incubated for 30 minutes on ice in the dark (Table 2.1). Cells were washed with PBS/Azide (section 2.1.1.11) then re-suspended in 200μl of PBS/Azide. Cells were then immediately acquired on a flow cytometer (section 2.3.2.7). If flow cytometric acquisition was not performed immediately cells were instead re-suspended in 200μl of PFA (1% in PBS) and stored in the dark at 4°C.

2.3.2.3 Intracellular cytokine staining
Single cell suspensions were generated as described in sections 2.3.1.4 and 2.3.1.5. In experiments where intracellular cytokines were detected, cells were activated for four hours at 37°C in RPMI 1640 media containing 20ng/ml PMA (Sigma), 1μM ionomycin (Invitrogen) and Golgistop™ (BD Biosciences). Cells were then washed with PBS/BSA/Azide (section 2.1.1.10), blocked with 1.25μl murine gamma-globulin (10mg/ml - Rockland D609-0100) for 20 minutes at room temperature and incubated with the anti-CD4:PECy7 antibody (Table 2.1) for 30 minutes at 4°C. Cells
were then washed with PBS/BSA/Azide and fixed and permeabilised using a Cytofix/Cytoperm kit (BD Biosciences # 554715) according to the manufacturer’s instructions. Intracellular IFN-γ and IL-17 was then stained for using the anti-IFN-γ:FITC and anti-IL-17:PE antibodies as described in Table 2.1. Cells were then washed in PBS/Azide and re-suspended in PBS/1% PFA and analysed on a BD LSR II flow cytometer (section 2.3.2.7). If flow cytometric acquisition was not performed immediately cells were stored in the dark at 4°C and acquired within 4 days of staining.

### 2.3.2.4 Intracellular BrdU staining

Mice were fed BrdU (Sigma) in their drinking water as described in section 2.3.5.2 before single cell suspensions were generated (sections 2.3.1.4). Cells were then re-suspended in PBS/BSA/Azide (section 2.1.1.10) at $4 \times 10^6$/ml and 50μl was added to wells of 96-well trays (Falcon # 353077). Fc receptors on cells were blocked by adding 1.25μl of murine gamma-globulin (10mg/ml - Rockland D609-0100) to each well and incubating at room temperature for 20 minutes. Ten microlitres of primary anti-CD4:PECy7 antibody (Table 2.1) was then added to each well and incubated at 4°C for 30 minutes. Following washing with PBS cells were transferred to tubes (Falcon # 352008), centrifuged and re-suspended in 500μl NaCl (0.15M) before 1.2ml of ice-cold 95% ethanol was added in a drop-wise manner whilst vortexing. Tubes were then incubated on ice for 30 minutes in the dark before cells were washed in 2ml of cold PBS. Cells were then re-suspended in 1ml of DNase solution (section 2.1.1.13), incubated for 30 minutes at 37°C with 5% CO₂. Following this incubation, cells were washed in 2ml of PBS then incubated for 30 minutes at 4°C with 10μl of anti-BrdU:FITC antibody (Table 2.1). Cells were then suspended in 200μl of PBS and transferred to a 96-well tray which was centrifuged. Cells were then resuspended in 200μl of PBS/1% PFA and acquired on a BD LSR II flow cytometer (section 2.3.2.7). If flow cytometric acquisition was not performed immediately cells were stored in the dark at 4°C and acquired within 4 days of staining.
2.3.2.5 Intracellular FoxP3 staining

Single cell suspensions were generated as described in section 2.3.1.4. Cells were then re-suspended in PBS/BSA/Azide (section 2.1.1.10) at 4 x 10^6/ml and 50μl was added to wells of 96-well trays (Falcon # 353077). Fc receptors on cells were blocked by adding 1.25μl of murine gamma-globulin (10mg/ml - Rockland D609-0100) to each well and incubating at room temperature for 20 minutes. Ten microlitres of both anti-CD4:PECy7 and anti-CD25:FITC antibodies (Table 2.1) were then added to each well and incubated at 4°C for 30 minutes. Cells were then washed with PBS/BSA/Azide before being suspended in 200μl of Fix/Perm solution (eBioscience # 00-5121-00) and incubated overnight at 4°C. In the morning, cells were washed once with 200μl Permeabilisation buffer (eBioscience # 00-8333-56) then incubated with 10μl of anti-FoxP3:PE antibody (Table 2.1) for 30 minutes on ice in the dark. Cells were then washed once in Permeabilisation buffer before being suspended in 200μl of PBS/1% PFA for acquisition on a BD LSR II flow cytometer (section 2.3.2.7). If flow cytometric acquisition was not performed immediately cells were stored in the dark at 4°C and acquired within 4 days of staining.

2.3.2.6 Annexin V and Propidium Iodide staining

Single cell suspensions were generated as described in section 2.3.1.4. Cells were then re-suspended in PBS/BSA/Azide (section 2.1.1.10) at 4 x 10^6/ml and 50μl was added to wells of 96-well trays (Falcon # 353077). Fc receptors on cells were blocked by adding 1.25μl of murine gamma-globulin (10mg/ml - Rockland D609-0100) to each well and incubating at room temperature for 20 minutes. Ten microlitres of either anti-CD4:PECy7 or anti-B220:PECy7 antibodies (Table 2.1) was then added to wells and incubated at 4°C for 30 minutes. Cells were then washed with Annexin V staining buffer (section 2.1.1.14) and 100μl of Annexin V staining buffer, containing 2μl of Annexin V:FITC (MBL # BV-1001-5) and 1μl of propidium iodide (Sigma # P4864), was added to each well. Cells were incubated at room temperature in the dark for 10-15 minutes before being centrifuged at 400 x g for 1 minute and re-suspended in 200μl of Annexin V staining buffer. Cells were immediately acquired on a BD LSR II flow cytometer (section 2.3.2.7).
2.3.2.7 Flow cytometric analysis
Cell fluorescence data was acquired on a Becton Dickinson LSRII or FACSCanto flow cytometer and data were analysed using either BD FACSDiva or FlowJo software. Lymphocytes were gated using forward- and side-scatter characteristics and isotype controls were used to determine gating for cell surface and intracellular antibodies.

2.3.3 Immunostaining of tissues

2.3.3.1 Preparation of spinal cord sections
Spinal cords were collected and frozen in Tissue-Tek® OCT as described in section 2.3.1.6. Before sectioning, blocks were allowed to equilibrate to a temperature of -20°C. Ten micron cryostat sections were cut from embedded tissue, air dried on microscope slides, then stored at -20°C until they were used for immunostaining (section 2.3.3.2).

2.3.3.2 Immunohistochemical staining of tissue sections
Frozen sections were prepared as described in section 2.3.3.1 and stored at -20°C. Before use, slides were equilibrated to room temperature for 1 hour prior to use. Slides were then fixed in 100% ice-cold acetone for 10 minutes before being rehydrated in PBS. Endogenous peroxidise activity was blocked with 0.3% hydrogen peroxide for 10 minutes before slides were washed through 3 changes of PBS. Sections were then blocked with 100μg/ml murine gamma globulin (Rockland D609-0100) for 30 minutes in a humid chamber at room temperature. After 3 washes in PBS, slides were incubated with primary antibody (Table 2.1) for 1 hour in a humid chamber at 4°C. After 3 washes in PBS, slides were incubated with HRP-conjugated anti-Rat secondary antibody for 1 hour in a humid chamber at 4°C. Slides were then washed 3 times with PBS and binding of antibodies was revealed by incubation with 3,3’-diaminobenzidine (DAB) substrate (DAKO, NSW, Australia). Sections were counterstained with haematoxylin (section 2.3.3.3), mounted and examined by routine light microscopy.
2.3.3.3 Haematoxylin staining

Slides were stained with antibodies as described in section 2.3.3.2. Slides were then immersed in Gill’s haematoxylin (section 2.1.1.18) for approximately 4 minutes (depending on the strength and age of the haematoxylin). Slides were then immersed in Scott’s tapwater substitute (section 2.1.1.16) for 1 minute then distilled water for 1 minute. Slides were then subjected to ethanol (70% ethanol for 2 minutes, 95% ethanol for 2 minutes, 100% ethanol for 2 minutes, followed by immersion in clean 100% ethanol for another 2 minutes) before being immersed in two batches of Safsolvent (Ajax Finechem # A2537) for 5 minutes each. Slides were then allowed to briefly air-dry before being mounted with DePeX (BDH # 361254D) and examined by routine light microscopy.

2.3.4 Cell culture

2.3.4.1 Overnight culture of cells for chemotaxis assays

Mice were euthanased and primary cells were isolated as described in section 2.3.1.4. Cells were cultured overnight in 4ml of complete media (section 2.1.5.3) at a concentration of $2 \times 10^7$ cells per well in a 6 well tissue culture tray.

2.3.4.2 In vitro culture of immature dendritic cells from bone marrow

Dendritic cells were removed from the bone marrow and single cell suspensions were generated as described in section 2.3.1.7. Bone marrow cells were then suspended to $2 \times 10^5$ cells/ml in 2ml of warm BMDC media (section 2.1.5.4) with 20ng/ml of recombinant GM-CSF (rmGM-CSF) (Biosource # PMC 2015) and added to a single well of a six well tissue culture dish (Falcon # 353046). Cells were cultured at 37ºC in 5% CO₂ for 10 days. On day 3 another 2ml of BMDC media with 20ng/ml rmGM-CSF was added. On day 6 and 8, 2ml of cell suspension was removed and centrifuged at 300 x g for 4 minutes before the supernatant was removed and cells re-suspended in 2ml of fresh BMDC media with 20ng/ml rmGM-CSF and added back to the plate. On day 10, immature BMDCs were used for DC antigen presentation assays (section 2.3.8) or subjected to maturation culture conditions (section 2.3.4.3).
2.3.4.3 Maturation of BMDCs in vitro

To produce a mature phenotype in BMDCs, cells were cultured for 10 days in conditions described in section 2.3.4.2 to generate immature BMDCs. On day 10, the 2ml cell suspension was removed, cells were centrifuged at 300 x g for 5 minutes and resuspended in 2ml of fresh BMDC media with 10ng/ml rmGM-CSF (Biosource # PMC 2015), 1μg/ml LPS (made in-house) and 8.5ng/ml TNF-α (Biosource # PMC 3014). Cells were then added back to the well and cultured overnight. On day 11, cells which had adhered to the plastic were gently loosened using a cell scraper before supernatant was removed from the wells. Cells were then washed and re-suspended at the required concentration for in vitro chemotaxis assays (section 2.3.7.2).

2.3.4.4 Anti-CD3/anti-CD28 stimulated culture conditions

Cells were isolated from the spleens of mice as described in section 2.3.1.4. In some cases cells were also stained with CFSE (section 2.3.2.1). During the process of isolating the cells, 96 well plates (Falcon # 353077) were coated with 50μl of anti-CD3 antibody (clone #2C11, produced in-house and affinity purified) at 10μg/ml in PBS for 1 ½ hours at 37ºC. Following this incubation, plates were washed twice with PBS before 100μl of complete media containing 1μg/ml anti-CD28 was added (Table 2.1) (antibody value indicates final concentration). Cells were re-suspended at a concentration of 2.5 x 10⁶ cells/ml before 100μl of cells were placed into each well. Plates were cultured for 4 days at 37ºC in 5% CO₂ before cells were subjected to flow cytometric analysis as described in section 2.3.2.7. In cases where cells were inhibited with IC87114, cells were pre-incubated with the inhibitor or controls (section 2.1.4) for at least 15 minutes prior to being added to the well.

2.3.4.5 PHA stimulated culture conditions

Cells were isolated from the spleens of mice as described in section 2.3.1.4. In some cases cells were also stained with CFSE (section 2.3.2.1). 100μl of complete media containing 100μg/ml PHA (Sigma # L9132) as well as 100μl of cells suspended to a concentration of 2.5 x 10⁶ cells/ml were added to relevant wells (therefore PHA was at a final concentration of 50μg/ml). Plates were cultured for 4 days at 37ºC in 5%
CO₂ before cells were subjected to flow cytometric analysis as described in section 2.3.2.7. In cases where cells were inhibited with IC87114, cells were pre-incubated with the inhibitor or controls (section 2.1.4) for at least 15 minutes prior to being added to the well.

2.3.4.6 Th1-skewing culture conditions
Cells were isolated from the spleens of mice as described in section 2.3.1.4. During the process of isolating the cells, 96 well plates (Falcon # 353077) were coated with 50μl of anti-CD3 (clone #2C11, produced in-house and affinity purified) antibody at 10μg/ml in PBS for 2 hours at 37ºC. Following this incubation, plates were washed twice with PBS before 100μl of complete media containing the following additives: 1μg/ml anti-CD28 and 10ng/ml IL-12 (cytokine and antibody values indicate final concentration) (Table 2.3). Cells were re-suspended at a concentration of 2.5 x 10⁶ cells/ml before 100μl of cells were placed into each well. In cases where cells were inhibited with IC87114, cells were pre-incubated with the inhibitor or controls (section 2.1.4) for at least 15 minutes prior to being added to the well. Plates were cultured for 4 days at 37ºC in 5% CO₂ before cells were stained for flow cytometric analysis with anti-CD4, anti-IFN-γ and anti-IL-17 antibodies as described in sections 2.3.2.2 and 2.3.2.3.

2.3.4.7 Th17-skewing culture conditions
Cells were isolated from the spleens of mice as described in section 2.3.1.4. During the process of isolating the cells, 96 well plates (Falcon # 353077) were coated with 50μl of anti-CD3 (clone #2C11, produced in-house and affinity purified) antibody at 10μg/ml in PBS for 2 hours at 37ºC. Following this incubation, plates were washed twice with PBS before 100μl of complete media containing the following additives was added: 1μg/ml anti-CD28 and 10μg/ml anti-IFN-γ, 10μg/ml anti-IL-4, 10ng/ml IL-1β, 5ng/ml TGF-β, 20ng/ml IL-6 and 10ng/ml IL-23 (cytokine and antibody values indicate final concentration) (Table 2.3). Cells were re-suspended at a concentration of 2.5 x 10⁶ cells/ml before 100μl of cells were placed into each well. In cases where cells were inhibited with IC87114, cells were pre-incubated with the inhibitor or controls (section 2.1.4) for at least 15 minutes prior to being added to the well.
well. Plates were cultured for 4 days at 37°C in 5% CO₂ before cells were stained for flow cytometric analysis with anti-CD4, anti-IFN-γ and anti-IL-17 antibodies as described in sections 2.3.2.2 and 2.3.2.3.

2.3.5 Proliferation assays

2.3.5.1 Analysis of cell division ex vivo by CFSE dye dilution
Mice immunised with MOG₃₅₋₅₅ in CFA (section 2.2.3.1) were euthanased 9 days post-immunisation and the draining inguinal and brachial lymph nodes were taken for proliferation assays (section 2.3.1.4). Cells were labelled with CFSE as described in section 2.3.2.1 before being suspended at a final concentration of 2.5 x 10⁶ cells/ml. In a 200μl volume, 2.5 x 10⁵ cells were cultured in 96-well round-bottom trays with added MOG₃₅₋₅₅ at a concentration of 25 or 50μg/ml, or with 1.5μg/ml Concanavalin A (ConA; Amersham Pharmica Biotech, Australia). Wells that contained no stimulating antigen were included as a negative control. After 3 days of culture at 37°C in a humidified atmosphere containing 5% CO₂, 50μl of complete media was added to each well to replenish nutrients. On the fourth day of culture cells were harvested and analysed by flow cytometry (section 2.3.2.7). Cell division (proliferation) was determined as a progressive halving of CFSE fluorescence intensity.

2.3.5.2 Detection of in vivo cellular proliferation by BrdU incorporation
BrdU was dissolved to 0.8mg/ml in the drinking water of the animals starting at day 6 post-immunisation. The drinking bottles were covered in aluminium foil to prevent light-induced degradation of BrdU. Mice were culled at day 9 post-immunisation, single cell suspensions of lymphocytes from draining inguinal and brachial lymph nodes were generated (section 2.3.1.4) and flow cytometric analysis was performed to analyse BrdU incorporation to DNA (section 2.3.2.4).
2.3.6 ELISA

2.3.6.1 Two-site (sandwich) ELISA for IgG detection

Neuro-antigen (MOG<sub>35-55</sub>) specific IgG was measured through two-site (sandwich) peroxidise ELISA assays. High-protein-binding ELISA plates (Corning) were incubated overnight at 4°C with 200ng/well MOG<sub>35-55</sub> in 200μl of ELISA coating buffer (section 2.1.1.3). Plates were then washed three times with 200μl PBS/Tween per well (section 2.1.1.2) before being blocked for 2 hours with 3% BSA (Sigma) in PBS at room temperature. Following three more washes with PBS/Tween, 100μl of serum at a 1:5 dilution in PBS/BSA 1% was added and incubated for 90 minutes at room temperature. Plates were washed four more times with PBS/Tween before 100μl of 1:5,000 anti-mouse IgG:HRP (Table 2.1) in PBS/BSA 1% was added. After incubation for 45 minutes at room temperature plates were washed five times with PBS/Tween. All detection plates were then developed using 200μl/well o-Phenlenediamine dihydrochloride (Sigma Fast<sup>TM</sup> OPD; Sigma) as per the manufacturer’s specifications. Reactions were stopped with 50μl 3M HCl and the intensity was read at 490nm with a Bio-Rad plate reader. Serum from naïve mice was used as a negative control.

2.3.6.2 Two-site (sandwich) ELISA for cytokine detection

High protein-binding ELISA plates (Costar # 3590, Corning International, NY, USA) were coated with either anti-IFN-γ or anti-IL-17 capture antibodies in ELISA coating buffer (section 2.1.1.3) overnight at 4°C. Plates were washed three times in PBS/Tween (section 2.1.1.2) before being blocked with 3% BSA (Sigma) in PBS and incubated at room temperature for 2 hours. Following this incubation, plates were washed three times and samples (culture supernatants at a 1:15 dilution in 1% BSA/PBS solution) were added and incubated for 1 ½ hours at room temperature. A standard curve was generated by using known concentrations of either recombinant IFN-γ (R&D # 485 MI-100) or IL-17 (R&D # 421 ML-025). Plates were then washed three times and biotinylated secondary antibodies were added for 45 minutes. Plates were then re-washed four times and SA:HRP (Rockland # S00-03) was added for 30 minutes at room temperature before washing five times. Detection was
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performed by adding 200μl of Sigma Fast™ OPD (Sigma # P9187) to each well for up to 15 minutes. Reactions were stopped with 50μl of 3M HCl and read on a Biotrack II plate reader at 490nm. ELISA assays were analysed using the GraphPad Prism program. A standard curve, and all experimental samples, were analysed using several equations. First, data was transformed using the following equation: 

\[ X = \log[X] \]

Then, the standard curve was determined by performing a sigmoidal dose-response (variable slope) where the goodness of fit for the standard curve was deemed sufficient if it was over \( R^2 = 0.98 \). Unknown X values were then transformed using the following equation: 

\[ Y = 10^Y \]

to obtain a final concentration of pg/ml. Data were then normalised depending on the initial sample dilution during incubation in the ELISA assay.

2.3.7 Transwell chemotaxis assays

2.3.7.1 Transwell chemotaxis assay with splenocytes

Following isolation of splenocytes from mice (section 2.3.1.4) and overnight incubation (section 2.3.4.1), cells were washed in PBS before being resuspended to \( 1 \times 10^7 \) cells/ml and subjected to transwell chemotaxis assays. Chemokines, in 600μl of chemotaxis buffer, were added to the lower chambers of a transwell plate (6.5mm diameter filter, 5μm pore size; Corning, NY, USA). After adding 100μl of cells to the upper chambers, the assay was incubated for 3 hours at 37º and 5% CO₂. Controls included media only in the lower transwell (to assess spontaneous migration of cells through the filter) and wells which included 100% of input cells were used as a control to enumerate migrated cells. Following the 3 hour incubation, cells were collected from the lower chamber after extensive washing of the filter underside with the chemotaxis buffer from the lower chamber. Collected cells were centrifuged at 8,000 rpm for 4 minutes. Cells were then subjected to staining with anti-CD4 and anti-B220 antibodies (Table 2.1) and flow cytometric analysis (section 2.3.2.7). Unlabelled Calibrite beads (BD # 349502) were used to enumerate the number of cells per 5,000 beads and this was compared to the standard curve values to determine the total number of migrated cells.
2.3.7.2 Transwell chemotaxis assay with dendritic cells

Mature BMDCs were generated as described in section 2.3.4.3. Following the 11 day culture, BMDCs were washed in PBS before being labelled with CFSE (section 2.3.2.1), re-washed and resuspended to 5 x 10^6 cells/ml and subjected to transwell chemotaxis assays. The chemokine CCL19, in 600μl of chemotaxis buffer, was added to the lower chambers of a transwell plate (6.5mm diameter filter, 5μm pore size; Corning, NY, USA) at final concentrations of 0.3μg/ml or 10μg/ml. After adding 100μl of cells to the upper chambers, the assay was incubated for 3 hours at 37º and 5% CO₂. In cases where cells were inhibited with IC87114, cells were pre-incubated with the inhibitor or controls (section 2.1.4) for at least 30 minutes prior to being added to the well. Controls included media only in the lower transwell (to assess spontaneous migration of cells through the filter) and wells which included 100% of input cells were used to enumerate migrated cells. Following the 3 hour incubation, cells were collected from the lower chamber after extensive washing of the filter underside with the chemotaxis buffer from the lower chamber. Collected cells were centrifuged at 8,000 rpm for 4 minutes before being suspended in 100μl of PBS and placed into wells of a black 96 well tray (Costar # 3631). Migration was measured using a Molecular Imager FX (BioRad) with excitation at 488nm and emission at 494nm. The migration index was determined by dividing the value obtained for the experimental sample by the value of the no-chemokine negative control.

2.3.8 Dendritic cell antigen presentation assay

Immature BMDCs were generated from naïve C57BL/6 mice as described in section 2.3.4.2. To pulse BMDCs with antigen, 10^4 immature BMDCs per well of 96 well trays were incubated for a period of 2 hours in the presence of OVA323-339 (Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University) in complete media. During this incubation, spleens were isolated from OT-II mice (section 2.3.1.4) and were labelled with CFSE (section 2.3.2.1). Following the 2 hour DC antigen-pulsing incubation, cells were washed twice with PBS before 2.5 x 10^5 CFSE-labelled OT-II lymphocytes were added to each well. Cells were cultured for 4 days at 37ºC and 5% CO₂. On the third
day of the culture, 50μl of complete media was added to each well to replenish nutrients. Following the culture period, cells were labelled with anti-CD4 antibodies (Table 2.1) before cell division (proliferation) was determined as a progressive halving of CFSE fluorescence intensity by flow cytometry (section 2.3.2.7). In cases where DCs were treated with IC87114 or DMSO, the inhibitor (10μM IC87114) or an equal volume of DMSO control were added for the 2 hour DC pulsing step before being washed away during the PBS washes. When responding OT-II cells were treated with IC87114 or DMSO the inhibitor or equal volumes of DMSO were added to cells at least 15 minutes prior to cells being added to wells containing DCs.

2.3.9 GC/MS analysis of IC87114 in plasma
All GC/MS analysis of IC87114 in plasma was carried out at Calistoga Pharmaceuticals (Seattle, USA). Plasma from animals that had not received IC87114 was used as a negative control.

2.3.10 Statistical analysis
All statistical analysis was performed in GraphPad Prism 3 or GraphPad Prism 5 software using students t tests where *$P<0.05$, **$P<0.01$ and ***$P<0.001$. 
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### Table 2.1: Antibodies used in this study

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**SECONDARY ANTIBODIES/REAGENTS**

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**CONTROL ANTIBODIES**

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### Table 2.2: Chemokines used in this study

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* From Ian Clark-Lewis (Biomedical Research Centre, University of British Colombia, Vancouver, Canada)
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### Table 2.4: Inhibitors used in this study

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Table 2.5: Primers used in p110⁰̵̵¹⁰̵̵⁰⁰¹⁰⁰ genotyping PCR

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CHAPTER 3

Characterisation of p110δ^{D910A/D910A} mice and analysis of EAE disease pathogenesis
3.1 OVERVIEW
In normal biology, the p110δ protein binds to one of several regulatory proteins to form the heterodimeric PI3Kδ, ultimately regulating the activation of many different proteins that are involved in a wide range of cellular processes. However, many of the precise functions of PI3Kδ are still elusive. The ultimate aim of this project is to further understand the role that p110δ plays within the immune system in order to indicate whether p110δ may be useful as a therapeutic target in autoimmune disease. This was addressed using both p110δ<sup>D910A/D910A</sup> mice and by using the highly selective p110δ inhibitor, IC87114. These initial experiments both generally characterise the p110δ<sup>D910A/D910A</sup> mice and address the ability of these animals to develop autoimmune responses in EAE, a model of the human disease multiple sclerosis.

3.2 CHARACTERISATION OF p110δ<sup>D910A/D910A</sup> MICE
The p110δ<sup>D910A/D910A</sup> mice have been previously described (248). They were generated by gene targeting the p110δ gene, Pik3cd, with a knock-in vector that ultimately changed an aspartate residue at amino acid position 910 to an alanine, rendering the protein catalytically inactive. Mice were then back-crossed on to a C57BL/6 background. To begin this study, the p110δ<sup>D910A/D910A</sup> mice were characterised in regards to their genotype, lymphocyte marker expression and the potential of their lymphocytes to migrate towards homeostatic chemokines.

3.2.1 Genotyping of p110δ<sup>D910A/D910A</sup> mice
Breeding pairs of p110δ<sup>D910A/D910A</sup> mice were obtained from the Ludwig Institute (section 2.2.1). As breeding of homozygous animals was unsuccessful (Hibbs, personal communication), p110δ<sup>D910A/D910A</sup> mice were instead bred with p110δ<sup>D910A/WT</sup> mice (heterozygous) to generate offspring. Genotyping was routinely carried out as described in the materials and methods (section 2.2.2) (Figure 3.1A). Analysis of the percentage of each genotype of mouse (i.e. p110δ<sup>D910A/WT</sup> or p110δ<sup>D910A/D910A</sup>, male or female) per 100 mice born was analysed for over 400 mice. These data show that normal Mendelian breeding ratios were observed in these
animals and there were no significant differences between the sex or genotype of the mice born (Figure 3.1B).

3.2.2 Surface marker expression on splenocytes from p110δD910A/D910A mice

An in-depth study of surface marker expression on naïve lymphocytes from p110δD910A/D910A mice has not yet been performed. Therefore the expression of several different leukocyte markers, including homeostatic chemokine receptors and adhesion molecules on the surface of splenocytes from p110δD910A/D910A mice and wild-type C57BL/6 animals, was investigated. Lymphocytes were isolated from the spleen of p110δD910A/D910A and wild-type mice, single cell suspensions were generated and several cell surface markers were probed with antibodies and analysed by flow cytometry. It was found that there was no significant difference between the proportions of CD4+ and CD8+ T cells, nor B220+ B cells, in the spleen of p110δD910A/D910A mice when compared with wild-type animals (Figure 3.2A). Cells that were CD4+ did not show any difference in the levels of expression of the homeostatic chemokine receptors CCR7 or CXCR4 (Figure 3.2B). Furthermore, there was no difference in the proportion of lymphocytes isolated from the spleens of wild-type versus p110δD910A/D910A mice that expressed either the integrin α4 or β1 chains (CD49d and CD29 respectively) (Figure 3.2C). Previous reports have however demonstrated that the p110δD910A/D910A mice have fewer cells in the secondary lymphoid organs when compared with wild-type mice (248, 462). This phenomenon (in the spleen and inguinal and brachial lymph nodes) was also routinely observed throughout this study.

3.2.3 Chemotaxis of p110δD910A/D910A splenocytes towards homeostatic chemokines

It has been previously reported that p110δ inactivation does not affect T cell migration in vitro, however it does reduce the capacity for B cells to migrate towards CXCL13, albeit in p110δ−/−, not p110δD910A/D910A mice (432). Migration of B and T cells from p110δD910A/D910A mice was assessed by performing Transwell chemotaxis assays as described in section 2.3.7.1. The concentrations of stimulating chemokines that were used were previously optimised (432, 496). B cell migration towards
CXCL13 was reduced when p110δ catalytic function was abrogated (Figure 3.3A). However, the capacity of CD4+ T cells to migrate towards the homeostatic chemokines CCL19 and CCL21 was reduced by approximately 50% in splenocytes from p110δ<sup>D910A/D910A</sup> mice when compared with those from wild-type animals (Figures 3.3B and 3.3C respectively).

### 3.3 DETERMINATION OF A FUNCTIONAL ROLE FOR p110δ IN EAE

As discussed at length in the introduction, it has been previously demonstrated that the efficient function of the p110δ protein is important for the activation, differentiation, survival and general function of a number of different cell types both in vitro and, whilst it has been studied to a lesser extent, in vivo. Considering the important role that p110δ plays in leukocytes, it was considered that p110δ<sup>D910A/D910A</sup> mice may display differences in their ability to mount autoimmune responses in disease models. EAE was chosen as a model as it is an inducible autoimmune disease whose pathogenesis can be measured visually by observing the extent and severity of paralysis, as well as by a range of well-documented common analytical immunological methods.

#### 3.3.1 Effects of p110δ inactivation on EAE disease pathogenesis

To determine whether p110δ plays a role in EAE, both p110δ<sup>D910A/D910A</sup> mice and wild-type C57BL/6 mice were immunised with MOG<sub>35-55</sub> emulsified in CFA and pertussis toxin (as described in 2.2.3.1). Mice were evaluated for clinical disease signs from day 9 post-immunisation (section 2.2.3.2). Three independent experiments were performed and are shown here separately (Figure 3.4A-C) as well as pooled (Figure 3.4D). In both experiment 1 and 3 (figures 3.4A and 3.4C respectively) it was observed that p110δ<sup>D910A/D910A</sup> mice initially developed EAE almost to the same extent as their wild-type counterparts (with clinical disease scores of approximately 2-2.5 representing a flaccid tail and partial hind limb paralysis), before their clinical disease scores dropped to around 1-1.5 (indicating that they had a partially flaccid tail). Disease scores were significantly different from day 19 and day 16 post-immunisation respectively. Mice that were immunised in experiment number 2 (Figure 3.4B) did not develop EAE disease as severely as wild-type
C57BL/6 animals and there was a significant difference between the two groups from day 13 post-immunisation. When these experiments were pooled (Figure 3.4D) it was clear that, on average, EAE was reduced in p110\(^{\text{D910A/D910A}}\) mice when compared with wild-type mice. This difference was statistically-significant from day 15 post-immunisation. Cumulated EAE disease scores where all three experiments are pooled and accumulated daily are also shown (Figure 3.4E) and there is a statistically-significant difference between the two groups from day 16 post-immunisation.

There was no difference between animal cohorts with respect to either disease incidence, the day of disease onset, or the day of peak EAE disease when the data were pooled (Figures 3.4F-H). However, the pooled peak EAE clinical disease score of the p110\(^{\text{D910A/D910A}}\) mice was significantly lower when compared with that of wild type mice (Figure 3.4I). The mean peak disease score of p110\(^{\text{D910A/D910A}}\) mice was 2.152 (± 0.231), indicating a paralysed tail only, whereas the mean peak disease score of wild type mice was 2.988 (± 0.062), which correlated with complete hind-limb paralysis (P <0.01).

### 3.3.2 Heterozygous (p110\(^{\text{D910A/WT}}\)) mice develop EAE disease in the same manner as wild-type C57BL/6 mice

To determine whether the severity of EAE that develops in heterozygous (p110\(^{\text{D910A/WT}}\)) mice is reduced when compared to wild-type C57BL/6 animals, both groups were immunised with MOG\(_{35-55}\) emulsified in CFA and pertussis toxin as described in 2.2.3.1. Mice were evaluated for clinical disease signs from day 9 post-immunisation (section 2.2.3.2). Data shown are pooled from two independent experiments (Figure 3.5A) and cumulative disease scores are also shown (Figure 3.5B). There was no significant difference in the disease incidence, day of onset, day of peak disease or peak disease score between the two cohorts (Figures 3.5C-F respectively). Therefore there is no difference in the EAE disease that develops in wild-type animals when compared with p110\(^{\text{D910A/WT}}\) mice.
3.4 ANALYSIS OF SPINAL CORD PATHOLOGY DURING EAE

To begin the analysis of the mechanism behind the reduction of EAE disease in the p110δ\(^{D910A/D910A}\) mice, signs of pathology within the CNS of these animals and wild-type animals were compared. This was done by generating frozen sections of spinal cords at different time-points through the disease course and analysing them by IHC.

3.4.1 Immunohistochemical analysis of lesions in the spinal cords of p110δ\(^{D910A/D910A}\) mice

Cross-sections from the spinal cord of p110δ\(^{D910A/D910A}\) and wild-type mice at day 15 and 28 post-immunisation for EAE were cut and immunohistochemical staining was performed with an anti-CD45 antibody (section 2.3.3). CD45, otherwise known as ‘leukocyte common antigen’, is expressed at high levels on the surface of all cells of haematopoietic origin, except erythrocytes (497). Probing spinal cord sections for CD45\(^+\) cells was therefore used as a method of determining leukocyte influx to the CNS during EAE. Spinal cords from mice displaying representative EAE disease scores were used. Immunohistochemical staining on spinal cord sections clearly showed fewer CD45\(^+\) leukocytes in the CNS of p110δ\(^{D910A/D910A}\) mice on day 28 post-immunisation (Figure 3.6A). The reduced disease scores by the later phases of EAE in p110δ\(^{D910A/D910A}\) mice correlated with a reduction in the number of CD45\(^+\) leukocytes in lesions in the spinal cords of these animals. These results were commonly observed in both lumbar and thoracic regions of the spinal cord.

3.4.2 Lesions in the spinal cords of p110δ\(^{D910A/D910A}\) mice

Areas containing at least ten CD45\(^+\) cells (‘lesions’) were enumerated on both day 15 and day 28 post-immunisation (Figure 3.6B). It was observed that at day 15 there was a strong trend towards fewer lesions in the CNS of p110δ\(^{D910A/D910A}\) mice. However, by day 28 post-immunisation there was a statistically significant difference between the two groups.

3.5 SUMMARY

In summary, p110δ\(^{D910A/D910A}\) mice were successfully bred and births occurred at expected Mendelian ratios. As far as homeostasis of the immune system is
concerned, there was no difference in the proportion of CD4⁺ or CD8⁺ T cells, nor B220⁺ B cells, in the spleens of p110δ<sup>D910A/D910A</sup> mice when compared to wild-type animals. Similar levels of the homeostatic chemokine receptors CCR7 and CXCR4, as well as the α4 and β1 integrins (CD49d and CD29 respectively), were also observed on the surface of p110δ<sup>D910A/D910A</sup> mice when compared with wild-type mice. Taking all of these data into account, it appears that inactivation of p110δ does not overtly affect homeostasis of the immune system, at least with respect to the parameters investigated. Despite this, migration of both B cells towards CXCL13 and T cells towards CCL19 and CCL21 was reduced in cells in which p110δ had been catalytically inactivated. While it has previously been demonstrated that B cells require functional p110δ to effectively migrate towards CXCL13 <em>in vitro</em> (432), it had not previously been shown that p110δ plays a role in T cell migration <em>in vitro</em>. This is discussed further in chapter 6 of this thesis. In a novel finding, it was observed that complete genetic inactivation of the p110δ protein clearly resulted in reduced EAE pathogenesis. This was further supported by the finding that there were fewer lesions consisting of CD45⁺ leukocytes in the spinal cord of p110δ<sup>D910A/D910A</sup> mice when compared with wild-type animals. No differences in EAE pathogenesis were observed when comparing wild-type C57BL/6 and heterozygous p110δ<sup>D910A/WT</sup> mice. These findings define a clear role for p110δ in EAE pathogenesis, but demonstrate that complete inactivation of p110δ is required for this to occur. A complete picture of the mechanism behind this reduction in CNS leukocytes and EAE pathology remains to be addressed. The following chapter describes experiments that characterise the reduced autoimmune response in p110δ<sup>D910A/D910A</sup> mice.
Figure 3.1: Genetic characterisation of the p110δ knock-out/knock-in mutant mice

(A) Genomic DNA was extracted from the tail tips of mice overnight as described in section 2.2.2. The resulting DNA was subjected to 35 cycles of PCR with previously genotyped heterozygous (p110δ^{D190A/WT}) and homozygous (p110δ^{D910A/D910A}) mouse DNA as controls. Homozygous and heterozygous mice were characterised by the visualisation of either a single band at 500bp or one band at 500bp and another at 300bp respectively. Representative genotyping results for over 400 mice are shown.

(B) Following genotyping of animals, typical breeding ratios were determined by observing the percentage of each possible genotype (i.e. heterozygous male, heterozygous female, homozygous male or homozygous female) per 100 mice counted (400 mice represented in total). Data are expressed as mean ± SEM.
Figure 3.2: Surface phenotyping of lymphocytes from p110δ<sup>Δ910A/Δ910A</sup> and wild-type mice

Spleens were isolated from naïve p110δ<sup>Δ910A/Δ910A</sup> and wild-type mice and single cell suspensions were prepared and analysed by flow cytometry (section 2.3.2.2). Representative flow cytometric histograms are shown in each figure. In each case the lines representing samples are as follows: isotype control (green), wild-type (blue) and p110δ<sup>Δ910A/Δ910A</sup> (red). **(A)** Lymphocytes were stained with anti-CD4, anti-CD8 and anti-B220 antibodies. **(B)** Lymphocytes were stained with anti-CD4, anti-CCR7 and anti-CXCR4 antibodies. CD4<sup>+</sup> cells were gated upon (shown in the histogram on the left) and CCR7 or CXCR4 expression on their cell surface was determined. **(C)** CD29 (β1 integrin chain) and CD49d (α4 integrin) expression on the surface of lymphocytes. Data points represent the mean percentage of positive lymphocytes ± SEM (n = 3 mice per group) and represent two independent experiments.
CHAPTER 3: Characterisation of p110δ<sup>D910A/D910A</sup> mice and analysis of EAE pathogenesis

A

![Graph showing % cells positive for CD4, CD8, and B220 for Wild-type and p110δ<sup>D910A/D910A</sup> mice.](image)

- **Wild-type**
- **p110δ<sup>D910A/D910A</sup>**

**Cell surface marker**

- CD4<sup>+</sup>
- CD8<sup>+</sup>
- B220<sup>+</sup>
CHAPTER 3: Characterisation of p110δ<sup>D910A/D910A</sup> mice and analysis of EAE pathogenesis

B

% CD4<sup>+</sup> cells positive

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B

Chemokine receptor

CD4

CCR7

CXCR4

CD4

CXCR4

Chemokine receptor

% CD4<sup>+</sup> cells positive

0 10 20 30 40 50 60 70 80 90 100

CCR7

CXCR4

CD4

CCR7

CXCR4

CD4

CXCR4

Chemokine receptor

% CD4<sup>+</sup> cells positive

0 10 20 30 40 50 60 70 80 90 100

CCR7

CXCR4

CD4

CCR7

CXCR4

CD4

CXCR4
CHAPTER 3: Characterisation of p110$^\delta^{D910A/D910A}$ mice and analysis of EAE pathogenesis

C

![Bar chart showing % cells positive for CD29$^+$ (β1) and CD49d$^+$ (α4) integrin chains in Wild-type and p110$^\delta^{D910A/D910A}$ mice.]

- **Wild-type**
  - CD29$^+$ (β1)
  - CD49d$^+$ (α4)

- **p110$^\delta^{D910A/D910A}$**
  - CD29$^+$ (β1)
  - CD49d$^+$ (α4)
CHAPTER 3: Characterisation of p110δ<sup>D910A/D910A</sup> mice and analysis of EAE pathogenesis

A

![Graph showing the number of B220<sup>+</sup> cells migrated to 1μg/ml CXCL13](image)

B

![Graph showing the number of CD4<sup>+</sup> cells migrated to 0.5μg/ml CCL19](image)
CHAPTER 3: Characterisation of p110<sup>D910A/D910A</sup> mice and analysis of EAE pathogenesis

Figure 3.3: Chemotaxis of p110<sup>D910A/D910A</sup> splenocytes towards homeostatic chemokines

Lymphocytes were isolated from the spleen of naive wild-type C57BL/6 mice and p110<sup>D910A/D910A</sup> animals, stained with Calcein and subjected to Transwell chemotaxis assays as described in section 2.3.7.1. Following a three hour incubation, cells were isolated from the lower chamber of the chemotaxis tray and subjected to staining with anti-CD4 and anti-B220 antibodies and analysed by flow cytometry. Total numbers of migrated cells were calculated as described (section 2.3.7.1). (A) B220<sup>+</sup> cell migration in response to 1μg/ml CXCL13. Data represent the mean number of migrated cells ± SEM (n = 2). *P <0.05. (B) CD4<sup>+</sup> cell migration in response to 0.5μg/ml CCL19. Data represent the mean number of migrated cells ± SEM (n = 2). **P <0.01. (C) CD4<sup>+</sup> cell migration in response to 4μg/ml CCL21. Data represent the mean number of migrated cells ± SEM (n = 2). ***P <0.001.
Figure 3.4: Effects of p110δ inactivation on EAE pathogenesis

Mice were immunised with MOG_{35-55} and pertussis toxin as described in section 2.2.3.1. Animals were monitored for clinical disease from day 9 post-immunisation. EAE disease scores were evaluated as described in section 2.2.3.2. Wild-type C57BL/6 (blue squares) and age-matched p110δ_{D910A/D910A} (red squares) mice were immunised to induce EAE. (A-C) Data from three independent experiments are shown. Data points represent the mean EAE disease score ± SEM (*$P<$0.05). (D) Pooled disease scores from the three independent experiments shown in Figure 3.4 A-C. (Wild-type C57BL/6 n = 22, p110δ_{D910A/D910A} n = 23. *$P<$0.05). (E) Cumulative EAE disease scores of pooled EAE experiments with p110δ_{D910A/D910A} (red squares) and age-matched C57BL/6 (blue squares) mice throughout the disease course. Data are mean pooled EAE disease score ± SEM. *$P<$0.05. (F) EAE disease incidence was 100% in both experimental groups. (G) Day of EAE onset. Data represent the mean day of EAE onset ± SEM. (H) Day of peak EAE disease. Data represent the mean day of peak EAE disease ± SEM. (I) Peak EAE disease score. Data represent the mean peak EAE disease score ± SEM. **$P<$0.01.
CHAPTER 3: Characterisation of p110<sup>D910A/D910A</sup> mice and analysis of EAE pathogenesis

**A**

EAE study #1

**B**

EAE study #2

**C**

EAE study #3
CHAPTER 3: Characterisation of p110δ<sup>D910A/D910A</sup> mice and analysis of EAE pathogenesis

![Graph D](image1)

**EAE disease score**

- **Wild-type**
- **p110δ<sup>D910A/D910A</sup>**

![Graph E](image2)

**Cumulative disease score**

- **Wild-type**
- **p110δ<sup>D910A/D910A</sup>**
CHAPTER 3: Characterisation of p110δD910A/D910A mice and analysis of EAE pathogenesis

F

G

H

I

EAE incidence

Day of onset

Day of peak disease

Peak disease score

Wild-type p110δ D910A/D910A

Wild-type p110δ D910A/D910A

Wild-type p110δ D910A/D910A

Wild-type p110δ D910A/D910A
CHAPTER 3: Characterisation of p110\textsubscript{\(\delta^{D910A/D910A}\)} mice and analysis of EAE pathogenesis

A

![Graph showing EAE disease score over time for Wild-type and p110\textsubscript{\(\delta^{D910A/WT}\)} mice.]

B

![Graph showing cumulative disease score over time for Wild-type and p110\textsubscript{\(\delta^{D910A/WT}\)} mice.]

---

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Figure 3.5: Mice heterozygous for the p110δ mutation develop EAE in the same manner as wild-type C57BL/6 mice
Mice were immunised with MOG\textsubscript{35-55} emulsified in complete Freund’s adjuvant as described in section 2.2.3.1. Pertussis toxin was also administered at the time of immunisation and on the second day post-immunisation. Mice were evaluated for clinical disease symptoms from day 9 post immunisation. (A) EAE disease scores observed in wild-type and p110$^\delta$\textsuperscript{D910A/WT} mice were evaluated as described in section 2.2.3.2. Data points represent the mean EAE disease score ± SEM (Wild-type $n$ = 15 mice, p110$^\delta$\textsuperscript{D910A/WT} $n$ = 14 mice). (B) Cumulative EAE disease scores (where each day’s score is added to that from the previous day) throughout the disease course. (C) Disease incidence. (D) Day of EAE disease onset. (E) Day of peak EAE disease. (F) Peak EAE disease score. All statistics are mean ± SEM (wild-type $n$ = 15, p110$^\delta$\textsuperscript{D910A/WT} $n$ = 14 mice).
Figure 3.6: Lesions in the spinal cord of mice immunised for EAE
Spinal cords were removed from mice (as described in section 2.3.1.6) at day 15 and day 28 post-immunisation for EAE. Sections were cut and stained with rat anti-mouse CD45 antibody and haematoxylin (section 2.3.3). (A) CD45⁺ cells in sections of spinal cords from p110δ<sup>D910A/D910A</sup> and age-matched C57BL/6 mice at day 15 and 28 post-immunisation for EAE. Top panels show lumbar sections from spinal cords obtained at day 15 post-immunisation; bottom panels are thoracic sections of spinal cords extracted at day 28 post-immunisation for EAE. Sections are representative of several sections from at least 3-5 mice per group. (B) The number of lesions on day 15 and 28 post-immunisation in cross sections of thoracic and lumbar spinal cords from p110δ<sup>D910A/D910A</sup> and age-matched C57BL/6 mice. Lesions in 12-18 sections from 3-5 mice per group were counted. Data are representative of the mean lesion number ± SEM. **p < 0.01.
CHAPTER 3: Characterisation of $p110^{\text{D910A/D910A}}$ mice and analysis of EAE pathogenesis

A

Day 15

Wild-type

p$110^{\text{D910A/D910A}}$

Day 28
CHAPTER 3: Characterisation of p110δ^{D910A/D910A} mice and analysis of EAE pathogenesis

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CHAPTER 3: Characterisation of p110δ^{D910A/D910A} mice and analysis of EAE pathogenesis

B

Day post-immunisation

<table>
<thead>
<tr>
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<th>Day 15</th>
<th>Day 28</th>
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<tbody>
<tr>
<td># of lesions containing &gt;10 CD45+ cells</td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>7 ± 1</td>
<td>4 ± 1</td>
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<tr>
<td>p110δ^{D910A/D910A}</td>
<td>5 ± 1</td>
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n.s. **
CHAPTER 3: Characterisation of p110δ

D910A/D910A mice and analysis of EAE pathogenesis
CHAPTER 4

The effect of p110δ inactivation on cells of the immune system during EAE
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE
4.1 OVERVIEW

In the previous chapter it was demonstrated that p110δ<sup>D910A/D910A</sup> mice do not develop EAE to the same extent as wild-type animals. It is possible that p110δ is playing a variety of roles in different cell types that govern EAE pathogenesis. This chapter focuses on investigating the mechanisms behind the reduced EAE pathology observed in the p110δ<sup>D910A/D910A</sup> mice.

4.2 PRIMING AND SURVIVAL OF CD4<sup>+</sup> T LYMPHOCYTES IS REDUCED IN p110δ<sup>D910A/D910A</sup> MICE

As discussed in the introduction, CD4<sup>+</sup> T cells have been widely implicated in MS and EAE. Furthermore, it has been demonstrated that p110δ function influences effector T cell trafficking to antigenic tissue <i>in vivo</i> as well as the differentiation and sustained activation of both Th1- and Th2-type CD4<sup>+</sup> T cells. To this end, the effect of p110δ inactivation on the CD4<sup>+</sup> T cell compartment in EAE was investigated, with initial studies focussing on the priming of CD4<sup>+</sup> T cells during EAE. Furthermore, p110δ has been implicated in cell survival of a number of other cell types, particularly B cells (238, 239, 416, 448). Since T cells share the same signalling pathways that govern this process (in particular the Akt/PKB initiated survival pathways) the impact of p110δ inactivation on T cell survival during the immune priming phase of EAE was also investigated.

4.2.1 CD4<sup>+</sup> T lymphocytes in the draining lymph nodes of p110δ<sup>D910A/D910A</sup> mice display a more naïve phenotype than those from wild-type counterparts

To determine whether CD4<sup>+</sup> T cells from p110δ<sup>D910A/D910A</sup> mice were being activated as efficiently as those from wild-type counterparts, a detailed study of the expression of classical activation markers on the surface of cells from the draining inguinal and brachial lymph nodes throughout the EAE disease course was performed. These activation markers included CD62L (L-selectin) and the chemokine receptors CCR7, CXCR3 and CCR6. CD62L and CCR7 are both highly expressed on naïve T lymphocytes (44, 498-500), whereas CXCR3 and CCR6 are up-regulated on the surface of T cells following activation, and are therefore indicative of activated T lymphocytes (501-504). Lymphocytes were isolated from the inguinal and brachial
lymph nodes of p110δ^{D910A/D910A} and wild-type mice at days 9 and 15 post-immunisation, single cell suspensions were generated and the expression of the aforementioned surface markers was assessed by flow cytometry (section 2.3.2.2). It was found that, when compared with wild-type mice, there was a higher proportion of CD4+ cells from p110δ^{D910A/D910A} mice that express the naïve cell markers CD62L and CCR7 at both days 9 and 15 post-immunisation (Figures 4.1A and 4.1B respectively). Complementary to this, CD4+ T cells from the draining lymph nodes of p110δ^{D910A/D910A} mice expressed lower levels of the activation markers CXCR3 and CCR6 at both time-points compared to that observed on the surface of CD4+ cells from wild-type animals (Figures 4.1C and 4.1D respectively). These data indicate that the CD4+ T cells in the draining inguinal and brachial lymph nodes of p110δ^{D910A/D910A} mice are not activated as efficiently as T cells in wild-type animals.

4.2.2 There are fewer T effector memory cells in the draining lymph nodes of p110δ^{D910A/D910A} mice throughout EAE

The proportion of effector memory T cells in the draining inguinal and brachial lymph nodes of p110δ^{D910A/D910A} and wild-type mice was assessed by flow cytometry. It was observed that at both day 9 and day 15 post-immunisation the p110δ^{D910A/D910A} mice had a lower proportion of CD4+ T cells that were CD62L^{lo}/CD44^{hi}, indicating that there were fewer effector memory T cells in the draining lymph nodes of these animals compared with wild-type mice (Figure 4.2).

4.2.3 The ex vivo and in vivo proliferative response of p110δ^{D910A/D910A} CD4+ T cells following stimulation with the neuroantigen MOG\textsubscript{35-55}

Once T cells become activated following antigen presentation they undergo a rapid proliferation. It was observed that fewer T cells from p110δ^{D910A/D910A} mice express markers of activation following immunisation with MOG\textsubscript{35-55} in CFA, suggesting that there was less priming of the autoimmune response in these animals. Therefore, the ability of lymphocytes to proliferate ex vivo and in vivo was assessed.

To determine whether p110δ is important for proliferation of primed T cells ex vivo, animals were immunised with MOG\textsubscript{35-55} in CFA before the draining inguinal and
brachial lymph nodes were extracted on day 9 post-immunisation (sections 2.2.3.1). Single cell suspensions were generated and cells were labelled with CFSE before being incubated for four days in the presence of MOG, as described in section 2.3.5.1. Concanavalin A (ConA) was used as a positive control to induce high levels of cell proliferation, while cells that were incubated with media only were used as a negative control for non-antigen-specific proliferation that may occur during the incubation period. It was observed that lymphocytes from p110δ\textsuperscript{D910A/D910A} mice underwent significantly less proliferation in response to MOG\textsubscript{35-55}-stimulation than cells from wild-type animals (Figure 4.3). There was no difference in background proliferation observed between the two cohorts (i.e. media only-treated cells). In addition, it was observed that cells from p110δ\textsuperscript{D910A/D910A} mice did not proliferate in response to ConA stimulation as efficiently as wild-type cells. Together, these data indicate that lymphocytes which lack functional p110δ are less capable of undergoing proliferation, and are less activated in response to MOG\textsubscript{35-55}. However, the observed reduction in cell division following stimulation with ConA indicates that this reduction in proliferation was not antigen-specific.

While assessment of proliferation of MOG\textsubscript{35-55}-stimulated cells \textit{ex vivo} is useful to indicate the level of activation of the stimulated cells, immune priming \textit{in vivo} is a much more complex process which requires a number of cellular and molecular interactions not attainable \textit{in vitro}. These include the provision of growth factors, cytokines and chemokines that are required for efficient activation of cells \textit{in vivo}, as well as co-stimulation by APCs. Therefore, to investigate whether p110δ is important for cell activation/proliferation \textit{in vivo}, priming of immune cells was further investigated using BrdU proliferation assays as described in section 2.3.5.2. Briefly, animals were fed BrdU in their drinking water from day six post-immunisation before draining inguinal and brachial lymph nodes were removed at day nine post-immunisation. BrdU incorporation into the DNA of CD4\textsuperscript{+} cells was assessed by flow cytometry after lymphocytes were labelled with anti-CD4 and anti-BrdU antibodies. Consistent with the results of the surface marker staining and \textit{ex vivo} proliferation assay it was observed that CD4\textsuperscript{+} cells from p110δ\textsuperscript{D910A/D910A} mice had incorporated
less BrdU into their DNA compared with wild-type mice, indicating less immune priming was occurring in these animals (Figure 4.4).

4.2.4 Apoptosis is increased in CD4+ cells from p110δ<sup>D910A/D910A</sup> mice

Previous reports have demonstrated that several different types of cells which lack p110δ undergo increased levels of apoptosis (238, 239, 416, 448). However, the impact of p110δ inactivation on antigen-activated T cell survival has yet to be reported. Propidium iodide and annexin V staining was performed to assess the level of cell death in lymphocytes from p110δ<sup>D910A/D910A</sup> and wild-type mice throughout the EAE disease course (section 2.3.2.6). It was observed that at day 6 and 9 post-immunisation there was a lower proportion of apoptotic cells in the draining lymph nodes of wild-type mice than in p110δ<sup>D910A/D910A</sup> mice (Figure 4.5A). When this was analysed in more detail, focusing on the CD4+ T cell population, it was observed that CD4+ cells in the draining lymph nodes of p110δ<sup>D910A/D910A</sup> mice were undergoing more apoptosis at day 9 post-immunisation (Figure 4.5B). These data indicate that p110δ function is imperative for CD4+ T cell survival during EAE immune priming.

4.3 B CELL ACTIVATION AND FUNCTION IS REDUCED IN p110δ<sup>D910A/D910A</sup> MICE DURING EAE

While T cells are critical for the induction of disease in MS/EAE, it has been demonstrated that B cells also play an important role in the pathology and clinical outcomes of these diseases. Furthermore, the development, survival, activation and function of B cells are demonstrably reliant on p110δ (238, 239). Therefore, the presence of B cells in the CNS, antibody production and B cell survival in p110δ<sup>D910A/D910A</sup> mice during EAE was investigated.

4.3.1 B220<sup>+</sup> cells do not enter the central nervous system during EAE

To examine the extent of B220<sup>+</sup> B cell infiltration to the CNS of p110δ<sup>D910A/D910A</sup> and wild-type mice during EAE, spinal cords were extracted from both cohorts at day 15 post-immunisation and cross-sections of these spinal cords were cut and stained with anti-B220 antibodies (section 2.3.3). Spinal cords from mice displaying representative EAE disease scores were used. Immunohistochemical staining on
spinal cords showed that, while wild-type mice had B220\(^+\) cells in the spinal cord at day 15 post-immunisation, there were no detectable B220\(^+\) cells in the spinal cord of p110\(\delta^{D910A/D910A}\) mice (Figure 4.6). These results were commonly observed in both the lumbar and thoracic regions of the spinal cord.

4.3.2 Antigen-specific antibody production does not occur in p110\(\delta^{D910A/D910A}\) mice throughout EAE

MOG\(_{35-55}\)-specific antibody production by p110\(\delta^{D910A/D910A}\) and wild-type mice was subsequently investigated. Serum was isolated from the mice at days 9, 15 and 28 post-immunisation for EAE and ELISA assays were performed as described in section 2.3.6.1. Regardless of the severity of the clinical disease scores throughout the experiments there was no increase in the levels of MOG\(_{35-55}\)-specific IgG in the serum of p110\(\delta^{D910A/D910A}\) mice at days 9, 15 and 28 post-immunisation (Figure 4.7). Wild-type mice, however, displayed significantly increasing levels of MOG\(_{35-55}\)-specific IgG in the serum throughout the disease course, indicating that B cells were maturing, undergoing isotype-switching and increasing their IgG output. There were particularly high levels of MOG\(_{35-55}\)-specific antibodies in the serum of wild-type mice at day 28 post-immunisation, which correlates with a typical time-course for IgG production in immune challenges (505, 506).

4.3.3 Apoptosis is increased in B220\(^+\) cells from p110\(\delta^{D910A/D910A}\) mice at EAE disease onset

It has been widely reported that p110\(\delta\) plays an important role in B cell survival \textit{in vitro} (238, 239). The defects observed in B cell antibody production and trafficking to the CNS of p110\(\delta^{D910A/D910A}\) mice during EAE may not only be due to defects in these particular processes, but also due to B cells lacking functional p110\(\delta\) undergoing higher levels of apoptosis. To test this, the levels of apoptotic B cells in the draining lymph nodes at EAE onset were investigated. Lymphocytes were extracted from draining brachial and inguinal lymph nodes at day 9 post-immunisation and stained with anti-B220 antibodies as well as Annexin V and propidium iodide before being analysed by flow cytometry (section 2.3.2.6). It was observed that while approximately 10\% of B220\(^+\) cells extracted from wild-type
lymph nodes were AV+/PI+, more than 30% of B220+ cells from p110δD910A/D910A mice were undergoing apoptosis (Figure 4.8). Therefore, without p110δ, a significantly higher proportion of B220+ cells from p110δD910A/D910A mice are apoptotic during early stages of EAE.

4.4 p110δ INACTIVATION DOES NOT AFFECT DENDRITIC CELL MIGRATION OR ACTIVATION

Cell-mediated immunity relies on efficient antigen processing and presentation by APCs such as dendritic cells. Without functional p110δ, T cell responses have been shown to be reduced (present study and others (248, 249, 381, 464)). However, the observed reduction in T cell activation in the p110δD910A/D910A mouse could be due to a T cell intrinsic defect, or a defect in DC function, or both. In spite of this, the role of p110δ in the function of DCs has not yet been reported.

4.4.1 Ex vivo migration of dendritic cells is not affected by p110δD910A/D910A inactivation

The capacity of BMDCs to migrate towards chemokines in vitro was assessed. DCs were isolated from the bone marrow of p110δD910A/D910A and wild-type mice (section 2.3.1.7) before being cultured in the presence of GM-CSF for 10 days. Following this incubation, medium containing GM-CSF, LPS and TNFα was added to the DCs in order to produce a mature phenotype (section 2.3.4.3). There was no difference between the numbers of DCs that were isolated from the bone marrow of wild-type and p110δD910A/D910A mice (Figure 4.9A), nor was there a difference between the numbers of mature DCs generated in culture (Figure 4.9B). Transwell chemotaxis assays were then performed to assess the ability of mature DCs to migrate in vitro towards the homeostatic chemokine CCL19. It was observed that mature BMDCs lacking functional p110δ migrated in vitro towards the chemokine CCL19 as effectively as mature BMDCs from wild-type mice (Figure 4.9C).

4.4.2 Migration of dendritic cells in vivo is not reliant on p110δ

Migration of DCs from the site of immunisation to the draining lymph nodes is imperative for efficient antigen-presentation to T cells and subsequent disease
induction during EAE. Therefore, \textit{in vivo} DC migration was assessed. The abdomens of both p110δ\textsuperscript{D910A/D910A} and wild-type mice were shaved and painted with a 1% FITC solution (section 2.2.6). Forty-eight hours later the inguinal and brachial lymph nodes were removed, and single cell suspensions were generated and co-stained with anti-CD11c antibodies before being analysed by flow cytometry. There was no difference in the proportion of CD11c\textsuperscript{+} cells that were isolated from the draining lymph nodes of p110δ\textsuperscript{D910A/D910A} mice when compared with wild-type mice (Figure 4.10A), nor was there a difference in the proportion of CD11c\textsuperscript{+} cells that were FITC\textsuperscript{+} in the draining lymph nodes of the two groups (Figure 4.10B). Therefore, p110δ does not play a role in the migration of activated DCs from the skin to the draining lymph nodes.

4.4.2 Dendritic cells from CFA-immunised p110δ\textsuperscript{D910A/D910A} mice display the same phenotype as wild-type mice

To assess the maturation of DCs, both p110δ\textsuperscript{D910A/D910A} and wild-type mice were immunised in the hind flank with CFA and draining inguinal lymph nodes were extracted 48 hours later and stained with DC markers. It was observed that CD11c\textsuperscript{+} DCs had similar levels of the activation markers CD86 and MHC class II on the cell surface (Figure 4.11). Therefore p110δ inactivation does not affect the activation of DCs following CFA immunisation \textit{in vivo}.

4.5 T CELL DIFFERENTIATION TO T REGULATORY, Th1- AND Th17-TYPES IS SIGNIFICANTLY REDUCED UPON p110δ INACTIVATION

It has been demonstrated both in the present study and in the published literature (248, 249, 381, 464) that inactivation of p110δ results in inefficient T cell activation. As discussed in the introduction to this thesis (section 1.3.4.1), Th17 cells have been recently implicated as the driving force behind the severe pathogenesis observed in both EAE and MS. While p110δ has been shown to be important for the function and development of Th1, Th2 and Treg cells (381, 464), the role of p110δ in Th17 cell differentiation has not yet been reported. Due to the importance of Th17 cells in EAE/MS pathogenesis, the role of p110δ in Th17 cell biology was investigated. As there has been a strong link between p110δ and Th1 and Treg cell differentiation
(both of which have been implicated in MS/EAE pathogenesis), the effect of genetic p110δ inactivation in these cell types was also further examined throughout the EAE disease course.

4.5.1 p110δD910A/D910A mice have fewer regulatory T cells in draining lymph nodes at disease onset and peak disease time-points

Treg cells are important for the regulation of the immune response. Inefficient Treg function often results in autoimmunity and Treg function has been shown to be compromised in p110δD910A/D910A mice in a colitis model (464). Therefore, the presence of Treg cells in the draining lymph nodes of p110δD910A/D910A mice throughout EAE was investigated. Draining inguinal and brachial lymph nodes were extracted at days 6 and 15 post-immunisation and cells were stained with anti-CD4, anti-CD25 and anti-FoxP3 antibodies and analysed by flow cytometry. It was observed that on day 6 post-immunisation there was a significantly higher proportion of CD4+/CD25+/FoxP3+ Treg cells in the draining lymph nodes of p110δD910A/D910A mice when compared with wild-type animals (Figure 4.12). Despite this, by day 15 post-immunisation a significantly lower proportion of cells in the draining lymph nodes were Tregs. This indicates that p110δ is involved in the differentiation and development of Treg cells during EAE.

4.5.2 In vitro differentiation of CD4+ cells the Th1- and Th17- types is affected by p110δ inactivation

To determine the role of p110δ in the differentiation of Th1 and Th17 cells in vitro naïve splenocytes were isolated from p110δD910A/D910A and wild-type mice and cultured for four days in the presence of anti-CD3 and anti-CD28 under either Th1-skewing or Th17-skewing culture conditions (sections 2.3.4.6 and 2.3.4.7 respectively). Briefly, cells were exposed to IL-12 to induce differentiation to the Th1-type or TGF-β, IL-6, IL-1β and IL-23 in the presence of neutralising anti-IFN-γ and IL-4 antibodies to induce differentiation to the Th17-type. Following the four day culture, cells were stained for the expression of surface CD4 and intracellular IFN-γ and IL-17 before flow cytometric analysis. It was observed that cells from p110δD910A/D910A mice were less capable of differentiating to either IFN-γ-producing
Th1 cells (Figure 4.13A) or IL-17-producing Th17 cells (Figure 4.13B). Therefore, p110δ inactivation results in a reduction in differentiation of both Th1 and Th17 cells in vitro.

### 4.5.3 Differentiation of CD4⁺ cells to the Th1-type throughout EAE is affected by p110δ inactivation

Th1 cells have been shown to be involved in EAE pathogenesis (155, 162, 163) and to require p110δ for efficient differentiation (present study and (381)). Therefore, the presence of Th1 cells in the draining lymph nodes and CNS of p110δ<sup>D910A/D910A</sup> and wild-type mice throughout EAE was assessed. To do this, cells were isolated from the spinal cords and draining inguinal and brachial lymph nodes of p110δ<sup>D910A/D910A</sup> and wild-type animals at days 15 and 28 post-immunisation, as described in section 2.3.4.6. Following this, lymphocytes were stained for expression of surface CD4 and intracellular IFN-γ and IL-17 and analysed by flow cytometry. On day 15 post-immunisation, the p110δ<sup>D910A/D910A</sup> mice had a higher proportion of Th1 cells in their draining lymph nodes than did wild-type mice (Figure 4.14A). The proportion of IFN-γ-producing CD4⁺ cells in the lymph nodes was greatly reduced by day 28 post-immunisation, particularly in the p110δ<sup>D910A/D910A</sup> mice. On both days 15 and 28 post-immunisation there was no statistically significant difference in the proportion of Th1 cells in the spinal cords of the two cohorts (Figure 4.14B). Representative flow cytometric plots are shown in Figure 4.14E. Taken together, the data observed from both the lymph nodes and spinal cord indicate that p110δ<sup>D910A/D910A</sup> mice are capable of mounting a Th1 response during EAE and that the proportion of Th1 cells in the draining lymph nodes is increased at peak disease.

### 4.5.4 p110δ is imperative for the development of Th17 cells in vivo during EAE

In contrast to the results observed when investigating the ability of p110δ<sup>D910A/D910A</sup> mice to mount a Th1 response during EAE, mice lacking functional p110δ were not as capable of mounting a Th17 response. At the later time-point investigated (day 28 post-immunisation), the proportion of CD4⁺/IL-17⁺/IFNγ⁻ Th17 cells in the draining lymph nodes of p110δ<sup>D910A/D910A</sup> mice was significantly lower than that observed in wild-type mice (Figure 4.14C). This phenomenon was also observed in the spinal
cord of p110δ<sup>D910A/D910A</sup> mice at day 28 post-immunisation, with a strong trend towards proportionally less Th17 cells in the spinal cord of these animals at day 15 post-immunisation also (Figure 4.14D). Representative flow cytometric plots are shown in Figure 4.14E. Mice lacking functional p110δ therefore mount a less efficient Th17 response in EAE.

### 4.5.5 There is a strong bias towards a Th1-type immune response in p110δ<sup>D910A/D910A</sup> mice

When the data shown in Figure 4.14 are expressed as a ratio (i.e. the number of Th1 cells per Th17 cell), p110δ<sup>D910A/D910A</sup> mice had a higher Th1:Th17 ratio in the draining lymph nodes at all disease stages examined (Figure 4.15A). Whereas wild-type mice typically had a Th1:Th17 ratio of between 1:1 and 2:1, p110δ<sup>D910A/D910A</sup> mice had a Th1:Th17 ratio of around 5:1 and 6:1 at both day 15 and 28 post-immunisation. This difference was also observed in the spinal cord of p110δ<sup>D910A/D910A</sup> mice at the day 28 post-immunisation time point, with a trend towards the same phenomena at peak disease (day 15 post-immunisation) (Figure 4.15B). This indicates that while wild-type C57BL/6 mice mount both Th1 and Th17 immune responses, p110δ<sup>D910A/D910A</sup> mice produce an autoimmune response that is strongly skewed towards Th1.

### 4.5.6 CNS infiltration of F4/80<sup>+</sup> macrophages and Ly6G<sup>+</sup> neutrophils is altered in the absence of functional p110δ

It has been previously reported that adoptive transfer of encephalitogenic cells, which have been skewed <i>ex vivo</i> to the IL-12-driven Th1-type, results in an autoimmune response characterised by macrophage-rich CNS infiltrates. Conversely, when IL-23-driven Th17-type cells are transferred, a neutrophil-rich CNS infiltrate occurs (169). In those previous reports, in spite of a difference in the cells that made up the CNS infiltrates, the resulting paralysis was clinically indistinguishable between the two groups. However those data demonstrate that Th1- and Th17-type autoimmune responses can drive the activation and recruitment of different cell-types to the CNS.
To investigate the effect of reduced Th17-type responses on the cellular infiltrates in the CNS of $p110\delta^{D910A/D910A}$ mice, the proportion and number of F4/80$^+$ macrophages and Ly6G$^+$ neutrophils in the CNS of $p110\delta^{D910A/D910A}$ and wild-type mice at days 6 and 15 post-immunisation for EAE were determined by flow cytometry (section 2.3.2.2). There was no significant difference in the number of cells isolated from the CNS of $p110\delta^{D910A/D910A}$ mice when compared with wild-type mice at day 6 and day 15 post-immunisation (Figure 4.16A). At day 6 post-immunisation wild-type mice had a significantly higher proportion of F4/80$^+$ macrophages in the CNS when compared with $p110\delta^{D910A/D910A}$ mice (Figure 4.16B). At both time points there were significantly fewer F4/80$^+$ cells in the CNS of $p110\delta^{D910A/D910A}$ mice (Figure 4.16C). While there was a significantly higher proportion of Ly6G$^+$ cells in the CNS of $p110\delta^{D910A/D910A}$ mice at day 6 post-immunisation when compared with wild-type counterparts (Figure 4.16D), there was no difference in the proportion of Ly6G$^+$ cells between the two cohorts at day 15 post-immunisation, nor was there a difference between the numbers of Ly6G$^+$ cells isolated from the CNS at either time-point investigated (Figure 4.16E). When expressed as a ratio of the number of F4/80$^+$ macrophages to Ly6G$^+$ neutrophils in the CNS, wild-type mice had a higher macrophage to neutrophil ratio at day 6 post-immunisation (approximately 2.5:1) and an equal number of macrophages and neutrophils in the CNS at day 15 post-immunisation (Figure 4.16F). In contrast, $p110\delta^{D910A/D910A}$ mice had equal numbers of macrophages and neutrophils in the CNS six days post-immunisation and a macrophage to neutrophil ratio of approximately 0.5:1 at day 15 post-immunisation. These data together indicate that while Ly6G$^+$ neutrophils from $p110\delta^{D910A/D910A}$ mice seem capable of migrating to the CNS, there appears to be a defect in recruitment of F4/80$^+$ macrophages to the CNS during EAE in these animals.

4.6 SUMMARY

As described in chapter 3 of this thesis, $p110\delta$ function is imperative for the development of an efficient autoimmune response in EAE. This chapter has further examined the cellular basis for this. Firstly, the work presented here demonstrates that $p110\delta$ is required for efficient T cell priming in EAE. CD4$^+$ T cells from $p110\delta$-
inactivated mice have a more naïve phenotype when compared with wild-type T cells and they do not undergo as much proliferation \textit{ex vivo} or \textit{in vivo} as do wild-type T cells. In addition, p110δD910A/D910A mice have fewer effector memory T cells at EAE onset and at peak disease. Despite a reduction in Th1 cell generation \textit{in vitro}, the proportion of Th1 cells in the CNS of p110δD910A/D910A mice was observed to be similar to that observed in wild-type animals. In a novel finding, the differentiation of CD4+ T cells to the highly-pathogenic Th17-type was demonstrated to be significantly reduced both \textit{in vitro} and \textit{in vivo} when p110δ is inactive. The reduction in T cell activation and differentiation observed in p110δ-deficient animals was not due to a disruption in DC migration or activation (the ability of DCs to effectively process and present antigen is addressed in chapter 5 of this thesis). While the proportion of Tregs in draining lymph nodes in p110δD910A/D910A mice was shown to be elevated at day 6 post-immunisation when compared with wild-type animals, by peak disease there was a lower proportion of Tregs in p110δ-deficient mice. Previous reports have shown that p110δD910A/D910A mice have reduced Treg function which correlated with a lack of protection from experimental colitis (464). However, in the present study it appears that the reduced T cell activation and differentiation may be sufficient to reduce EAE disease in these animals, despite the lower proportion of Tregs. Alternatively, the increased proportion of Tregs in the p110δD910A/D910A mice prior to disease onset may be sufficient to confer some protection against the development of severe EAE. In addition to the reduced T cell function observed in this study, the capacity of B cells to enter the CNS and produce MOG35-55-specific IgG was inhibited. Because anti-MOG antibodies have been implicated in EAE pathogenesis these data indicate that B cells are reliant on p110δ function to be pathogenic in EAE. Levels of apoptosis in lymphocytes from the two experimental cohorts were also tested and it was observed that a higher proportion of both B and T cells lacking functional p110δ were undergoing apoptosis at day 9 post-immunisation. Together these data indicate that not only is the activation of p110δ-deficient antigen-specific B and T cells reduced but that these cell types are incapable of maintaining efficient survival signals. Both of these phenomena are likely to be contributing to the reduced EAE pathogenesis observed in the p110δD910A/D910A mice. Finally, while equal proportions of Ly6G+ neutrophils were
observed in the CNS of both cohorts during EAE, proportionally fewer F4/80+ macrophages were observed in the CNS of p110δD910A/D910A mice when compared with wild-type animals, indicating that p110δ may play an important role in macrophage biology. In summary, this chapter shows that, primarily through its role in T and B cell activation and survival, p110δ is unequivocally linked to the development of EAE.
Figure 4.1: Characterisation of CD4+ T lymphocytes in the draining lymph nodes of p110δ^{D910A/D910A} or wild-type mice throughout EAE.

Draining lymph nodes from wild-type and p110δ^{D910A/D910A} mice on either day 9 or 15 post-immunisation for EAE were analysed for expression of CD4, CD62L (A), CCR7 (B), CXCR3 (C) and CCR6 (D). Representative flow cytometry plots are shown. Data are representative of at least 3 independent experiments. *P <0.05, **P <0.01, ***P <0.005. All data are mean ± SEM (n = 6-8 mice per group).
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

A

Day 9

Wild-type

p110δ\textsuperscript{D910A/D910A}

% CD4\textsuperscript{+} cells that are CD62L\textsuperscript{+}

Day 15

***

Day post-immunisation

Day 9

Day 15

***

B

Day 9

Wild-type

p110δ\textsuperscript{D910A/D910A}

% CD4\textsuperscript{+} cells that are CCR7\textsuperscript{+}

Day 15

***

Day post-immunisation

Day 9

Day 15

***
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C

% CD4+ cells that are CXCR3+

Day 9 Day 15

Wild-type p110δD910A/D910A

Day post-immunisation

Wild-type p110δD910A/D910A

Day 9 Day 15

CD4

CXCR3

21.48 28.52

30.68 29.09

9.02 5.75

2.21 1.52

D

% CD4+ cells that are CCR6+

Day 9 Day 15

Wild-type p110δD910A/D910A

Day post-immunisation

Wild-type p110δD910A/D910A

Day 9 Day 15

CD4

CCR6

21.59 23.21 34.2 37.8 16.7 4.12 4.57 5.4

34.2 4.57 37.8 5.4

142
Figure 4.2: Effector memory T cells in the draining lymph nodes throughout EAE.

CD4^+ cells from the draining lymph nodes of p110δ^{D910A/D910A} mice at both day 9 and day 15 post-immunisation were analysed for expression of CD44 and CD62L to identify memory CD4^+ T cells. Representative flow cytometry plots gating on CD4^+ cells are shown. Data are representative of at least 3 independent experiments. *P <0.05, **P <0.01, ***P <0.005. All data are mean ± SEM (n = 6-8 mice per group).
Figure 4.3: Ex vivo antigen-specific proliferation of encephalitogenic cells.

Proliferation of encephalitogenic cells ex vivo was determined by immunising wild-type and p110δD910A/D910A mice with MOG35-55 emulsified in CFA as described in section 2.2.3. At day 9 post-immunisation draining inguinal and brachial lymph nodes were extracted and single cell suspensions were generated (section 2.3.1.4) and stained with CFSE (section 2.3.2.1). Cells were then cultured for 4 days in the presence of 25μg/ml MOG35-55, 50μg/ml MOG35-55 or 1.5μg/ml ConA (section 2.3.5.1). Cultures that contained media and cells alone were used as negative controls. Following the 4 day culture, cells were washed, resuspended in PBS/Azide and proliferation of wild-type (blue bar) and p110δD910A/D910A (red bar) cells was analysed by assessing the progressive halving of CFSE fluorescence. A representative flow cytometry histogram overlay is shown for cell division in response to ConA (wild-type = blue line, p110δD910A/D910A = red line). *P <0.05, ***P <0.005. All data are mean ± SEM (n = 8-9 mice per group).
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

Cells Only

% cells divided

Wild-type

p110δ^{D910A/D910A}

Cells Only, 25μg/ml MOG, 50μg/ml MOG, 1.5μg/ml ConA

**% cells divided**

**Cell #**

CFSE

10^1 10^2 10^3 10^4 10^5 10^6
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

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Figure 4.4: Proliferation of CD4+ T cells in vivo following MOG\textsubscript{35-55} immunisation.

Mice were immunised with MOG\textsubscript{35-55} emulsified in CFA as described in section 2.2.3. From day 6 post-immunisation mice were fed BrdU in their drinking water (section 2.3.5.2). At day 9 post-immunisation, mice were euthanased and draining inguinal and brachial lymph nodes were extracted (section 2.3.1.4). Single cell suspensions were generated and stained with antibodies specific for surface CD4 and intracellular BrdU (section 2.3.2.4). Representative flow cytometry plots are shown. Data are representative of at least 3 independent experiments. **$P<0.01$. All data are mean ± SEM (n = 5).
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

Figure 4.5: CD4⁺ T cells that lack p110δ undergo higher levels of apoptosis throughout EAE than wild-type cells.

Mice were immunised to develop EAE as described in section 2.2.3. At days 6 and 9 post-immunisation, animals were euthanased and draining inguinal and brachial lymph nodes were extracted (section 2.3.1.4). Single cell suspensions were generated, stained with Annexin V and propidium iodide and analysed by flow cytometry (section 2.3.2.6). (A) The proportion of apoptotic (Annexin V⁺/Propidium iodide⁺) cells in the draining lymph nodes of p110δ<sup>D910A/D910A</sup> mice (red bars) compared with wild-type mice (blue bars). Representative FACS plots are also shown. (B) Cells were labelled with anti-CD4 antibodies before Annexin V and propidium iodide staining and the proportion of apoptotic (AnnexinV⁺/Propidium iodide⁺) CD4⁺ cells in the draining lymph nodes of p110δ<sup>D910A/D910A</sup> (red bar) and wild-type (blue bar) mice at day 9 post-immunisation for EAE was calculated. Representative flow cytometry plots are also shown. Results are representative of at least two independent experiments. ***P <0.005. All data are mean ± SEM (n = 6-8 mice per group).
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

A

% cells AV⁺/PI⁺

Day 6 Day 9

Day post-immunisation

Wild-type p110δ/D910A/D910A

***

% CD4⁺ cells that are AV⁺/PI⁺

Day 6 Day 9

Wild-type p110δ/D910A/D910A

4.53 6.04

4.26 6.77

Annexin V

Propidium Iodide

B

% CD4⁺ cells that are AV⁺/PI⁺

Wild-type p110δ/D910A/D910A

***

5.13 16.68

6.04

5.13

6.04

15.0

16.68

15.0

20.0

Wild-type p110δ/D910A/D910A

Annexin V

Propidium Iodide
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

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CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

Figure 4.6: Reduced B cell infiltration of the CNS of p110δ^{D910A/D910A} mice.
Spinal cords were removed from mice (section 2.3.1.6) at day 15 post-immunisation for EAE. Cross sections were cut and stained with rat anti-mouse B220 antibody and haematoxylin (section 2.3.3). B220+ cells in representative thoracic sections of spinal cords from p110δ^{D910A/D910A} and age-matched C57BL/6 mice at day 15 post-immunisation for EAE are shown. Isotype control antibody used was a RatIgG2a monoclonal. Arrow indicates B220+ cells. Sections are representative of several sections from at least 3-5 mice per group.
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Figure 4.7: MOG\textsubscript{35-55}-specific IgG is not detectable in the serum of p110δ\textsuperscript{D910A/D910A} mice.

Direct ELISAs were performed to determine the levels of MOG\textsubscript{35-55}-specific IgG in the serum of mice throughout the EAE disease course. Serum was isolated from wild-type or p110δ\textsuperscript{D910A/D910A} mice at days 9, 15 and 28 post-immunisation. Serum from naïve (non-immunised) animals was used as a negative control. ELISAs were performed as described in section 2.3.6.1. Data are mean ± SEM and are representative of at least 2 independent experiments (n = 6-8).
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Figure 4.8: B220+ in the draining lymph nodes of mice without functional p110δ undergo higher levels of apoptosis than wild-type counterparts.

Mice were immunised for EAE as described in section 2.2.3. At day 9 post-immunisation draining inguinal and brachial lymph nodes were extracted and single cell suspensions generated (section 2.3.1.4). Cells were stained with B220 antibodies (section 2.3.2.2) before being stained with Annexin V and propidium iodide (section 2.3.2.6) and analysed by flow cytometry (section 2.3.2.7). The proportion of apoptotic (Annexin V+/Propidium iodide+) B220+ B cells in the draining lymph nodes of p110δ<sub>D910A/D910A</sub> mice (red bars) and wild-type mice (blue bars) is shown. Representative flow cytometry dot plots are also shown. Data are representative of two independent experiments. ***P <0.005. All data are mean ± SEM (n = 5).
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Figure 4.9: In vitro migration of BMDCs to CCL19 is not reliant on p110δ.

Bone-marrow was isolated from the femurs of naïve wild-type C57BL/6 (blue bar) and p110δ<sup>D910A/D910A</sup> (red bar) mice (section 2.3.1.7). **(A)** Cell numbers extracted from the bone marrow of the two cohorts were calculated. Data are representative of at least five independent experiments and shown is the mean cell number ± SEM (n = 4). **(B)** Bone marrow-derived cells were cultured to generate mature DCs (section 2.3.4.3). The number of mature DCs generated in cultures was determined by trypan blue staining and counting cells with a typical DC phenotype. Data are representative of five independent experiments (n = 4). **(C)** Mature DCs were subjected to in vitro transwell chemotaxis assays as described in section 2.3.7.2. Data are pooled from three independent experiments (n = 10). All data are mean ± SEM.
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

A. Number of cells isolated from one femur (x 10⁶)

B. Number of cells isolated from culture (x 10⁷)

C. Migration index

- No Chemokine
- Wild-type
- Wild-type p110δ D910A/D910A
- Wild-type p110δ D910A/D910A

CCL19 concentration

- 0.3 μg/ml
- 10 μg/ml
Figure 4.10: Dendritic cell migration *in vivo* is not affected by genetic inactivation of p110δ.

DC migration *in vivo* was assessed by painting the abdomen of mice with FITC two days prior to removal of the draining lymph nodes (section 2.2.6). Single cell suspensions were then stained with anti-CD11c antibodies and analysed by flow cytometry. (A) The proportion of CD11c⁺ DCs isolated from p110δ<sup>D910A/D910A</sup> (red bar) or wild-type (blue bar) mice. (B) The proportion of CD11c⁺ cells that were FITC⁺ in the draining lymph nodes of p110δ<sup>D910A/D910A</sup> (red bar) and wild-type (blue bar) mice. Figures show pooled data from two independent experiments (n = 12). All data are mean ± SEM.
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A

% CD11c+ cells

Wild-type  p110δ<sub>D910A/D910A</sub>

B

% CD11c<sup>+</sup> cells that are FITC<sup>+</sup>

Wild-type  p110δ<sub>D910A/D910A</sub>
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Figure 4.11: Dendritic cell activation following CFA immunisation.

To assess the activation status of DCs in p110δ<sup>D910A/D910A</sup> versus wild-type mice, animals were immunised with CFA in the hind flank 7 days before inguinal lymph nodes were removed, and the proportion of active DCs was also assessed by comparing the proportion of CD11c<sup>+</sup> cells that were also positive for CD86 or MHC class II. The levels of CD11c<sup>+</sup> cells that were either CD86<sup>+</sup> or MHC II<sup>+</sup> in p110δ<sup>D910A/D910A</sup> (red bars) and wild-type (blue bars) mice are shown. Data are representative of two independent experiments (n = 8). All data are mean ± SEM.
Figure 4.12: Regulatory T cell generation is disrupted in p110δ<sup>D910A/D910A</sup> mice at peak EAE disease.

P110δ<sup>D910A/D910A</sup> and wild-type animals were immunised as described in section 2.2.3 and draining lymph nodes were extracted at day 6 and 15 post-immunisation (section 2.3.1.4). Single cell suspensions were stained with antibodies against surface CD4, CD25 and intracellular FoxP3 (sections 2.3.2.2 and 2.3.2.5). The proportion of CD4<sup>+</sup> cells that were CD25<sup>+</sup>/FoxP3<sup>+</sup> in the lymph nodes of p110δ<sup>D910A/D910A</sup> (red bars) and wild-type (blue bars) was then analysed by flow cytometry. Representative flow cytometry plots are shown. Figures are representative of two independent experiments (n = 8). Data are mean ± SEM.
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

![Graph showing the effect of p110δ inactivation on CD4+ cells that are CD25+/FoxP3+](image)

- **Wild-type**
- **p110δ<sup>D910A/D910A</sup>**

<table>
<thead>
<tr>
<th></th>
<th>Day 6</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD4+ cells that are CD25+/FoxP3+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>7.11</td>
<td>4.13</td>
</tr>
<tr>
<td>p110δ&lt;sup&gt;D910A/D910A&lt;/sup&gt;</td>
<td>11.17</td>
<td>14.57</td>
</tr>
</tbody>
</table>

*Significant difference (p < 0.05)*
**Significant difference (p < 0.01)**
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

Figure 4.13: Differentiation of cells from p110δ^{D910A/D910A} and wild-type mice ex vivo under Th1- and Th17-skewing culture conditions.

Lymphocytes from p110δ^{D910A/D910A} (red bars) and wild-type (blue bars) mice were incubated under Th1- or Th17-skewing culture conditions (sections 2.3.4.6 and 2.3.4.7) for 4 days before being assessed for (A) Th1 (CD4^{+}/IFN-γ^{+}/IL-17^{−}) or (B) Th17 (CD4^{+}/IFN-γ^{−}/IL-17^{+}) cell production. To assess Th1 and Th17 cell production, cells were stained for surface CD4 and intracellular IFN-γ (Th1 cells) or IL-17 (Th17 cells) and analysed by flow cytometry (sections 2.3.2.2 and 2.3.2.3). Representative flow cytometric dot plots are also shown. Data are representative of two independent experiments (n = 9). ***P <0.005. All data are shown as mean ± SEM.
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

A

**Wild-type**

**p110δ<sup> D910A/D910A</sup>**

% Th1 cells

0 1 2 3 4 5 6 7

***

B

**Wild-type**

**p110δ<sup> D910A/D910A</sup>**

% Th17 cells

0 2 4 6 8 10

***

IL-17

IFNγ

5.99 3.18

8.28 3.24
Figure 4.14: Th17 responses are significantly reduced in p110δ$^{D910A/D910A}$ mice.

The proportion of Th1 (IFN-γ+/IL-17-/CD4+ cells) cells in (A) the draining lymph nodes and (B) spinal cords of p110δ$^{D910A/D910A}$ (red bars) and wild-type (blue bars) mice throughout the EAE disease course was assessed by isolating lymphocytes at days 15 and 28 post-immunisation for EAE and staining for surface CD4 and intracellular IFN-γ (sections 2.3.2.2 and 2.3.2.3). Proportions of Th17 (IFN-γ-/IL-17+/CD4+ cells) cells were also assessed in (C) the draining lymph nodes and (D) spinal cords by staining for surface CD4 and intracellular IL-17 (sections 2.3.2.2 and 2.3.2.3). (E) Representative flow cytometric dot plots of lymph node and spinal cord cells are also shown. *P <0.05, **P <0.01, ***P <0.005. Data are expressed as mean ± SEM and are representative of at least two independent experiments per time-point. Lymph node n = 5-8, spinal cord n = 4-6.
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

A

Day post-immunisation

% Th1 cells in the draining lymph nodes

Wild-type

p110δ^{D910A/D910A}

**

Day 15

Day 28

B

Day post-immunisation

% Th1 cells in the spinal cord

Wild-type

p110δ^{D910A/D910A}

**

Day 15

Day 28

...
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

C

% Th17 cells in the draining lymph nodes

Day 15 Day 28

Day post-immunisation

Wild-type
p110δ^{D910A/D910A}

***

*


D

% Th17 cells in the spinal cord

Day 15 Day 28

Day post-immunisation

*
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

Wild-type

**Lymph node**

Day 15

IL-17

p110δ

D910A/D910A

Day 28

IFNγ

Wild-type

0.70

1.26

0.79

3.36

12.84

39.08

4.42

41.90

0.56

1.10

0.11

0.44

2.68

19.01

0.91

16.15

Spinal cord

E Day 15 Day 28
Figure 4.15: The autoimmune response in \( \text{p110}\delta^{D910A/D910A} \) is skewed towards a Th1-type and away from the more pathogenic Th17-type.

Flow cytometric data from Figure 4.14 was also used to determine the relative ratio of Th1 (CD4\(^+\)/IFN-\(\gamma\)/IL-17\(^+\)) to Th17 (CD4\(^+\)/IFN-\(\gamma\)/IL-17\(^-\)) cells in the draining lymph nodes and spinal cords of \( \text{p110}\delta^{D910A/D910A} \) (red bars) and wild-type (blue bars) mice throughout the EAE. These data represent the number of Th1 cells per Th17 cells in (A) the draining lymph nodes or (B) the spinal cord throughout the disease course. *\( P < 0.05 \), **\( P < 0.01 \). Data are expressed as mean ± SEM and are representative of at least two independent experiments per time-point. Lymph node \( n = 5-8 \), spinal cord \( n = 4-6 \).
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A

**# Th1 cells per Th17 cell in the draining lymph nodes**

- **Wild-type**
- **p110δD910A/D910A**

Day post-immunisation:
- **Day 15**
- **Day 28**

B

**# Th1 cells per Th17 cell in the spinal cord**

Day post-immunisation:
- **Day 15**
- **Day 28**

**Legend:**
- ****
- ****
Figure 4.16: F4/80* macrophage infiltration to the CNS is affected by p110δ inactivation.

Infiltration of macrophages and neutrophils to the CNS was assessed by extracting leukocytes from spinal cords from p110δ<sup>D910A/D910A</sup> (red bars) and wild-type (blue bars) mice at day 6 and 15 post-immunisation for EAE and staining cells for expression of surface F4/80 and Ly6G before flow cytometric analysis (section 2.3.2.2). (A) Total cell numbers recovered from spinal cords is shown. The proportion and number of F4/80<sup>+</sup> cells (B and C respectively) and Ly6G<sup>+</sup> cells (D and E respectively) in the CNS of p110δ<sup>D910A/D910A</sup> and wild-type was assessed by flow cytometry. (F) Flow cytometric data was also used to determine the relative ratio of F4/80<sup>+</sup> macrophages to Ly6G<sup>+</sup> neutrophils in the spinal cords of p110δ<sup>D910A/D910A</sup> (red bars) and wild-type (blue bars) mice at days 6 and 15 post-immunisation for EAE. These data represent the number of F4/80<sup>+</sup> cells per Ly6G<sup>+</sup> cell in the spinal cord throughout the disease course. *P <0.05, **P <0.01, ***P <0.005. Data are expressed as mean ± SEM (n = 7-8 mice per group).
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

A

Day post-immunisation

Number of cells isolated from the spinal cord

Wild-type

p110δ<sup>D910A/D910A</sup>

Day 6

Day 15

B

% F4/80<sup>+</sup> cells

Day post-immunisation

Day 6

Day 15

C

# F4/80<sup>+</sup> cells

Day post-immunisation

Day 6

Day 15

D

% Ly6G<sup>+</sup> cells

Day post-immunisation

Day 6

Day 15

E

# Ly6G<sup>+</sup> cells

Day post-immunisation

Day 6

Day 15
CHAPTER 4: The effect of p110δ inactivation on cells of the immune system during EAE

![Graph showing the effect of p110δ inactivation on cells of the immune system during EAE.](image-url)

- **Wild-type**
- **p110δD910A/D910A**

The graph illustrates the number of F480+ cells per Ly6G+ cell over the days post-immunisation. On Day 6, there is a significant difference (indicated by ***) between the wild-type and p110δD910A/D910A groups. On Day 15, the difference is also significant (indicated by **).
CHAPTER 5

Investigation into the efficacy of the p110δ inhibitor IC87114 as a therapeutic for EAE
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CHAPTER 5: The efficacy of the p110δ inhibitor IC87114 as a therapeutic for EAE

5.1 OVERVIEW
In the past, studies into PI3K function were performed using pan-PI3K inhibitors such as Wortmannin and LY294002. However in recent years small molecule, isoform-specific inhibitors for PI3K catalytic subunits have been developed (352-361). One of these inhibitors, IC87114, is highly specific for the class IA p110δ catalytic isoform (359). IC87114 has no known off-target effects on other protein kinases such as Akt1 (PKBα), PKCα, PKCβII, p38 MAPK, DNA-PK, c-Src, casein kinase I and checkpoint kinase I and significantly inhibits p110δ catalytic function and the generation of PIP3 (359). Published studies using IC87114 have demonstrated that this compound is capable of reducing in vitro activation, function and/or trafficking in a number of different cell types including neutrophils, mast cells, NK cells and B and T cells (238, 240, 359, 360, 362-372). IC87114 treatment has also been shown to reduce pathology in a model of rheumatoid arthritis (370). Isoform-specific PI3K inhibitors are currently undergoing clinical development for cancer treatment and in the near future they may also be adapted to treat autoimmune diseases. Therefore, due to the high specificity of IC87114 towards p110δ, and in view of the data presented in the previous two chapters implicating p110δ in the immune response occurring in EAE, an initial investigation into the potential of this compound to reduce clinical disease during EAE was conducted and is reported in this chapter.

5.2 CHARACTERISATION OF THE EFFECT OF IC87114 ON T CELL DIFFERENTIATION IN VITRO
The p110δ-specific inhibitor IC87114 has been demonstrated to reduce the activation and function of several different cell types (238, 240, 359, 360, 362-372). While genetic inactivation of p110δ reduces IFN-γ-production by Th1-skewed cells in vitro (249), pharmacological inhibition of p110δ function has not yet been demonstrated to reduce the differentiation of Th1 or Th17 cells. As both Th1 and Th17 cells are important for EAE pathogenesis, initial studies were undertaken to compare the effects of the pan-PI3K inhibitor LY294002 and the p110δ-specific inhibitor IC87114 on T cell differentiation in vitro.
5.2.1 The pan-PI3K inhibitor LY294002 reduces differentiation of naïve T cells to the Th1-type

PI3Ks have been shown to be important for Th1 differentiation (present study and (249)). To examine the effect of the pan-PI3K inhibitor LY294002 on Th1 cell differentiation, spleens were extracted from wild-type C57BL/6 mice and single cell suspensions were generated (section 2.3.1.4). Cells were then cultured for four days under Th1-skewing culture conditions (media containing IL-12 - section 2.3.4.6) in the presence of LY294002 or a diluent control (section 2.1.4.1) before being analysed by flow cytometry. Concentrations of LY294002 used in the present study have been used previously and have been shown to inhibit PI3K activation (440). Th1 cells were defined as CD4+ cells that were IFN-γ+/IL-17-. CD4+ cells that were cultured with the highest concentration of LY294002 (100μM) had very little intracellular IFN-γ when compared to diluent controls (Figure 5.1A). Culturing cells with lower concentrations of LY294002 (2.5μM and 5μM) did not affect the proportion of cells that were CD4+/IFN-γ+/IL-17- when compared with the negative controls. Despite this, when culture supernatants were analysed by ELISA, there were lower levels of IFN-γ detectable in the supernatants of all of the LY294002-treated samples when compared to the diluent control (Figure 5.1B). This indicates that, even at the lower concentrations, LY294002 inhibits secretion of IFN-γ by IL-12-driven cells in vitro. Therefore PI3Ks may not only be important for Th1 differentiation but also for cytokine secretion in vitro.

5.2.2 IC87114 treatment reduces differentiation of Th1 cells

It is clear that pan-PI3K inhibition results in reduced Th1 differentiation under the culture conditions used for this study. The efficacy of the p110δ inhibitor IC87114 to inhibit this process was then assessed as described above. It was observed that cells which were cultured with 5μM or 10μM of IC87114 showed a marked reduction in their capacity to differentiate to IFN-γ-producing Th1 cells (Figure 5.2A). Furthermore, levels of IFN-γ in the culture supernatant of IC87114-treated cells were significantly lower than that observed in the supernatant of diluent controls (Figure 5.2B). These findings demonstrate that IC87114 inhibits the process of Th1 differentiation and IFN-γ production/secretion in vitro. As observed with LY294002,
cytokine secretion was more sensitive to inhibition of p110δ than was Th1 differentiation.

5.2.3 LY294002 reduces Th17 cell differentiation
The results of experiments using p110δ<sup>D910A/D910A</sup> mice indicate a role for p110δ in Th17 generation (see chapter 4). To determine whether pharmacological inhibition of p110δ similarly inhibits Th17 production, lymphocytes were cultured for four days under Th17-skewing culture conditions (media containing IL-6, TGF-β, IL-1β, IL-23, anti-IFN-γ and anti-IL-4 - section 2.3.4.7), in the presence of LY294002 or diluent (as a negative control). It was observed that at all concentrations of LY294002 tested (2.5μM, 5μM and 100μM) there was a significant reduction in the proportion of CD4<sup>+</sup> cells that were IL-17<sup>+</sup>/IFN-γ<sup>−</sup> (Th17 cells) when compared with the samples cultured in the presence of the diluent negative control (Figure 5.3A). When analysed by ELISA, it was observed that cells which were exposed to LY294002 also secreted lower levels of IL-17 into the culture supernatant; IL-17 was undetectable in the wells where cells were exposed to 100μM LY294002 (Figure 5.3B). These findings show that PI3Ks are important for Th17 cell differentiation and possibly for IL-17 production/secretion.

5.2.4 CD4<sup>+</sup> cell differentiation to a Th17-type is significantly impacted by IC87114
To assess whether IC87114 inhibits Th17 differentiation in vitro, lymphocytes were cultured in Th17-skewing culture conditions in the presence or absence of IC87114 suspended in DMSO (section 2.1.4.2). CD4<sup>+</sup> cells were examined for intracellular IL-17 by flow cytometry (sections 2.3.2.2 and 2.3.2.3). It was observed that CD4<sup>+</sup> cells which were exposed to optimal concentrations of IC87114 (5μM and 10μM) failed to effectively differentiate to the IL-17-producing Th17-type (Figure 5.4A). There was very little IL-17 detectable by ELISA in the supernatant of these cultures (Figure 5.4B). Therefore the p110δ-specific inhibitor IC87114 can significantly reduce Th17 cell differentiation when administered at concentrations of 5μM and 10μM in vitro, verifying an important role for p110δ in Th17 differentiation.
5.3 IC87114 DOES NOT AFFECT DC FUNCTION

In chapter 4 of this thesis the role of p110δ in DC migration and activation was addressed. Here, the ability of the p110δ inhibitor IC87114 to affect DC migration and activation is also tested. Antigen processing and presentation by DCs is integral for efficient immune responses. Therefore IC87114 was also used to inhibit p110δ in either DCs or responding T cells to determine whether pharmacological inhibition of p110δ affects DC antigen processing and presentation, as well as T cell proliferation in response to presentation of antigen by DCs.

5.3.1 IC87114 treatment does not affect DC migration

To test whether IC87114 affects DC migration, BMDCs were isolated from wild-type C57BL/6 mice (section 2.3.1.7) before being cultured in the presence of GM-CSF for 10 days. Following this incubation, media containing GM-CSF, LPS and TNFα was added to the DC cultures in order to produce a mature phenotype (section 2.3.4.3). DCs were then labelled with CFSE, pre-treated with IC87114 or diluent, and subjected to Transwell chemotaxis assays to assess the ability of mature DCs to migrate towards the chemokine CCL19 in vitro (section 2.3.7.2). Consistent with that observed in p110δ<sup>D910A/D910A</sup> mice (Figure 4.9B), pharmacological inhibition of p110δ with IC87114 did not affect the capacity of DCs to migrate towards CCL19 in vitro (Figure 5.5).

5.3.2 IC87114 treatment of DCs does not affect antigen processing and presentation

Antigen presentation by DCs to cells within the lymph nodes is important for the efficient activation of the immune response. The ability of OVA-pulsed, IC87114-treated, DCs to present antigen to CFSE-labeled OT-II lymphocytes was examined to determine if p110δ plays a role in DC antigen processing and presentation. Immature wild-type DCs were pulsed with OVA for a period of two hours, during which time they were treated with IC87114 or diluent (section 2.1.4.2). Following this, DCs were washed and subsequently incubated with CFSE-labelled OT-II splenocytes, and CD4<sup>+</sup> OT-II cell proliferation was assessed by flow cytometry 4 days later (section 2.3.8). There was no difference in the ability of IC87114 treated DCs to induce
proliferation in OT-II CD4⁺ T cells when compared with diluent control-treated DCs (Figure 5.6).

5.3.3 IC87114 treatment of responding OT-II cells inhibits proliferation in response to antigen presentation by DCs
To further examine the role of p110δ in the dynamic T cell/DC interaction, the DC cell antigen presentation assay described above was performed without p110δ inhibition of DCs but with IC87114-treatment of responding OT-II T cells. When responding OT-II cells were treated with IC87114 for the 4-day incubation period there was a significant reduction in their ability to proliferate in response to OVA presented by DCs (Figure 5.7). This indicates that p110δ inhibition induces an intrinsic defect in T cell activation, thereby reducing the proliferative response.

5.3.4 IC87114 is detectable in plasma following administration via oral gavage
Before any in vivo experiments using IC87114 were commenced, initial tests were performed to ensure that administration of IC87114 resulted in the compound being effectively delivered to the blood stream. The most effective in vivo dosing strategy of IC87114 has been previously devised (Kamal Puri, Calistoga Pharmaceuticals, personal communication). This involves a twice daily administration of 30mg/kg of IC87114 (suspended in vehicle) (sections 2.1.4.2 and 2.1.4.3) to mice via oral gavage (section 2.2.5). To test the levels of IC87114 in the bloodstream of mice, blood was collected one, two or four hours following administration of IC87114 (section 2.3.1.3). Plasma samples were then analysed by gas chromatography – mass spectrometry (GC-MS) to examine IC87114 levels (section 2.3.9). It was observed that at one, two and four hours post-administration of IC87114 there were appropriate levels of IC87114 for pharmacological activity detectable in the plasma of animals (Figure 5.8) (Kamal Puri, Calistoga Pharmaceuticals, personal communication).
5.3.5 IC87114 treatment does not affect DC activation following CFA immunisation

To examine whether IC87114 treatment in vivo affects the proportion of active DCs in the draining lymph nodes, mice were treated with IC87114 or vehicle alone for 48 hours following immunisation with CFA (section 2.2.4). Draining inguinal and brachial lymph nodes were then extracted and treated with collagenase (section 2.3.1.4) before cells were stained with antibodies against CD11c, CD86 and MHC class II and analysed by flow cytometry (section 2.3.2.2). It was observed that there was an equal proportion of CD11c+ cells in the draining lymph nodes of mice treated with IC87114 when compared with animals treated with the vehicle only (Figure 5.9A). The CD11c+ DCs from mice treated with IC87114 in vivo expressed levels of the activation markers CD86 and MHC II that were comparable to that observed from vehicle-treated mice (Figure 5.9B). Therefore, IC87114 treatment in vivo does not affect the proportion of active DCs in draining lymph nodes following CFA immunisation, thereby confirming the observed lack of effect of genetic inactivation of p110δ on these DC functions.

5.4 IC87114 TREATMENT IN VIVO REDUCES CD4+ CELL PROLIFERATION

Before considering IC87114 treatment in vivo it was important to investigate whether the inhibitor is capable of inhibiting T cell responses after in vivo administration. Therefore, preliminary experiments were performed to examine this.

5.4.1 IC87114 treatment in vivo reduces the ex vivo proliferative capacity of naïve CD4+ cells

In a first series of experiments, the proliferative capacity of T cells with ex vivo inhibition of p110δ was assessed. Cells taken from the spleen of mice with no prior treatment in vivo were labelled with CFSE (section 2.3.2.1) before being incubated for four days in the presence of anti-CD3/anti-CD28 antibodies (section 2.3.4.4) with the addition of IC87114 or diluent (section 2.1.4.2). At the end of the four day culture, cells were harvested and analysed by flow cytometry. Cell proliferation was assessed by comparing experimental samples with cells that were incubated in the
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presence of culture media alone (cells only negative controls); a progressive halving of CFSE fluorescence intensity was considered a marker of cell proliferation. It was observed that 5% of CD4+ T cells treated in vitro with the diluent control divided in response to the anti-CD3/anti-CD28 stimulation whereas only 2.5% of CD4+ T cells treated in vitro with IC87114 were capable of proliferating in response to anti-CD3/anti-CD28 (Figure 5.10A).

To assess whether IC87114 treatment in vivo can reduce the proliferative capacity of naïve CD4+ T cells, wild-type C57BL/6 mice were administered either IC87114 or the vehicle control via oral gavage in the morning and evening (section 2.2.5). The following morning, mice were again administered IC87114 or vehicle before the spleen of the mice was removed one hour later and single cell suspensions were generated and set up with the same culture conditions described above (without inhibitor or diluent). It was observed that approximately 8% of the CD4+ T cells taken from the spleen of vehicle control-treated mice proliferated in response to anti-CD3/anti-CD28 stimulation during the culture period. However only 5% of CD4+ T cells from mice treated with IC87114 underwent cell division during this time (Figure 5.10B). Therefore, these data show that in vivo treatment with the p110δ inhibitor IC87114 reduces the ex vivo proliferative capacity of naïve T cells. These data indicate that the IC87114 treatment in vivo can reduce the ex vivo proliferative capacity of naïve T cells.

5.5 IC87114 ADMINISTRATION DURING EAE

IC87114 is a potent inhibitor of p110δ and initial published experiments show that this compound may prove to be a useful therapeutic in diseases such as cancer (particularly leukaemia) and rheumatoid arthritis (370, 371). The autoimmune disease MS is characterised by an influx of both antigen-specific and non-specific cells to the CNS where they cause local inflammation and myelin and axon destruction. This damage results in the progressive neurological defects observed in MS patients. Due to the role that cells of the immune system play in MS, it was thought that administration of IC87114 may aid in down-regulating the autoimmune response in MS patients by inhibiting the activation and effector function of key
disease mediators such as T and B cells. IC87114 may therefore provide some therapeutic relief to RR-MS patients by limiting the frequency and severity of relapses, and it may also prove useful for slowing the degeneration observed in PP-MS patients.

The experiments already shown in this chapter show that IC87114 is capable of almost completely inhibiting Th17 differentiation in vitro, reducing Th1 differentiation in vitro and reducing the proliferative capacity of CD4^+ T cells following in vivo treatment. IC87114 does not affect DC migration, activation or antigen processing and presentation. Therefore, due to the capacity of IC87114 to limit T cell function, and the proven importance of T cells in EAE, IC87114 was administered to wild-type C57BL/6 throughout EAE with two different dosing strategies. The first was a ‘preventative’ strategy where animals were administered IC87114 via oral gavage (section 2.2.5) from the day prior to EAE immunisation until the experimental end-point (section 5.5.1). The second dosing strategy was a ‘therapeutic’ approach where animals were only administered the compound from the time that they were showing symptoms of established clinical disease (section 5.5.2).

5.5.1 Preventative dosing of IC87114 to mice immunised with MOG_{35-55}

To determine the efficacy of IC87114 to prevent EAE, mice were administered IC87114 twice daily via oral gavage from one day prior to EAE immunisation. This represents the ‘preventative’ dosing strategy. As an added control, plasma samples taken throughout this experimental procedure were analysed by GC-MS to determine the levels of IC87114 in the blood of mice. This was done at various time-points post-IC87114 dosing. It was observed that the animals being treated with IC87114 had high levels of the compound in their bloodstream at most of the time-points tested throughout the disease course. However, by 12 hours post-IC87114 administration there were only low levels of IC87114 detectable in the bloodstream (Figure 5.11). Animals receiving 30mg/kg of IC87114 twice daily throughout the course of EAE exhibited a similar EAE disease course when compared with mice receiving the vehicle control (Figure 5.12A). There was no significant difference
between the average day of disease onset and the average day of peak disease between the two cohorts (Figures 5.12B and 5.12C respectively). The average peak disease score was however significantly higher in animals treated with IC87114 when compared with the vehicle control-treated animals (Figure 5.12D). In addition, there were high levels of animal morbidity in both the IC87114 and vehicle control-treated groups; this is has not been previously observed in other EAE experiments performed in the laboratory in which this work was conducted. Kaplan-Meyer survival charts show that the rate of death in the IC87114-treated group was much higher than that observed in the vehicle control-treated animals (Figure 5.12E). The reason for this is as yet unclear. However, these data indicate that IC87114 treatment from the time of EAE immunisation (under these conditions) does not reduce EAE pathogenesis.

### 5.5.2 Therapeutic dosing of IC87114 to mice immunised with MOG\textsubscript{35-55}

In addition to preventative dosing experiments, IC87114 treatment following EAE disease onset was also performed to assess the potential of this inhibitor to reduce disease in animals showing physical disease symptoms. Mice were immunised and EAE was allowed to develop. Two days following EAE disease onset (where animals had a clinical disease score of 2 or higher) twice-daily oral gavage administration of IC87114 or the vehicle control was commenced. It was observed that there was no difference between the EAE disease scores observed in IC87114 treated mice when compared with vehicle control-treated mice (Figure 5.13A). In addition, there was no difference in the peak disease scores observed in the two cohorts (Figure 5.13B). While there was some morbidity observed in animals in both treatment groups it was not as distinct as that observed with the preventative dosing strategy (Figure 5.13C). These data show that, at least under the conditions tested and with this dosing strategy, therapeutic dosing of mice with IC87114 does not reduce EAE pathogenesis.
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5.6 IC87114 TREATMENT IN VIVO DOES NOT AFFECT THE EX VIVO PROLIFERATION OF CFA-ACTIVATED CD4⁺ T CELLS OR B220⁺ B CELLS

The initial studies described in section 5.4 demonstrate that IC87114 treatment in vivo results in reduced ex vivo proliferative capacity of naïve CD4⁺ T cells (section 5.4.1). However, during EAE, the immune system is activated by MOG₃₅-₅₅ emulsified in CFA. Due to the lack of reduction in EAE pathogenesis following IC87114 administration in both preventative and therapeutic dosing strategies, the ability of IC87114 treatment to inhibit ex vivo proliferation of both CFA-activated CD4⁺ T cells and B220⁺ B cells was examined.

5.6.1 IC87114 treatment in vivo does not reduce ex vivo proliferation of CFA-activated CD4⁺ T cells

To assess whether IC87114 treatment in vivo can reduce the proliferation of activated CD4⁺ T cells ex vivo, mice were immunised with CFA in the hind flanks and scruff of the neck (section 2.2.4) and treated with either IC87114 or the vehicle control for 4 days in the mornings and evenings. On the final day, IC87114 or vehicle control were administered in the morning 30 minutes before draining inguinal and brachial lymph nodes were extracted, and cells were stained with CFSE and cultured with anti-CD3/anti-CD28 as described in section 2.3.4.4. Following the four day culture period, cell proliferation was assessed by comparing experimental samples with cells that were incubated in the presence of culture media alone (cells only negative controls). There was no difference in the levels of proliferation observed in cells isolated from mice that had received IC87114 in vivo when compared with cells isolated from vehicle control-treated animals (Figure 5.14A). As a control, cells from un-treated animals were cultured in the presence of IC87114 or a diluent control. As observed previously with naïve T cells (Figure 5.10A), in vitro treatment of CFA-activated CD4⁺ T cells with IC87114 reduced their proliferative capacity when compared with diluent controls (Figure 5.14B). Therefore, IC87114 treatment in vivo does not reduce the proliferative capacity of CD4⁺ T cells that have been previously activated as a result of CFA immunisation.
5.6.2 In vivo IC87114 treatment does not reduce the ex vivo proliferative capacity of CFA-activated B220⁺ cells

B cells are also important for EAE pathogenesis (115, 127, 188-191, 226-231) and it has been clearly shown that p110δ is integral for efficient B cell function (238-241, 243, 244, 247, 372, 398, 401, 432, 459, 507). Therefore, the ability of p110δ-inhibited B220⁺ B cells to proliferate in response to stimulation with PHA following CFA-activation in vivo was assessed. Mice were treated with IC87114 or the vehicle control for 4 days following CFA immunisation and then single cell suspensions were generated as described in section 2.3.1.4. Cells were labelled with CFSE and cultured for 4 days in the presence of PHA (section 2.3.4.5). At the end of the culture period, division of B220⁺ cells was assessed by flow cytometry (section 2.3.2.2). It was observed that there was no difference in levels of proliferation of B220⁺ cells from mice treated with IC87114 in vivo when compared to cells from vehicle control treated animals (Figure 5.15A). When cells from CFA-immunised mice were cultured in the presence of IC87114 in vitro there was a significant reduction in the ability of B220⁺ cells to proliferate in response to PHA stimulation when compared with diluent-treated controls (Figure 5.15B). These data demonstrate that in vivo treatment of CFA-immunised mice with the p110δ inhibitor IC87114 does not lead to a reduction in the ability of B cells to proliferate when stimulated with PHA ex vivo.

5.7 SUMMARY

In chapter 4 of this thesis it was demonstrated that genetic inactivation of p110δ results in reduced EAE pathogenesis. IC87114 is a highly specific inhibitor of p110δ that has no known off-target effects on other protein kinases and has been demonstrated to significantly inhibit p110δ function in vitro (238, 240, 359, 360, 362-366, 368-372, 458). It has also been shown to reduce pathology in a model of RA (370). Due to this, the ability of IC87114 to reduce activation and differentiation of Th1 and Th17 cells in vitro, as well as to influence EAE pathogenesis in vivo, was examined. The results presented in this chapter demonstrate that IC87114 is a potent inhibitor of Th1 and Th17 differentiation in vitro. However Th17, more so than Th1, cells appear to be highly reliant on p110δ for efficient differentiation and cytokine production. IC87114 was also observed to inhibit CD4⁺ T cell and B220⁺ B cell
proliferation \textit{in vitro}. Even though T cell activation \textit{in vivo} is reduced when p110δ is genetically inactivated (chapter 4), IC87114 has no effect on DC migration, activation and antigen processing and presentation. This is consistent with that observed in the p110δ$^{D910A/D910A}$ mice. Despite the finding that IC87114 reduces Th1 and Th17 differentiation \textit{in vitro}, and despite that observed in the p110δ$^{D910A/D910A}$ mice, IC87114 administration to mice did not affect EAE pathogenesis. There are a number of potential reasons for this observation. IC87114 is known to have a short half-life \textit{in vivo} and the adopted IC87114 dosing strategy may have been inefficient, or the doses of MOG$_{35-55}$ and CFA used to induce EAE may have activated the immune system with such potency that inhibition of p110δ did not affect the disease pathogenesis. Also, the inhibitor may be blocking function of cells such as regulatory T and B cells, both of which play an important role in negatively regulating autoimmunity. As the effects of IC87114 \textit{in vivo} are only beginning to be investigated, it is possible that there are as yet unknown effects on many different cell types which are responsible for EAE pathology. This is discussed further in chapter 6 of this thesis and future studies will endeavour to address these issues.
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Figure 5.1: Inhibition of Th1-type cell differentiation and IFN-γ production and secretion by LY294002.

Lymphocytes were isolated from the spleen of naïve wild-type C57BL/6 mice (section 2.3.1.4) and cultured for four days in Th1-skewing culture conditions (section 2.3.4.6) in the presence of 2.5μM, 5μM or 100μM LY294002 or a diluent control (section 2.1.4.1). Cells were then stained with antibodies against surface CD4 and intracellular IFN-γ and IL-17. (A) The proportion of Th1 (CD4⁺ cells that are IFN-γ⁺/IL-17⁺) cells that were isolated from the cultures was determined by flow cytometric analysis (sections 2.3.2.2 and 2.3.2.3). (B) Levels of IFN-γ in the supernatant of cultures were also analysed by ELISA (section 2.3.6.2). Representative flow cytometry plots are also shown. Data are mean ± SEM and are representative of three independent experiments (n = 3).
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![Graph A]}

**% Th1 cells**

- DMSO
- 2.5 μM
- 5 μM
- 100 μM

**LY294002 concentration**

![Graph B]}

**IFNγ (pg/ml)**

- DMSO
- 2.5 μM
- 5 μM
- 100 μM

**LY294002 concentration**
CHAPTER 5: The efficacy of the p110δ inhibitor IC87114 as a therapeutic for EAE

Figure 5.2: Inhibition of Th1-type cell differentiation and IFN-γ production and secretion by the p110δ inhibitor IC87114.

Lymphocytes were isolated from the spleen of naïve wild-type C57BL/6 mice (section 2.3.1.4) and cultured for four days in Th1-skewing culture conditions (section 2.3.4.6) in the presence of 1μM, 5μM or 10μM IC87114 or a diluent control (section 2.1.4.2). Cells were then stained with antibodies against surface CD4 and intracellular IFN-γ and IL-17. (A) The proportion of Th1 (CD4⁺ cells that are IFN-γ⁺/IL-17⁻) cells that were isolated from the cultures was determined by flow cytometric analysis (sections 2.3.2.2 and 2.3.2.3). (B) Levels of IFN-γ in the supernatant of cultures were also analysed by ELISA (section 2.3.6.2). Representative flow cytometry plots are also shown. Data are mean ± SEM and are representative of three independent experiments (n = 3).
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A

% Th1 cells

DMSO 1μM 5μM 10μM

IC87114 concentration

* *

IFNγ (pg/ml)

DMSO 1μM 5μM 10μM

IC87114 concentration

B

IFNγ (pg/ml)

DMSO 1μM 5μM 10μM

IC87114 concentration

** **

IL-17
Figure 5.3: Inhibition of Th17-type cell differentiation and IL-17 production and secretion by LY294002.

Lymphocytes were isolated from the spleen of naïve wild-type C57BL/6 mice (section 2.3.1.4) and cultured for four days in Th17-skewing culture conditions (section 2.3.4.7) in the presence of 2.5μM, 5μM or 100μM LY294002 or a diluent control (section 2.1.4.1). Cells were then stained with antibodies against surface CD4 and intracellular IFN-γ and IL-17. (A) The proportion of Th17 (CD4+ cells that are IFN-γ/IL-17+) cells that were isolated from the cultures was determined by flow cytometric analysis (sections 2.3.2.2 and 2.3.2.3). (B) Levels of IL-17 in the supernatant of cultures were also analysed by ELISA (section 2.3.6.2). Representative flow cytometry plots are also shown. Data are mean ± SEM and are representative of three independent experiments (n = 3).
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A

% Th17 cells

LY294002 concentration

DMSO 2.5μM 5μM 100μM

% Th17 cells

IL-17A (pg/ml)

LY294002 concentration

DMSO 2.5μM 5μM 100μM

IL-17A (pg/ml)

B

---

---
Figure 5.4: Inhibition of Th17-type cell differentiation and IL-17 production and secretion by the p110δ inhibitor IC87114.

Lymphocytes were isolated from the spleen of naïve wild-type C57BL/6 mice (section 2.3.1.4) and cultured for four days in Th17-skewing culture conditions (section 2.3.4.7) in the presence of 1μM, 5μM or 10μM IC87114 or a diluent control (section 2.1.4.2). Cells were then stained with antibodies against surface CD4 and intracellular IFN-γ and IL-17. (A) The proportion of Th17 (CD4+ cells that are IFN-γ/IL-17+) cells that were isolated from the cultures was determined by flow cytometric analysis (sections 2.3.2.2 and 2.3.2.3). (B) Levels of IL-17 in the supernatant of cultures were also analysed by ELISA (section 2.3.6.2). Representative flow cytometry plots are also shown. Data are mean ± SEM and are representative of three independent experiments (n = 3).
CHAPTER 5: The efficacy of the p110δ inhibitor IC87114 as a therapeutic for EAE

A

% Th17 cells

IC87114 concentration

DMSO 1μM 5μM 10μM

B

IL-17 (pg/ml)

IC87114 concentration

DMSO 1μM 5μM 10μM
Figure 5.5: IC87114 does not affect BMDC migration towards CCL19 in vitro. 

*In vitro* transwell chemotaxis assays were used to investigate the capacity of p110δ-inactivated mature BMDCs to migrate towards the chemokine CCL19. Bone-marrow cells were isolated from the femurs of naïve wild-type C57BL/6 mice (section 2.3.1.7) and were cultured for 11 days to generate mature DCs (section 2.3.4.3). Mature DCs, which were pre-treated with either IC87114 or diluent, were subjected to *in vitro* transwell chemotaxis assays as described in section 2.3.7.2. Data are mean ± SEM (n = 4).
Figure 5.6: P110δ inhibition does not affect antigen uptake and presentation by dendritic cells.

The ability of DCs lacking p110δ function was examined by pulsing BMDCs with OVA in the presence of the p110δ inhibitor, IC87114 (10μM - purple bars), or diluent (DMSO) control (pale green bars) before washing and incubating with CFSE-labelled OT-II lymphocytes (section 2.3.8). Four days later proliferation of CD4+ OT-II T cells was determined by flow cytometry (sections 2.3.2.2 and 2.3.2.7). Data are mean ± SEM and are representative of three independent experiments (n = 4).
Figure 5.7: Functional p110δ is required for proliferation of OT-II CD4⁺ T cells in response to OVA-presentation by dendritic cells.

Immature BMDCs were pulsed with OVA, washed and then incubated with OT-II cells that had been pre-treated with IC87114 (purple bars) or diluent (DMSO) control (pale green bars) (section 2.3.8). Four days later cell division of CD4⁺ OT-II cells was assessed by flow cytometry (sections 2.3.2.2 and 2.3.2.7). Data are mean ± SEM and are representative of three independent experiments (n = 4).
Figure 5.8: IC87114 is detectable in plasma following oral gavage.
Mice were administered IC87114 at a concentration of 30mg/kg via oral gavage (section 2.2.5). One, two and four hours later mice were euthanased and plasma was collected as described in section 2.3.1.3. Samples were sent to Calistoga Pharmaceuticals where GC-MS analysis was carried out to determine levels of IC87114 in the plasma. Data are mean ± SEM (n = 4).
Figure 5.9: IC87114 treatment in vivo does not affect DC activation

Mice were immunised at the scruff of the neck and in the hind flank with CFA and treated morning and evening with IC87114 or the vehicle control (section 2.2.5). Forty-eight hours after CFA immunisation mice were euthanased and draining lymph nodes were removed and treated with collagenase (section 2.3.1.4) before single cell suspensions were stained with anti-CD11c, anti-MHC II and anti-CD86 antibodies and analysed by flow cytometry (section 2.3.2.2). (A) Proportions of CD11c^+ cells isolated from the lymph nodes of IC87114 treated mice (purple bar) and vehicle control-treated animals (green bar) are shown. (B) The proportion of CD11c^+ cells from IC87114 treated (purple bars) and vehicle control-treated (dark green bars) mice that were either MHC II^+ or CD86^+ in is shown. Data are mean ± SEM (n = 6).
CHAPTEER 5: The efﬁcacy of the p110δ inhibitor IC87114 as a therapeutic for EAE

A

% cells CD11c+

Vehicle control IC87114

B

% CD11c+ cells positive for CD86 or MHC II

CD86 MHC II

Vehicle control IC87114
Figure 5.10: IC87114 treatment results in reduced *ex vivo* proliferation of naïve T cells.

(A) Splenocytes from naïve wild-type C57BL/6 mice were stained with CFSE, treated with IC87114 (purple bar) or diluent control (pale green bar) and subjected to stimulation with anti-CD3 and anti-CD28 for four days (section 2.3.4.4). Proliferation was determined by a progressive halving of CFSE fluorescence intensity by flow cytometry. (B) Mice were administered 30mg/kg of IC87114 (purple bar) or vehicle alone (dark green bar) via oral gavage in the morning and evening for one day and the morning of the next day before being euthanased one hour later (section 2.2.5). CD4\(^+\) T cell proliferation in response to anti-CD3/anti-CD28 stimulation was then assessed. Data are mean ± SEM and are representative of three independent experiments (n = 6).
CHAPTER 5: The efficacy of the p110δ inhibitor IC87114 as a therapeutic for EAE

A

% CD4+ cells divided

\[ \begin{array}{c}
\text{ Cells only } \\
\text{ αCD3/αCD28 }
\end{array} \]

DMSO

IC87114

* p < 0.05

B

% CD4+ cells divided

\[ \begin{array}{c}
\text{ Cells only } \\
\text{ αCD3/αCD28 }
\end{array} \]

Vehicle control

IC87114

* p < 0.05
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Figure 5.11: GC-MS analysis of IC87114 levels in the plasma of mice throughout the preventative EAE study. Mice were treated twice daily (approximately 9am and 5pm) with 30mg/kg of IC87114 or the vehicle control from the day before EAE immunisation (section 2.2.5). Throughout this study plasma samples were obtained from animals (section 2.3.1.3) and analysed using GC-MS by Calistoga Pharmaceuticals (section 2.3.9). The samples shown in this graph represent the μM concentration of IC87114 in the bloodstream at the indicated time-points. (Data represent the mean μM concentration ± SEM, n = 3-4 samples per point). In addition to the different post-dose time-points, the samples were taken on different days post-immunisation for EAE. These days were as follows: the 2 hours post-dose sample was taken on day 1 post-immunisation (n = 3), the 3 hours post-dose sample was taken on day 31 post-immunisation (n = 4), the 5 hours post-dose sample was taken on day 4 post-immunisation (n = 3) and the 12 hours post-dose sample was taken on day 31 post-immunisation (n = 3).
Figure 5.12: IC87114 treatment of EAE-immunised mice with a ‘preventative’ dosing strategy.

Mice were immunised with MOG_{35-55} and CFA as described in section 2.2.3. One day prior to immunisation, treatment with either the p110δ inhibitor IC87114 or vehicle control was begun and mice were administered with either treatment daily as described in section 2.2.5. (A) The mean clinical disease scores over time-course of disease. (B) Day of onset, (C) day of peak disease and (D) peak disease score. All data are representative of mean ± SEM (n = 14 vehicle control-treated mice, 16 IC87114 treated mice, except at time points where animals had died). (E) Kaplan-Meyer plots showing the percent survival of mice in each group following immunisation.
CHAPTER 5: The efficacy of the p110δ inhibitor IC87114 as a therapeutic for EAE

A

**Graph A:**
- **Y-axis:** EAE disease score
- **X-axis:** Day post-immunisation

**Legends:**
- Vehicle Control
- IC87114

B

**Graph B:**
- **Y-axis:** Day post-immunisation
- **X-axis:** Vehicle control vs. IC87114
- **Graph Description:**
  - **Day of onset**
  - **Bars:** Vehicle control vs. IC87114
  - **Day post-immunisation**

C

**Graph C:**
- **Y-axis:** Day post-immunisation
- **X-axis:** Vehicle control vs. IC87114
- **Graph Description:**
  - **Peak disease day**
  - **Bars:** Vehicle control vs. IC87114
  - **Day post-immunisation**

D

**Graph D:**
- **Y-axis:** EAE disease score
- **X-axis:** Vehicle control vs. IC87114
- **Graph Description:**
  - **Peak disease score**
  - **Bars:** Vehicle control vs. IC87114
  - **EAE disease score**

E

**Graph E:**
- **Y-axis:** % survival
- **X-axis:** Day post-immunisation
- **Legend:**
  - Vehicle control
  - IC87114
  - **Graph Description:**
  - **% survival**
  - **Day post-immunisation**

---

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Figure 5.13: IC87114 treatment of EAE-immunised mice with a ‘therapeutic’ dosing strategy
Mice were immunised with MOG\textsubscript{35-55} and CFA as described in section 2.2.3. On the second day after individual animals began showing signs of clinical disease (scores of 1.5-2.5), animals were treated with either IC87114 or vehicle control as described in section 2.2.5. (A) The mean clinical disease scores over time post-disease onset. The arrow indicates the day on which IC87114 or vehicle treatment was begun. (B) Peak disease score. All data are representative of mean ± SEM (n = 14 vehicle control-treated mice, 16 IC87114 treated mice, except at time-points where animals had died). (C) Kaplan-Meyer plots showing the percent survival of mice in each group following disease onset.
CHAPTER 5: The efficacy of the p110δ inhibitor IC87114 as a therapeutic for EAE

A

![Graph showing EAE disease score over time](image)

B

![Bar chart showing peak disease score](image)

C

![Graph showing % survival over time](image)
Figure 5.14: IC87114 treatment in vivo does not result in reduced ex vivo proliferation of CFA-activated CD4+ T cells.

(A) Mice were immunised with CFA and administered 30mg/kg of IC87114 or vehicle for four days as described in section 2.2.5. On the last morning mice were administered IC87114 or vehicle 30 minutes before being euthanased. Cells from the draining inguinal and brachial lymph nodes were then stained with CFSE and subjected to stimulation with anti-CD3 and anti-CD28 for four days (section 2.3.4.4). Proliferation of CD4+ cells was determined by a progressive halving of CFSE fluorescence intensity by flow cytometry. (B) Proliferation of CD4+ T cells that were treated with IC87114 or diluent control in vitro. Data are mean ± SEM (n = 6).
CHAPTER 5: The efficacy of the p110δ inhibitor IC87114 as a therapeutic for EAE

A

% CD4+ cells divided

Vehicle control
IC87114

Cells only αCD3/αCD28

B

% CD4+ cells divided

DMSO IC87114

Cells only αCD3/αCD28

***
Figure 5.15: IC87114 treatment in vivo does not result in reduced ex vivo proliferation of CFA-activated B220⁺ B cells.

(A) Mice were immunised with CFA and administered 30mg/kg of IC87114 or vehicle for four days as described in section 2.2.5. On the last morning mice were administered IC87114 or vehicle 30 minutes before being euthanased. Cells from the draining inguinal and brachial lymph nodes were then stained with CFSE and subjected to stimulation with anti-CD3 and anti-CD28 for four days (section 2.3.4.4). Proliferation of B220⁺ cells was determined by a progressive halving of CFSE fluorescence intensity by flow cytometry. (B) Proliferation of B220⁺ T cells that were treated with IC87114 or diluent control in vitro. Data are mean ± SEM (n = 6).
CHAPTER 5: The efficacy of the p110δ inhibitor IC87114 as a therapeutic for EAE

A

% B220+ cells divided

Vehicle control
IC87114

Cells only
PHA

0
2
4
6
8
10
12

B

% B220+ cells divided

DMSO
IC87114

Cells only
PHA

0
1
2
3
4
5
6
7
8

***
CHAPTER 6

Discussion
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The importance of the class IA PI3K catalytic subunit p110δ in the activation, differentiation and function of many different cell types has become increasingly apparent in recent years. Not only has p110δ been implicated in the activation, development and/or function of B cells (238-241, 243, 244, 248, 372, 398, 401, 432, 459), T cells (248, 249, 381, 464), mast cells (362, 363), NK cells (380, 434, 461, 462) and neutrophils (359, 360, 367-370), but inactivation of p110δ has also been demonstrated to be directly linked with a reduction in pathogenesis in a model for RA (370). However, while p110δ has been directly implicated in the function of these cell types, apart from one study in autoantibody-induced arthritis the effects of p110δ inactivation in models of autoimmune disease \textit{in vivo} are unexplored. Due to the specific role that p110δ plays in cells of haematopoietic origin (260), and the role that these cells play in autoimmune diseases such as MS and RA (29, 33, 115, 123, 127, 150, 151, 155, 156, 158, 159, 162, 164, 168-180, 182-185, 187-191, 404), it is possible that pharmacological inactivation of this protein may provide relief for people suffering from autoimmune pathologies.

6.1 KEY FINDINGS
The main aim of this study was to determine whether p110δ plays a role in experimental autoimmune encephalomyelitis, a model for the human autoimmune disease MS. This has been addressed by investigating the effect of genetic inactivation of p110δ on EAE. In addition, the ability of the highly selective p110δ inhibitor IC87114 to inhibit EAE was also investigated. The key findings of this study are:

- There were no gross differences between naïve p110δ\textsuperscript{D910A/D910A} mice and wild-type C57BL/6 animals in regards to the proportion of T and B cells in the lymph nodes, nor was there a difference in levels of adhesion molecules and homeostatic chemokine receptors on the surface of T cells from p110δ\textsuperscript{D910A/D910A} mice. However, T and B cells from p110δ\textsuperscript{D910A/D910A} mice were less capable of migrating towards homeostatic chemokines \textit{in vitro}.  

The p110δ\textsuperscript{D910A/D910A} mice showed significantly reduced EAE disease when compared with wild-type mice and this was characterised by fewer infiltrating leukocytes and lesions in the CNS of those animals.

p110δ\textsuperscript{D910A/D910A} mice had lower levels of activated CD4\textsuperscript{+} T cells in their draining lymph nodes and reduced proliferation of CD4\textsuperscript{+} T cells \textit{ex vivo} and \textit{in vivo} following EAE immunisation, indicating that p110δ inactivation significantly reduces T cell priming.

B cell function was also reduced in p110δ\textsuperscript{D910A/D910A} mice as demonstrated by a lack of detectable MOG-specific IgG in the serum, and an absence of B cells in the CNS of these animals.

Survival of both T and B cells was reduced at early EAE time-points when p110δ was genetically inactivated.

The differentiation of CD4\textsuperscript{+} T cells into the Th1- and Th17-type was significantly reduced when p110δ was inactivated (either genetically or pharmacologically) \textit{in vitro}.

Genetic inactivation of p110δ resulted in an EAE disease course that is biased towards the Th1-type due to a defect in Th17 cell generation.

p110δ inactivation did not affect dendritic cell migration, activation or antigen processing and presentation.

While the p110δ inhibitor IC87114 significantly reduced Th1 and Th17 differentiation \textit{in vitro}, as well as proliferation of naïve T cells \textit{ex vivo}, \textit{in vivo} administration of IC87114 did not reduce EAE pathogenesis with the dosing strategy that was performed in this study.
The following discussion will further address the major findings of this study, with a particular focus on how this study adds new insights to current paradigms in the field. Possible future directions for this study will also be discussed.

6.2 IMMUNE HOMEOSTASIS IN p110δ<sup>D910A/D910A</sup> MICE

An initial characterisation of the p110δ<sup>D910A/D910A</sup> mouse was undertaken for several reasons. Those included that they were a strain of mice that hadn’t previously been used in this laboratory and were being bred in a new animal house with potentially different microflora, and little documented information existed regarding basic immune homeostasis in these animals. The p110δ<sup>D910A/D910A</sup> mice were successfully bred and births occur at expected Mendelian ratios. The proportion of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and B220<sup>+</sup> B cells in the spleen was determined using flow cytometry. There was no difference in the proportion of CD4<sup>+</sup> or CD8<sup>+</sup> T cells, nor of B220<sup>+</sup> B cells, in the spleens of p110δ<sup>D910A/D910A</sup> mice when compared with wild-type animals.

Further characterisation of surface markers on splenic lymphocytes was carried out using flow cytometry. Homeostatic chemokine receptors CCR7 and CXCR4 are important for lymphocyte homing to designated microenvironments of secondary lymphoid organs (508). CCR7 and CXCR4 expression on the surface of CD4<sup>+</sup> T cells was the same in p110δ<sup>D910A/D910A</sup> mice and wild-type C57BL/6 mice. Cells were also screened for surface expression of α4 and β1 integrins (CD49d and CD29 respectively) as these molecules are involved in the adhesion of cells to various cell adhesion molecules such as VCAM-1 and MadCAM as well as fibronectin (509). Splenic lymphocytes from naïve p110δ<sup>D910A/D910A</sup> mice had the same level of both α4 and β1 integrins on their cell surface as did wild-type mice. These results are expected as it has been previously demonstrated that there is no reduction in the ability of anti-CD3-induced binding of p110δ<sup>D910A/D910A</sup> cells to fibronectin via β1 integrins (248). However, they also further demonstrate that there are not likely to be major defects in cell adhesion processes mediated by these molecules in the p110δ<sup>D910A/D910A</sup> mouse. It should be noted that there are other adhesion molecules that govern cell trafficking and several studies have reported reduced trafficking of p110δ-inactivated cells in vivo (241, 244, 248, 369, 370, 432, 434, 441). However, taking all of these data into account, it appears that genetic inactivation of p110δ
does not overtly affect homeostasis of the immune system, at least with respect to the parameters assessed.

6.3 MIGRATION OF NAÏVE p110δ-INACTIVATED LYMPHOCYTES IN RESPONSE TO HOMEOSTATIC CHEMOKINES

In vitro migration of both B cells towards CXCL13 and T cells towards CCL19 and CCL21 was reduced in cells that had catalytically inactive p110δ. Chemotaxis is generally thought to be induced via chemokines binding to G-protein-coupled receptors, thereby inducing signalling through the class IB PI3Kγ (3, 8, 253). However, increasing evidence is emerging to suggest that chemotaxis of B cells, T cells, NK cells and neutrophils may require p110δ for efficient activation of signalling events required for this response (241, 244, 248, 362, 363, 367-370, 432, 434, 441). It has previously been demonstrated that B cells require functional p110δ to effectively migrate towards CXCL13 in vitro (432) and CXCL13 has also been demonstrated to be important for EAE progression (140). However, it had not previously been shown that p110δ plays a role in T cell migration in vitro. In fact, the data presented in the present study contradicts previous reports in which T cell migration in response to the chemokines CXCL10, CXCL12, CCL5, CCL19 and CCL21 was not affected by loss of p110δ (432, 441). Although reasons for the differing outcomes are not clear, the studies were not performed in an identical manner; Reif and colleagues used cells from p110δ−/− mice (432), so differences between the p110δ-inactivation systems may account for the disparate results observed in this study, particularly since an up-regulation of other catalytic and regulatory subunits has been observed in animals in which other class IA subunits have been genetically deleted (373) and it is possible that this also occurs in p110δ−/− mice. Such compensation may rescue some of the lost p110δ function. Also, Jarmin and colleagues performed T cell migration assays towards CXCL10, CXCL12 and CCL5, which were not tested in the present study (441). However, it has been demonstrated that T cell receptor-induced p110δ activity is important for the efficient trafficking of activated T cells to antigenic tissue in vivo (441). Therefore, the results observed in the present work, whilst being converse to previous reports, could
indicate that p110δ plays a more important role in T cell migration than previously thought.

6.4 THE ROLE OF p110δ IN EAE PATHOGENESIS

In the present study, EAE was induced in gene targeted mice which express a kinase-dead form of p110δ (p110δ<sup>D910A/D910A</sup> mice) and it was observed that these animals developed significantly less severe disease pathologies compared with wild-type counterparts. While the three independent experiments presented show slightly different disease kinetics, it is clear that the physical symptoms of EAE are reduced in p110δ<sup>D910A/D910A</sup> mice, particularly at later disease time-points. This difference was dramatic; the wild-type animals displayed average disease scores of around 2.5-3, indicating at least partial, if not complete, hind-limb paralysis, whereas the p110δ<sup>D910A/D910A</sup> mice developed average disease scores between 1-1.5, indicating only a partially flaccid tail. Therefore, the reduction in EAE pathogenesis observed in p110δ<sup>D910A/D910A</sup> mice represents a significant inhibition of clinical manifestations of the disease. No significant differences in the day of onset or day of peak disease were observed. However, the average peak disease scores were significantly lower in p110δ<sup>D910A/D910A</sup> mice. Further investigation into CNS pathology by immunostaining of spinal cord cross-sections showed that p110δ<sup>D910A/D910A</sup> mice had a reduced number of lesions and fewer infiltrating CD45<sup>+</sup> leukocytes at late stages of EAE when compared with wild-type animals. The reasons for this were investigated in chapter 4 and are discussed in more detail below.

IC87114 is a highly specific inhibitor of p110δ that has no known off-target effects on other protein kinases and has been demonstrated to significantly inhibit p110δ function <i>in vitro</i> (238, 240, 359, 360, 362, 363, 365-370). It has also been shown to reduce pathology in a model of RA (370). Due to this, and the promising findings in the p110δ<sup>D910A/D910A</sup> mice, the ability of IC87114 to reduce EAE pathogenesis was examined. Unlike when p110δ was genetically inactivated, IC87114 treatment did not reduce EAE pathogenesis despite proving highly effective in inhibiting a number of relevant parameters <i>in vitro</i> and <i>ex vivo</i>. There are a number of possible explanations for this. In the p110δ<sup>D910A/D910A</sup> mice, p110δ has been genetically
inactivated in every cell, whereas the extent of the overall coverage of IC87114 \textit{in vivo}, and the level of inhibition of p110δ, has not yet been quantified. Furthermore, \( p110^\delta^{D910A/WT} \) mice, which express both wild-type and catalytically inactive forms of p110δ, do not display reduced EAE pathology, indicating that greater than 50% inhibition of p110δ is required to reduce EAE pathogenesis. The most likely explanation for the lack of efficacy of IC87114 is that the IC76114 dosing strategy used in this study was not optimal. For future research directions in regards to these issues, see section 6.9.1.

The present study has provided valuable and novel insight into the role of the p110δ protein in EAE by demonstrating that when p110δ is completely (genetically) inactivated, immune system activation and EAE pathology is reduced. It is therefore likely that therapeutically reducing p110δ function in MS patients could result in improved quality of life and reduced severity of relapses. However, further research, using different models of EAE, will be required to determine the efficacy of p110δ inhibition on different stages of MS pathology, such as during relapses and chronic stages of MS. The methods that may be undertaken to address this are discussed further in section 6.9.2.

To delineate the mechanism for the reduction in EAE pathogenesis observed in this study, the activation, survival and function of different cell types was investigated by observing the cellular consequences of p110δ inactivation, both \textit{in vitro} and \textit{in vivo}. These findings are discussed in the sections below.

\section*{6.5 THE EFFECT OF p110δ INACTIVATION ON CD4\(^{+}\) T CELL PRIMING, SURVIVAL AND DIFFERENTIATION}

CD4\(^{+}\) T cells have long been accepted to be the main cell-type responsible for driving MS/EAE pathology (29, 33, 115, 127, 150, 151, 155, 156, 158, 159, 162, 164, 175, 188-190). Following the observation that EAE was reduced in \( p110^\delta^{D910A/D910A} \) mice, the activation, differentiation and survival of CD4\(^{+}\) T cells in EAE-immunised, p110δ-inactive, animals was examined. It was observed that not only are there proportionally fewer T cells displaying markers of activation isolated

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from the draining lymph nodes of p110δ^{D910A/D910A} mice when compared with wild-type mice, but these T cells are also less capable of proliferating in response to antigen both _ex vivo_ and _in vivo_. These findings indicate that immune priming of T cells is not occurring efficiently in the p110δ^{D910A/D910A} mice, an outcome that may be due to inherent T cell defects or a disruption in antigen-presentation and subsequent T cell activation.

In the past, EAE was considered to be driven by Th1 cells secreting IFNγ. However, the recent discovery of a third major helper cell subclass that produce IL-17, called Th17 cells, has significantly added to the complexity of EAE (154, 159). It has now been shown that both Th1 and Th17 cells can drive EAE, but neither cell type can exclusively drive the pathogenic autoimmune response to the same extent without some participation from the other (154, 159). While it has been previously demonstrated that p110δ plays a role in Th1 differentiation _in vitro_ (249), the present study is the first to demonstrate that genetic or pharmacological inactivation of p110δ results in a reduction in both Th1 and Th17 differentiation _in vitro_ and that Th17-type cell production is reduced in p110δ^{D910A/D910A} mice throughout EAE. Moreover, it appears from these data that the generation of Th17 cells may be more reliant on p110δ than Th1 cells, as reflected by the more profound effect of p110δ inactivation on Th17 cell generation in the _in vitro_ skewing culture experiments. The reduction in EAE disease observed in p110δ^{D910A/D910A} mice can be attributed to ablated Th1 differentiation due to the autoimmune response being strongly skewed towards the less pathogenic Th1-type and away from the Th17-type when p110δ is inactive, whereas wild-type animals show an autoimmune response that is skewed towards the more pathogenic Th17-type. Functional p110δ was also necessary for cytokine secretion under both Th1- and Th17-skewing culture conditions. Cytokine production/secreation relies on processes such as up-regulation of transcription factors, protein synthesis and vesicle transport. PI3Ks have previously been directly and indirectly implicated in up-regulation of transcription factors as well as vesicle transport (510-514). As p110δ-inactivation resulted in reduced levels of IFN-γ and IL-17 in the supernatant of Th1- and Th17-skewed cultures respectively, it is possible that p110δ is also involved in this process in T cells. Together, this data
indicates that therapeutic intervention of p110δ signalling may prove to be beneficial in diseases where deregulation of Th17 immune responses is the underlying cause for pathology, such as in MS (34, 150, 151, 154, 156, 158, 159) and RA (30, 159, 199, 200). Despite this, Th17 differentiation appears to be governed by a number of different parameters in human versus mouse cells (29, 31-34, 63, 515-518). Therefore research on human samples will be necessary to further understand the role of this cell type in MS.

In addition to the reduced differentiation of Th1 and Th17 cells observed in the present study, p110δ was also shown to be important for the differentiation of regulatory T cells during EAE. While there was a higher proportion of Tregs in the draining lymph nodes of p110δ<sup>D910A/D910A</sup> mice at day 6 post-immunisation, by day 15 post-immunisation there was a significantly lower proportion of Tregs in the lymph nodes of p110δ<sup>D910A/D910A</sup> mice when compared with wild-type counterparts. Normally, Treg cells are responsible for the maintenance of peripheral tolerance and down-regulating the immune response, and therefore play an important role in preventing autoimmunity. The increased proportion of Tregs at day 6 post-immunisation may be contributing to regulation of the early autoimmune events in the p110δ<sup>D910A/D910A</sup> mice. However, a previous report has shown that Tregs from p110δ<sup>D910A/D910A</sup> mice are not capable of performing their suppressive function (464). Therefore it is likely that, in this model, Tregs may not be performing their effector function efficiently. Future work will need to be performed to confirm this. In addition, the reduction in T cell activation and differentiation may be sufficient to reduce EAE disease, despite the lower proportion of Tregs at peak disease, or the increased proportion of Tregs in the p110δ<sup>D910A/D910A</sup> mice prior to disease onset may be enough to confer some protection against the development of severe EAE. As it is probable that since a reduction in Treg function would result in the removal of one of the most important regulatory mechanisms in the immune system, therapies aimed at inhibiting p110δ would need to balance the importance of Treg function against the benefits of reducing Th17 function.
While it is clear that p110δ plays an important role in T cell differentiation and cytokine secretion, the mechanism remains to be elucidated. Previous reports indicate that p110δ has a significant role in TCR signalling (248, 249) therefore reduced signalling through the TCR is likely to be responsible at least in part for the reduction in T cell priming, survival and differentiation observed in this study. However, T cell differentiation is reliant on a number of different factors. In particular, inefficient signalling through cytokine receptors that initiate PI3Kδ intracellular signalling may be, at least in part, responsible for the reduced T cell differentiation observed. Future investigations into this theory may involve examining the TGF-β signalling pathway, as this cytokine has been demonstrated to drive differentiation of both Th17 and Treg cells (29, 519). In addition, intrinsic T cell pathways that involve p110δ may not only be responsible for reduced T cell differentiation. While the present study has not shown any differences in antigen processing and presentation by DCs, it has been previously reported that activated DCs lacking functional p110δ produce lower levels of IL-6 compared to wild-type DCs (463). Therefore, inactivation of p110δ may not only affect intrinsic T cell processes, but may reduce production of cytokines by APCs that are important for efficient T cell activation and differentiation. This is discussed further below (section 6.8).

In addition to the reductions in T cell activation and differentiation, p110δ-deficient CD4+ T cells undergo significantly higher levels of apoptosis during the priming phase of EAE. Primarily through its role in the activation of the pro-survival proteins Akt/PKB, p110δ has previously been demonstrated to be important for the survival of several different cell types (238, 239, 251, 416, 448). However, prior to the present study p110δ had not yet been implicated in the survival of primary, antigen-activated, T lymphocytes. As the immune system is controlled at many different levels (antigen processing and presentation, lymphocyte activation, differentiation and proliferation and a negative regulation of lymphocytes via apoptosis), the finding that p110δ is important for mature T cell survival has implications for our understanding of why the CD4+ T cell-driven autoimmune response is reduced in p110δ<sup>D910A/D910A</sup> mice. That is, not only is CD4+ T cell activation and differentiation
reduced, but these cells are also undergoing increased apoptosis. At this stage it is not possible to determine whether apoptosis is occurring due to inefficient T cell activation or if intrinsic defects in survival signals are resulting in higher levels of apoptosis, or both.

6.6 p110δ INACTIVATION AND B CELL FUNCTION

While T cells are generally accepted to be the predominant mediator of pathogenesis in EAE, B cells have also been unequivocally linked to MS/EAE pathology. This has primarily been through the observation of myelin-specific antibodies in the blood of MS patients (226-229), but also through studies in EAE models (127, 188-191, 230, 231). The role of p110δ in B cell function is also unambiguous; p110δ has been clearly shown to be required for efficient B cell development (239, 241, 244, 247, 248), trafficking (241, 244, 248, 432), BCR signalling (238-241, 243, 244, 248), cell-cycle regulation (398, 401), isotype class-switching (240, 372, 459), survival (238, 239) and T-dependent and -independent activation (239, 241, 244, 248) (see section 1.6). It was therefore hypothesised in the present study that B cell function would be reduced in EAE when p110δ was inactivated.

This study has shown that B cell function in EAE appears to be completely inhibited without p110δ. There were no detectable B cells in the CNS of p110δ

\[ D910A/D910A \] mice and there was no detectable MOG_{35-55}-specific IgG in the serum of p110δ

\[ D910A/D910A \] mice. This may be due to inefficient B cell activation, reduced B cell survival, inefficient trafficking of B cells to the CNS, or most likely a combination of all three.

The complete lack of detectable MOG_{35-55}-specific IgG in the serum of the p110δ

\[ D910A/D910A \] mice indicates that B cells are not being activated and/or class-switching after EAE immunisation. It has previously been demonstrated that B cells from p110δ

\[ D910A/D910A \] mice show uncontrolled class-switching to IgE and IgG1 under certain stimulatory conditions (240, 372, 507). The predominant immunoglobulin isoform usually observed in EAE is IgG1, so in view of the previous findings it is perhaps surprising that no antigen-specific IgG was detected in this study at all. However, B cells from p110δ

\[ D910A/D910A \] mice underwent higher levels of apoptosis than was observed in wild-type animals. Therefore, these results
indicate that B cells may not be generating IgG because they are either not being activated efficiently (and therefore do not reach a point where they undergo class-switching) or are undergoing apoptosis before they reach this point. Another reason for the lack of B cell activation may be the lack of observable T cell activation. An efficient B cell response to thymus-dependent antigens (such as that occurring in EAE) requires direct contact with Th cells and exposure to Th cell produced cytokines. Without this T cell help, B cell activation is reduced and isotype switching cannot occur. The reduction in T cell activation and cytokine production observed when p110δ is inactivated in the present study is therefore likely to also affect the activation of B cells. Therefore, together these findings indicate that, without functional p110δ, B cell responses are inefficient following EAE immunisation, not only due to reductions in p110δ-mediated BCR signalling (as has been previously reported (238-241, 243, 244, 248)) and increased B cell apoptosis (shown here and in previous studies (238, 239)) but possibly also due to reduced T cell help during B cell activation.

6.7 MACROPHAGES, NEUTROPHILS AND p110δ INACTIVATION

It has been reported previously that Th1- and Th17-driven EAE autoimmune responses result in a preferential recruitment of macrophages or neutrophils the CNS respectively (169). However, while it was clear that there was a reduction in the number of F4/80+ macrophages in the CNS of the p110δ<sup>D910A/D910A</sup> animals, on comparison to wild-type mice, there was no significant difference in the numbers of Ly6G+ neutrophils infiltrating the CNS of the p110δ<sup>D910A/D910A</sup> mice. Taking the previously published data into account, it could be assumed that p110δ<sup>D910A/D910A</sup> mice, which have a reduced Th17 response, would exhibit higher numbers of macrophages in the CNS, and that wild-type mice would have more neutrophils. However, the data described above indicate that p110δ may be more important for macrophage activation and/or migration than it was for neutrophil activation and entry to the CNS. Future work may involve focusing on the role of p110δ in this cell type.
6.8 DENDRITIC CELL FUNCTION AND p110δ INACTIVATION

CD4+ T cell activation requires presentation of antigen in the context of MHC class II by APCs such as DCs. As CD4+ T cell activation is so profoundly reduced in the p110δD910AD910A mice throughout EAE, the role of p110δ in DC trafficking, activation and function was investigated. Firstly, the capacity of DCs lacking functional p110δ to migrate to the chemokine CCL19 in vitro was assessed using DCs in which p110δ had been inactivated. CCL19 is expressed by interdigitating DCs and stromal cells in the T cell zones and assists in directing cells that express the chemokine receptor CCR7, such as naïve T cells and mature DCs, to these specific regions of lymph nodes (520). DC migration in vitro in response to CCL19 was not affected by either genetic or pharmacological inactivation of p110δ. In addition, DC migration from the skin to the draining lymph nodes in vivo was also not affected by p110δ-inactivation. Therefore, in contrast to p110γ (427), p110δ does not appear to play a role in DC trafficking.

DC activation and antigen processing and presentation were also shown to not be dependent on p110δ. The draining lymph nodes of CFA-immunised mice in which p110δ had been genetically or pharmacologically inactivated had the same proportions of CD11c+ DCs expressing the activation marker molecules CD86 or MHC II on their surface when compared with those isolated from control animals. As DC-mediated activation of T lymphocytes is integral for initiation of the autoimmune response in EAE, the ability of IC87114 to inhibit the DC-dependent activation and proliferation of T cells was investigated. It was observed that OVA-pulsed DCs that had been treated with IC87114 initiated equal levels of proliferation in OVA-specific T cells as did DCs from control-treated animals, indicating that p110δ inhibition in DCs does not affect their ability to process and present antigen. However, when non-treated, OVA-pulsed, DCs were cultured with IC87114 treated OT-II T cells there was a significant reduction in the ability of these T cells to proliferate, indicating that it is an intrinsic defect in T cell activation and proliferation caused by p110δ-inhibition that is responsible for reduced CD4+ OT-II T cell division. Taken together, these results indicate that p110δ-inhibition with IC87114 does not affect antigen processing and presentation by DCs, and that the
reduction in T cell activation in the p110δ<sup>D910A/D910A</sup> mice during EAE (which relies on antigen presentation by APCs) is probably due to intrinsic T cell defects as opposed to reduced DC function.

While it is clear that p110δ inactivation in DCs does not affect their ability to migrate in response to CCL19, to express activation markers or to process and present antigen, it has previously been reported that upon stimulation with cholera toxin <i>in vitro</i>, DCs from p110δ<sup>D910A/D910A</sup> mice do not secrete as much of the cytokine IL-6 as do wild-type mice (463). Lower levels of IL-6 secretion by mast cells with inactive p110δ has also been reported (362). Therefore, while it is unlikely that defects in the DC functions discussed above are responsible for the reduced T cell activation observed when p110δ was inactivated both genetically and pharmacologically in this study, reduced expression of IL-6 by these cells, as well as other cell types, may contribute to the impaired differentiation of cells when p110δ is inhibited <i>in vivo</i>. In particular, Th17 cells require stimulation with IL-6. Therefore, a reduction in IL-6 secretion by APCs and other leukocytes could play a role in the reduced levels of Th17 cells in the p110δ<sup>D910A/D910A</sup> mice. As the immune environment <i>in vivo</i> is highly complex and requires interaction between a variety of different cell types, future studies will need to delineate the effects of wide-spread p110δ inhibition so that any influence of p110δ-targeted therapy on immune biology can be fully understood.

6.9 FUTURE DIRECTIONS

This study has highlighted an important role for p110δ in the development of the autoimmune response in EAE. However, while the importance of p110δ in the efficient activation, function and survival of T and B cells was highlighted, only when p110δ was completely genetically inactivated was there a difference in EAE pathogenesis. While this study has firmly established a basis for investigating the use of p110δ inhibitors for therapeutic intervention of EAE and perhaps MS, future research must be undertaken to further delineate the role of p110δ in cells of the immune system and the efficacy of targeting p110δ as a therapeutic for autoimmunity. Initial studies should focus on three separate goals. While the p110δ
inhibitor IC87114 was demonstrated to be a potent inhibitor of T cell activation and differentiation \textit{in vitro}, it failed to reduce EAE pathogenesis \textit{in vivo}. It is possible that this may be improved with better coverage of the compound in mice, which will require that the dosing strategies used to administer the inhibitor are optimised. The present study was undertaken using a model of EAE that was induced with MOG\textsubscript{35-55} in mice on a C57BL/6 background, which results in a chronic disease course. It may be advantageous for future research to focus on the role of p110\(\delta\) in different EAE models. Lastly, before pharmacological targeting of p110\(\delta\) can be considered for humans, a thorough understanding of the effect of IC87114 on cells of the immune system must be achieved. All of these future directions are discussed further below.

6.9.1 IC87114 dosing strategy

Initial studies shown in chapter 5 of this thesis indicate that IC87114 treatment \textit{in vivo} reduces \textit{ex vivo} activation and proliferation of naïve CD4\(^+\) T cells. Inefficient activation of this cell type was demonstrated to be integral for the reduced EAE disease observed in p110\(\delta\)\textsuperscript{D910A/D910A} mice (chapter 4). Given the promising findings that IC87114 could reduce activation, proliferation and differentiation of CD4\(^+\) T cells, \textit{in vivo} administration of IC87114 to mice with EAE was undertaken. Both ‘preventative’ (where mice were administered IC87114 before EAE immunisation and throughout the disease course) and ‘therapeutic’ (where mice were administered IC87114 once they were showing clinical signs of disease and throughout the disease course) dosing strategies were trialled. However, there was no reduction in EAE pathogenesis observed with either dosing strategy. In fact, EAE pathogenesis appeared to be slightly enhanced in mice receiving IC87114 treatment when administered from the time of immunisation (i.e. ‘preventative’ dose). Despite these disappointing results, there are several avenues for future research that may be pursued to further investigate whether IC87114 may prove to have some therapeutic potential to reduce EAE.

To begin with, the \textit{in vivo} dosing regimen used in this study for IC87114 involved oral gavage of animals in the morning (9am) and afternoon (5pm) with 30mg/kg of IC87114 in vehicle. This regimen was suggested by Calistoga Pharmaceuticals, the
company which provided the compound for this study. From the in vitro studies presented in this chapter, it is evident that IC87114 can act as a potent inhibitor of p110δ function over four day culture periods. However, while GC-MS analysis of IC87114 levels in plasma throughout the preventative dosing EAE study generally displayed good coverage of the compound in the plasma of mice, at the 12 hour post-dose time-point it was evident that there was very little active compound in the plasma of these animals. This indicated that IC87114 is cleared quickly from the bloodstream of mice, in keeping with its known short half-life in vivo (Kamal Puri, Calistoga Pharmaceuticals, personal communication). As animals were administered IC87114 in the morning and evening of each day, the longest period between the dosing of the compound was typically 16-17 hours. It may be that during this time frame IC87114 was effectively cleared from the system of experimental animals and cell activation/function was allowed to progress. Randis and colleagues have used IC87114 during an in vivo model of rheumatoid arthritis and observed reduced disease pathology (370). In that study, IC87114 was used at 20mg/kg and administered three times daily at eight hour intervals. Therefore, while the same total daily amount of IC87114 was administered, it was done so at three different time-points as opposed to the two which were chosen for this study. It is therefore possible that altering the dosing regimen of IC87114 so that animals receive the compound three times daily may improve the overall coverage of the compound in the bloodstream and thereby have more of an effect on EAE disease.

6.9.2 Alternative EAE models

In this study, EAE was induced by immunising C57BL/6 (the background of the p110δD910A/D910A mice) mice with the MOG35-55 neuroantigen, which results in a progressive and chronic paralysis in animals. However, as discussed in the introduction of this thesis, there are several different models available for researching neuro-inflammation (section 1.3.3). One of these, induced by immunising SJL/J mice with the neuroantigen PLP139-151 (section 1.3.3.3), results in the development of an EAE disease course which more closely mimics RR-MS in that it is a remitting-relapsing disease course (120). Testing the effect of inhibition of p110δ during the relapse phase of EAE would be useful as this may lead to reduced immune cell
activation and trafficking to the CNS, thereby reducing the severity of relapses. This also has the added benefit of closely mimicking the way that p110δ-inhibition may therapeutically benefit MS patients. Future work may therefore elucidate the affects of IC87114 treatment on the progression of the relapse stage of EAE in this model. In addition, SJL/J mice are highly susceptible to EAE that is induced by adoptively transferring encephalitogenic cells, whereas C57BL/6 mice are not. Inducing adoptive EAE with either cells from p110δD910A/D910A mice that have been back-crossed on to a SJL/J background or in which encephalitogenic transferred cells have been inhibited with IC87114 may provide information on the role of p110δ in trafficking of activated cells to the CNS and the induction of CNS inflammation.

As well as using alternative EAE models, the cuprizone mediated model of demyelination, where mice are fed cuprizone in drinking water (which results in copper deficiency and the ablation of oligodendrocytes in the CNS), is another method of investigating neuro-inflammation (133). Cuprizone-mediated demyelination results from oligodendrocyte death and myelin is phagocytosed by microglia and peripheral macrophages, which therefore allows the study of demyelination within the CNS independently of myelin-specific cell-mediated immune responses. In addition, removal of cuprizone from the diet results in remyelination within the CNS. As p110δ has been shown to be expressed in the CNS (484), it will be important to observe whether inactivation of p110δ in CNS resident cells such as oligodendrocytes and microglia will affect CNS remyelination. This will be significant as loss of oligodendrocyte function, which could reduce remyelination, would not be a desirable outcome of p110δ inhibition that is otherwise intended to reduce the immune response.

In addition to using other models of neuroinflammation, it is possible that alteration of the immunisation protocol used in this study may lead to different experimental results. Here, EAE was induced by immunising mice with 100μg of the MOG35-55 neuroantigen, which results in the development of an ascending paralysis and a chronic and severe disease course whereby most animals experience complete hind-limb paralysis and some fore-limb paralysis. However, work that was ongoing in the
laboratory where this research was performed during the writing of this thesis has shown that if mice are immunised with reduced amounts of MOG\textsubscript{35-55} (as low as 25μg/mouse) they can still develop EAE that achieves a similar disease severity, albeit with a slightly delayed disease onset (Comerford, I., et. al., unpublished). In addition, it has been observed in this laboratory that immunising several gene knock-out strains of mice with lower doses of MOG\textsubscript{35-55} can afford a clearer distinction between differences in EAE pathogenesis in experimental cohorts. For example, in one strain, immunising with lower doses of MOG\textsubscript{35-55} has resulted in a complete lack of EAE disease developing in these animals, whereas wild-type mice still develop EAE with a similar severity as that observed when 100μg of MOG\textsubscript{35-55} is used for immunisation (S. McColl, I. Comerford, W. Litchfield, personal communication). These results are relevant as they highlight the potential importance of the immunisation method in the context of the p110δ inhibitor studies. It is possible that immunisation with 100μg of MOG\textsubscript{35-55} and CFA presents such a strong ongoing immunological challenge that it cannot be overcome by the inhibition of p110δ with IC87114. This is supported by the findings in chapter 5 that demonstrate that cells from naïve mice that were treated \textit{in vivo} with IC87114 did not proliferate in response to anti-CD3/anti-CD28 stimulation to the same level as vehicle control treated cells. However, when mice were immunised with CFA, thereby activating the immune system, IC87114 treatment \textit{in vivo} did not reduce the \textit{ex vivo} proliferation of CD\textsuperscript{4+} T cells or B220\textsuperscript{+} B cells. Therefore, activation of the immune system with CFA may be sufficient to override inhibitory affects of IC87114. Furthermore, whereas the p110δ\textsuperscript{D910A/D910A} animals have a complete genetic inhibition of p110δ function in every cell, IC87114 may only be capable of partially inhibiting p110δ function when administered \textit{in vivo} and may preferentially affect the function of p110δ in different cells (discussed more in 6.9.3). Therefore, IC87114 treatment may not cause sufficient inhibition of p110δ in relevant cells to allow the disease outcome to be significantly affected. Future studies may therefore focus on altering the dose of MOG\textsubscript{35-55}, and perhaps also CFA, used for initiating EAE and on determining whether this can affect the outcome of IC87114 administration to mice with the disease without compromising the EAE that develops in the control cohort.
6.9.3 p110δ attenuation and its impact on cells of the immune system

In addition to optimising both the IC87114 dosing strategy and the levels of MOG$_{35-55}$ required to induce disease in this model, it will be important to elucidate the effect of IC87114 on different cell populations in vivo. While IC87114 has already been demonstrated to inhibit neutrophil trafficking in vivo (368-370), there is minimal evidence in the literature reporting the consequences of p110δ inhibition by IC87114 on cells such as antigen-specific T and B cells and other non-antigen specific leukocytes in vivo. In addition, it is possible that IC87114 is inhibiting regulatory T as well as regulatory B cells, both of which have been implicated in the control and regulation of EAE (62, 191, 217-225). Studies into the differentiation and function of these cell types in p110δ-inhibited animals will be important to delineate the impact of p110δ inhibition on EAE progression, regulation and animal survival. Furthermore, it is known that the in vivo half-life of the p110δ inhibitor, IC87114, is higher in B cells than T cells (Kamal Puri, Calistoga Pharmaceuticals, personal communication). This may be an important distinction when considering targeting this protein to reduce pathologies such as autoimmune diseases. It will be important to determine the half-life of IC87114 in many immune cells, as well as the effects of p110δ inhibition on these cells, and tailor therapies towards diseases which are caused by the cell-types in which IC87114 inhibits p110δ and cellular function most effectively.

6.10 CONCLUDING REMARKS

Prior to this study, the role of p110δ in autoimmunity was not clear. Despite this, due to the fact that p110δ expression is largely limited to cells of the immune system and that these cells are responsible for a variety of different autoimmune pathologies, further research into the specific affect that p110δ inactivation could have on a model of autoimmunity was warranted. While future studies will be necessary, particularly into the efficacy of p110δ inhibitors, this study has provided novel insights into the importance of p110δ in immune cell function, and has established a basis for further research into targeting this protein as a therapeutic for pathologies such as autoimmune diseases.
CHAPTER 7

References


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487. Antonetti, D. A., P. Algenstaedt, and C. R. Kahn. 1996. Insulin receptor substrate 1 binds two novel splice variants of the regulatory subunit of


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CHAPTER 8

Appendix
Referee’s Comments and responses

1) p25, last sentence.
Please elaborate a little as to why lack of PTEN leads to diminished containment and clearance e.g indicate that this is thought to be due to lack of ability to prioritise movement to relevant chemoattractant cues.

The above sentence should read:
A lack of PTEN leads to diminished bacterial containment and clearance and reduced neutrophil-mediated arthritic inflammation respectively in these models (307). This is due to an inability of neutrophils to prioritise movement to relevant chemoattractant cues without the PTEN-mediated generation of a leading edge at the cell surface.

2) p27, para 1.
The candidate should state the key pharmacological difference between the modes of action of wortmannin and Ly294002 e.g covalent irreversible binding of wortmannin vs competitive/reversible action of Ly294002.

The first paragraph of section ‘1.4.4.1 Pan-PI3K inhibitors’ should read:
Two low-molecular-weight, cell-permeable pan-PI3K inhibitors, Wortmannin and LY294002, have been commercially available for a number of years and have enabled many initial studies into the function of PI3Ks (7, 339-343). These reagents have been important analytical tools for the development of the PI3K field and our current understanding of PI3K signalling. The chemical structures of Wortmannin and LY294002 are shown in Figures 1.9A and 1.9B respectively. Wortmannin binds covalently to PI3Ks whereas LY294002 binds in a competitive/reversible fashion. Both compounds potently inhibit class I PI3Ks at low concentrations by binding to the ATP binding pocket in the catalytic domain of the p110 subunits (344, 345). Wortmannin has a lower IC50 (Wortmannin IC50 = 4.2nM vs. LY294002 IC50 = 1.4μM) whereas LY294002 has a longer half-life and both have been used successfully, independently or in combination (344). It must be taken in to account
that both have off-target effects in that they both also inhibit the mammalian target of rapamycin (mTOR) and DNA-dependent protein kinase (DNA-PK). Wortmannin also inhibits ataxia telangiectasia mutated protein (ATM) and type II Proline-rich domain-containing inositol 5-phosphate kinases (PIPkins) α and β, whilst LY294002 can also inhibit casein kinase-2 (CK-2) (255, 346-350). However, if both Wortmannin and LY294002 are used at low concentrations (approximately 20-50nM and 10-100μM respectively) their specificity is largely restricted to PI3Ks.

3) p28, para. 1

Please elaborate a little on how the cited IC50 values for IC87114 were obtained e.g were the obtained using cell-based assays and if not, whether the values are accurate reflections of potency in cell-based assays. The IC50 values are a little different to ones I am familiar with, but the source reference is not immediately clear, so please clarify source of IC50 values.

The IC50 values mentioned in this paper are taken directly from the cited article (ref. 359: Sadhu, C., B. Masinovsky, K. Dick, C. G. Sowell, and D. E. Staunton. 2003. Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. J Immunol 170:2647-2654). The assay was performed in a cell-free system as described in the materials and methods section of this article. It is possible that this method of evaluating the IC50 for the IC87114 compound does not truly reflect the IC50 values that would be determined in cell-based assays. Despite this, it has been clearly demonstrated that, at similar concentrations to those used in our study, IC87114 can inhibit cell proliferation, migration, activation and function and reduce survival in several different cell types (238, 240, 359, 360, 362-372).

4) p30, para. 2

The candidate may want to cite recent papers concerning the distinct roles for p84 and p101 in mast cells (Bohnacker et al Science Signaling 2 (74) ra 27)

The abovementioned passage should read:
While there has been a plethora of research focusing on the class IA PI3K regulatory subunits, there has been less on the class IB regulatory subunits p101 and p84. The expression of both is limited mostly to cells of the immune system, however p84 is also expressed in cardiac tissue and has been demonstrated to be important for kinase-independent p110γ/PDE3B-mediated scaffolding in the heart (267, 270, 403, 404). Both subunits have been implicated in p110γ activation, and p101 over-expression has been demonstrated to enhance survival of T cells (405) as well as mast cell motility and activation (521). However, aside from this, very little is known about the specific function of p110 and p84.

The following reference is added:


5) p31, last sentence:
Other more relevant references should be included to support the role of PI3K gamma in T cell activation e.g Alcazar et al J Exp Med 2007 Nov 26; 204 (12):2977-87.

The abovementioned passage should read: PI3Kγ has also been implicated in the activation of T cells (252, 440), although its role in this respect is less well-defined.

4) p31, first para:
The candidate states that targeting of p110α and p110β in disease is unlikely to be therapeutically beneficial. However, several pharmaceutical companies are pursuing p110alpha as an anti-cancer target, so this statement needs to be revised accordingly.

The first paragraph of page 31 should read:
Despite the implication of both p110α and p110β in disease, due to their widespread expression and involvement in several critical cellular processes it will be difficult to therapeutically target these proteins without off-target affects. However, several pharmaceutical companies are currently developing p110α and p110β inhibitors, presumably with cell/tissue-specific targeting methods, as anti-cancer drugs. While this is promising, due to the more limited expression and function of the p110δ and p110γ catalytic PI3K subunits it may be more successful and less complicated to target these proteins as therapeutics for metastatic cancers and diseases where immune cells are implicated.

5) p74 and Fig 3.1 p107

On p74 (M&M) and legend for fig 3.1 (p107) the candidate refers to 500bp and 300bp products as denoting the heterozygous p110δ^{D910A/WT} mice while mice with only 500bp product represent the homozygous p110δ^{D910A/D910A} mice. However, in the figure on p107, the annotation indicates differently. Please clarify and correct accordingly.

The annotation on Figure 3.1A (p107) is incorrect. The band gel lane showing two bands (one at 500bp and one at 300bp) is representative of the p110δ^{D910A/WT} mice (not the p110δ^{D910A/D910A} mice) and the gel lane with only one band at 500bp is representative of the p110δ^{D910A/D910A} mice (not the p110δ^{D910A/WT} mice).

6) On p116, I am a little unclear as to what the candidate means by a cumulative disease score. Please clarify.

The cumulative disease scores were calculated by adding the scores in a cumulative fashion i.e. day 1 + day 2 + day 3 and so on. Therefore, for example, the ‘cumulative disease score’ shown at day 20 post-immunisation represents the sum total of the EAE disease scores from day 1-20.
7) p121, Fig 3.6
In the immunohistochemistry experiments it is not clear how the candidate defines a CD45+ lesion e.g. in fig 3.6 how many lesions are present in panel A? It would be useful to indicate individual lesions with arrows as in fig 4.6.

The lesions have been defined as areas where there are >10 CD45+ cells. Areas that had a large mass of CD45+ cells were counted as one lesion. Arrows on the above figure indicate areas which were defined as CD45+ lesions.
8) p141/fig 4.1
The percentage values in panels A and B are markedly higher than in the representative Facs data e.g. in fig 4.1B the histobars indicate that the % CD4+ T cells that are CCR7 in the p110δ<sup>D910A/D910A</sup> mice is around 80%, yet the representative data indicated it is only 22%. This is a massive difference and given the modest error bars, difficult to reconcile. Please comment accordingly/clarify exact “n” values for each histobar.

The percentage of CD4<sup>+</sup> cells that are CCR7<sup>+</sup> was calculated as follows:

\[
\frac{x}{(x+y)} \times 100
\]

where \(x\) = the upper right quadrant (CD4<sup>+</sup>/CCR7<sup>+</sup> cells) and \(y\) = the upper left quadrant (CD4<sup>+</sup> cells only)

Therefore, in the case of the abovementioned figure the calculation is as follows:

\[
\frac{22.57}{(22.57+4.74)} \times 100 = 82.6\%.
\]

\(n\) = 6-8 mice per group for these figures.

9) p156, fig 4.9
Please clarify the “typical DC phenotype” used to identify these cells in the legend and/or materials section.

A ‘typical’ DC was determined by observing the size and surface phenotype of the cells under a light microscope with trypan blue staining. Large cells with dendrite-like projections (as opposed to the smaller and smoother immature DCs and lymphocytes that can be found in such cultures) were considered to be mature DCs.

10) Fig 5.10 and 5.14
The overall percentage of cells responding to CD3/CD28 seems rather low in fig 5.10 compared to fig 5.14. The rationale for exploring the effect of in vivo pre-treatment of IC87114 under EAE conditions vs ex vivo CD3/CD28-stimulated proliferation in fig 5.14 is unclear. Would it not be better to study the effect of IC87114 on ex vivo proliferative responses to MOG peptide under these conditions? I’m a little unclear as to why in vivo administration of IC87114 under normal
conditions inhibits ex vivo CD3/CD28 responses in fig 5.10 but not in fig 5.14 after EAE immunisation? Does this reflect differences in use of splenocytes vs lymph node T cells in these two experiments?

The data shown in Figures 5.10 and 5.14 were generated from different experiments performed on different days. It is agreed that there was low levels of proliferation observed in the experiment presented in Figure 5.10A, however the nature of these experiments sometimes means that only low levels of proliferation is observed. This experiment was performed three times with similar results. It has been our experience that CD3/CD28 does not always generate high levels of naïve CD4+ T cell proliferation in vitro. The data shown in Figure 5.10 was generated by stimulating lymphocytes from a naïve mouse with anti-CD3 and anti-CD28 antibodies, whereas lymphocytes isolated from a mouse immunised with MOG and CFA were used to generate the data shown in Figure 5.14. It is hypothesised that the immunisation of MOG-CFA results in such a significant activation of the immune system that this overrides any inhibitory effects of IC87114, which may be why there were disparate results observed in Figure 5.10 (with naïve cells) and 5.14 (with activated cells). Future directions on how to address this are discussed in section 6.9 (with a particular focus in section 6.9.3).

As we have had difficulty with simulating T cells with the MOG_{35-55} peptide ex vivo, and to be consistent between the two experiments shown in the abovementioned figures, anti-CD3/anti-CD28 stimulation was used instead.

The reviewer notes that the differences observed may reflect the use of splenocytes or lymph node cells in the different experiments. It is also possible that this may have played a role. Naïve mice have very small lymph nodes so splenocytes were used so that sufficient yields of cells were obtained for the experiments in Figure 5.10. As mice are immunised with MOG-CFA in the hind flanks and scruff of the neck, lymphocytes from the inguinal and brachial lymph nodes were used for the experiment shown in Figure 5.14 as it was assumed that these would be more activated than splenocytes. It is possible that this difference has influenced the outcome of these experiments.
11) p207, fig 5.11
It is not clear why samples were taken on different days post-immunisation. Please clarify.

The purpose of taking these samples was to ensure that there was good coverage of the IC87114 compound in the mice throughout the disease course, hence the samples were taken at different days post-immunisation. Samples were also taken at different time-points as an added investigation into the half-life of IC87114 in the plasma of mice. While it would have been ideal to do these studies separately, these results still demonstrate that even though there are measurable IC87114 levels throughout disease at 2, 3 and 5 hours post-IC87114 administration, by 12 hours post-administration there is very little IC87114 detectable in the plasma of mice. Therefore, mice are presumed to have high levels of bioavailable IC87114 for most of the time, however it is assumed that the compound is routinely cleared from the blood of mice before the next dose was received.

12) The discussion is surprisingly short. I would like to see it improved by the provision of a schematic model(s) to help visualise the candidate’s theories and interpretation of the data in the context of existing knowledge. The applicant should consider revising her discussion accordingly.

Please see Figure 6.1 and the corresponding figure legend.

13) ref 468, p272: Remove the “t” typo.

This reference is correct, the authors name is “B.A. ‘t Hart”.

14) There is inconsistence in the use of “p110” and “PI3K” when referring to catalytic isoforms e.g “p110δ” and “p110γ” etc also referred to as “PI3Kδ” and “PI3Kγ”. Please be consistent.
It was the aim of the author to use the term “PI3K” when referring to the p110/regulatory subunit PI3K heterodimer complex. The “p110δ” terminology was used when specifically referring to the p110 catalytic subunit.
Figure 6.1: The role of p110δ in EAE. The pathogenesis of EAE is multifaceted. This study has indicated that there are several steps in which p110δ may be important. The p110δ protein was shown not to be important for antigen-uptake by DCs (A), DC trafficking to the draining lymph nodes (B) or presentation of antigen to T cells (C). However, p110δ inactivation results in intrinsic defects in T cell biology that may lead to reduced Th1 differentiation (D) in the lymph nodes as well as a profound reduction in Th17 cell differentiation (E). Production/secretion of the cytokines IL-17 and IFN-γ were reduced (F) and apoptosis of CD4+ T cells was increased without functional p110δ (G). There was a significant reduction in B cell function which may be a result of inefficient T cell-mediated activation (H) and apoptosis of B cells was observed to be increased (I). The p110δ protein is involved in B cell trafficking (J) and may also play a role in trafficking of T cells to the CNS (K). Animals lacking functional p110δ had fewer CD45+ cells (L), Th17 cells (M), B cells (N) and macrophages (O) in the CNS. There was no MOG-specific IgG detectable in p110δ-deficient mice (P). While the function of p110δ in oligodendrocytes (Q) and microglia (R) has not been addressed, future studies may elucidate a role for p110δ in microglia function in the CNS as well as remyelination by oligodendrocytes.
CHAPTER 8: Appendix

- Naïve T cell
- Activated Th1 cell
- Activated Th17 cell
- Activated CD8⁺ T cell
- Naïve B cell
- Plasma cell
- Dendritic cell
- Neuron
- Oligodendrocyte
- Microglia
- Myelin sheath (CNS-specific) antigen
- Cytokines/chemokines
- Macrophage
- IFN-γ
- IL-17
- Neutrophil
- NK cell
- Apoptotic T cell
- Apoptotic B cell