THE ROLE OF MORPHOGENS IN B CELL DEVELOPMENT

CYNTHIA ELOHO UMUKORO

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Abstract

Morphogens are signalling molecules that play a significant role in modulation of cell fate and development. Hedgehog proteins (Hh) are morphogens that have been shown to be involved in the development of immune cells. In this study, it is demonstrated that treatment of B cells with rShh, can increase B cell activation and also promote survival of B cells at 18hours post-stimulus. Also, at this time point, there was found to be an increase in secretion of antibody isotypes and IL-6. By 40hours post-stimulus, it was observed that the level of B cell activation was apparently arrested in treated B cells, whereas the level of activation continued to rise in untreated B cells. Interestingly, it was observed that there was an increase in the percentage of; CD23-CD25+ B cells when B cells were treated with rShh and this was accompanied by an increase in apoptosis. Consistent with this finding in relation to apoptosis, there was an increased expression of the pro-apoptotic protein *Bnip3* in B cells treated with rShh by 40hours post-stimulus. It was observed that there were three subsets of B cells arising in our culture at 40hours, which were all found to possess different characteristics. It was demonstrated that treatment with rShh can increase B cell differentiation towards FO-I at 18hours post-stimulus. By 40hours post-stimulus, Hh signalling can divert differentiation away from the FO-I B cell towards the T2-MZP, which was accompanied by an increase in IL-10 secretion. Gene expression analysis revealed that Hh signalling could modulate a number of molecules involved in delivering the BCR signal into the cells such as *Btk, Nfatc1* and *Traf2*. Additionally, deletion of *Dhh*, showed that there was a skewed peripheral B cell development in the Dhh-/- mice. Overall, our data demonstrate that Hh signalling can regulate the development of B cells in response to an activation stimulus by strengthening the BCR signalling pathway.

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Abbreviations

μg- Microgram

μl- Microlitre

-/- - Knockout

+/+ - Wildtype

APC- Antigen presenting cells

BAFF- B cell activating factor

BCR- B cell receptor

BM- Bone marrow cells

BMP- Bone morphogenic protein

Bnip3- Bcl2/ adenovirus E1B interacting protein 3

Bregs- Regulatory B cells

Btk- Bruton's tyrosine kinase

CD- Cluster of differentiation

CFSE- Carboxyfluoroescein diacetate succinimidyl ester

CLP- Common lymphoid progenitor

DC- Dendritic cell

DNA- Deoxyribonucleic acid

Dhh- Desert hedgehog protein

Dhh- Desert hedgehog protein gene

Dhh-/-- Desert hedgehog knockout mice

Dhh+/+- Desert hedgehog wild-type mice

ELISA- Enzyme linked immunoabsorbent assay

FO- Follicular B cells

FO-I –Follicular B cell type I

FO-II- Follicular B cell type II

FDCs- Follicular dendritic cells

FSC- Forward side scatter

GC- Germinal centre

Gli- Glioma transcription factor

Hh- Hedgehog protein

HSCs- Hematopoietic Stem cell

IL-10 – Interleukin 10

IL-6- Interleukin 6

IG- Immunoglobulin

Ihh- Indian hedgehog protein

ml- militer

MZ- Marginal zone B cells

Ng- nanogram

NK- Natural killer Cells

PCA-Principal component analysis

PBS- Phosphate buffered Saline

Ptch- Patched receptor

qRT-PCR- Quantitative real time polymerase chain reaction

rBMP-4- Recombinant bone morphogenic protein-4

rShh- Recombinant sonic hedgehog protein

RNA- Ribonucleuic acid

RT-PCR- Real time polymerase chain reaction

Shh- Sonic Hedgehog protein

Smo- Smoothened receptor

T1 – Transitional B cells Type 1

T2 – Transitional B cells Type 2

T2-MZP- Transitional B cells type 2- marginal zone precursors

TD- Thymus dependent

Th- T helper cells

TI- Thymus independent

TNF- Tumour necrosis factor

TNFR- Tumuor Necrosis factor receptor

TRAF- Tumour Necrosis factor receptor associated factor

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Chapter 1: Introduction

1.1. B cells

1.1.1. B cell development in the bone marrow

B-lymphocytes (B cells) are a population of cells that express clonally diverse cell surface immunoglobulin receptors recognising specific antigen epitopes and are capable of producing high affinity soluble antibodies (LeBien and Tedder, 2008, Cooper, 2015). They play a prominent role in preventing and combating infections. The development of B cells occurs in multiple stages that begin in the primary lymphoid tissue, the bone marrow, before subsequent developmental stages occur in the secondary lymphoid organs such as the spleen. The endpoint of the development of B cells is antibody production by terminally differentiated plasma cells as well as the differentiation of activated B cells into memory B cells (LeBien and Tedder, 2008).

In mammals, B cells are derived from hematopoietic stem cells (HSC) and are produced in the fetal liver, yolk sac and fetal marrow during gestation and in the bone marrow after birth. HSCs are pluripotent stem cells that gives rise to common lymphoid progenitor (CLP) which is restricted to all lymphoid cells (T cells, B cells and Natural killer cells) and common myeloid progenitor (CMP) which is restricted to all myeloid cells (erythrocytes, megakaryocytes) (Pieper *et al*., 2013, Nagasawa, 2006, Ichii *et al*., 2014, Hardy and Hayakawa, 2001, LeBien and Tedder, 2008, Tobon *et al*., 2013).

In the bone marrow, the early precursor of B cells, the CLP, lacks a B cell lineage marker and it is characterised by the expression of c-kit and IL-7 receptor α chain. Following CLP, B cell lineage restriction can be recognised by expression of the B220

isoform of CD45 (Hardy and Hayakawa, 2001, Matthias and Rolink, 2005). In the bone marrow, early development and commitment to the B cell lineage after CLP depends on the expression of several transcription factors including the early B cell factor (EBF), PU.1, E2A, IKAROS and paired box protein 5 (PAX5) (Shapiro-Shelef and Calame, 2005, Hardy and Hayakawa, 2001, Matthias and Rolink, 2005, Eibel *et al*., 2014, Smith and Sigvardsson, 2004). Pax5 is a fundamental transcription factor for commitment to the B cell lineage. Expression of this molecule is essential for immature and mature B cell development. Additionally, the bone marrow creates a microenvironmental niche that allows the development of B cells. It contains adherent stromal cells, which are crucial for B cell development by providing growth factors and cytokines such as interleukin 7 (IL-7) for the survival and differentiation of B cells as well as the Fims-like tyrosine kinase 3 (FI3-L) (Nagasawa, 2006, Eibel *et al*., 2014). IL-7 cytokine has been shown to be crucial in the development of B cells in mice but not in human (Busslinger, 2004). All these factors, facilitates CLP differentiation into precursor progenitor (pre-pro) B cells, which are B220+CD19-. These pre-pro B cells differentiate rapidly into CD19+ progenitor (pro-) B cells, which marks full commitment to the B cell lineage (Rumfelt *et al*., 2006).

B cell maturation depends on the successful rearrangement of the Immunoglobulin (Ig) DNA locus in the lymphoid stem cells. Expression of the immunoglobulin heavy and light chains ensures that the developing B cells express a membrane bound B cell receptor (BCR) which acts as an antigen receptor (LeBien and Tedder, 2008, Tobon *et al*., 2013). BCR is a membrane bound immunoglobulin consisting of a heterodimer composed of identical pairs of Ig heavy and light chains which are responsible for the clonal diversity of the B cell repertoire. The BCR complex also contains within it, a heterodimer of Igα (CD79a) and Igβ (CD79b), which is non-covalently attached with

the BCR. This Igα/Igβ heterodimer is required for the surface expression and signalling functions of the receptor (Perez-Vera *et al*., 2011, Matthias and Rolink, 2005, Kurosaki and Hikida, 2009, Eibel *et al*., 2014). In order to achieve BCR clonal diversity, the Ig heavy and light chain genes are composed of a constant and variable region with the variable region being responsible for the diversity of the antibody repertoire. In the case of the heavy chain, the variable region is formed by a series of segments termed variable (V), D (diversity) and J (Joining), which are brought together by site-specific recombinases in a process termed VDJ recombination. This is a highly regulated process that involves firstly the rearrangement of the D and J fragments before they join to the V fragment processed by recombinase associated genes 1 and 2 (RAG1 and RAG2). This process occurs in the heavy chain first prior to recombination in the light chain (Smith and Sigvardsson, 2004, Perez-Vera *et al*., 2011).

In the pro- B cell stage during development of B cells in the bone marrow, Igα and Igβ heterodimer together with chaperones such as calnexin are expressed on the surface of the cell before the Ig heavy chain gene locus rearrangement has taken place. Initially, expression of the heavy chain occurs intracellularly. After assembly, the heavy chain associates with components of the surrogate light chain, which include Lambda-5 (λ5) and VpreB, together these form the pre B cell receptor (pre-BCR) (Smith and Sigvardsson, 2004, Perez-Vera *et al*., 2011) and the cells are called pre- B cells at this stage. Expression of the pre-BCR is a crucial checkpoint in early B cell development at which the functional competence of the rearranged heavy chain is monitored in order to avoid immuno-incompetent B cells, which are removed by apoptosis. Only pre-B cells expressing a functional heavy chain are allowed to differentiate further. Signalling through the pre-BCR allows for allelic exclusion of the heavy chain locus thus preventing the expression of more than one receptor in a single B cell. Signalling via the

pre-BCR, induces a burst of proliferation allowing for clonal expansion of the large pre-B cells, which leads them to further differentiate into small pre-B cells (Matthias and Rolink, 2005, Tobon *et al*., 2013, Eibel *et al*., 2014). Small pre-B cells, up-regulate expression of the RAG1/2 genes required for the rearrangement of the Ig light chain thus allowing the production of a functional BCR with a unique specificity that is expressed as IgM on the surface of immature B cells.

Immature B cells are subjected to additional checkpoints, to test the BCR for functionality and autoreactivity. Potential autoreactive B cells are forced to a secondary immunoglobulin gene rearrangement process in a processing event called receptor editing (Matthias and Rolink, 2005, Tobon *et al*., 2013). Receptor editing involves reactivation of expression of the RAG genes and expression of a new Ig light chain to pair with the existing heavy chain in an effort to generate a non-self reactive BCR. B cells that fail this negative selection are either killed by apoptosis or inactivated by anergy (an induced state of unresponsiveness) (Cambier *et al*., 2007, Matthias and Rolink, 2005). B cells that pass through this tolerance undergo further differentiation resulting in the expression of IgD on their surface. These immature B cells exit the bone marrow and enter the periphery (Hardy and Hayakawa, 2001, LeBien and Tedder, 2008).

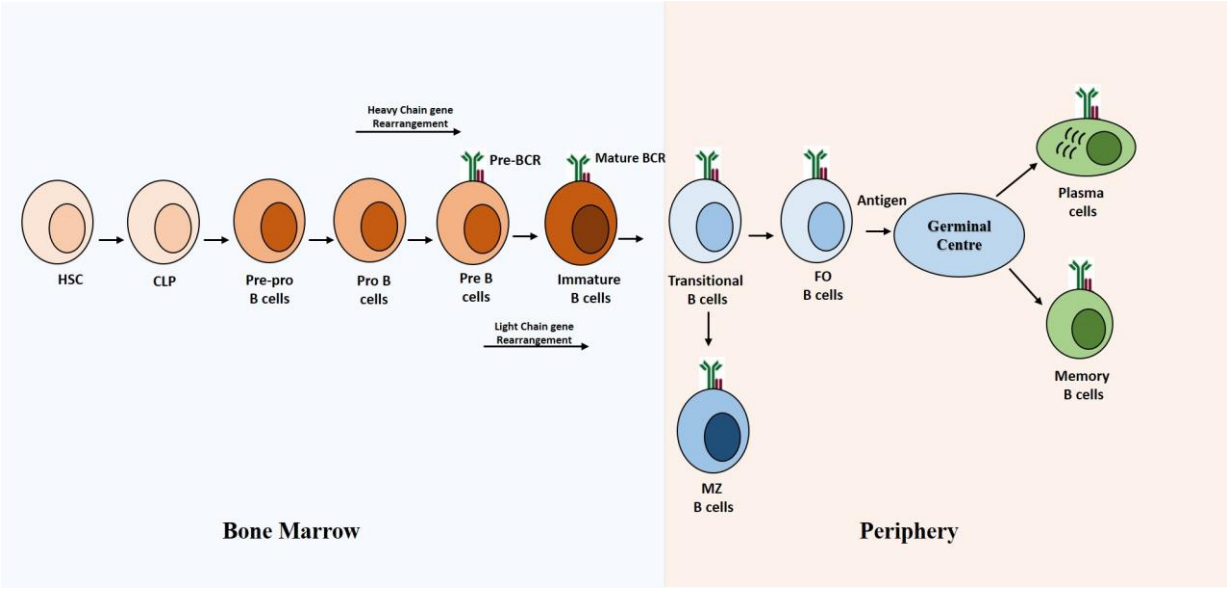


Figure 1.1. B cell development in mouse

B cells develop in the bone marrow in a culminated manner to become immature B cells. Immature B cells enter the periphery as transitional B cells. Transitional B cells can differentiate to become either FO or MZ B cells. Upon encounter with an antigen, B cells are activated to proliferate and form a germinal centre where they differentiate to become either plasma antibody producing cells or Memory B cells. Adapted from (Cambier *et al*., 2007).

1.1.2. Peripheral B cell development

Immature B cells migrate to a peripheral lymphoid organ such as the spleen via terminal branches of the central arterioles. Upon entry into the spleen, immature B cells differentiate from short-lived transitional B cells 1 (T1) to become T2 and, in certain circumstances, T3 (Matthias and Rolink, 2005, Pillia and Cariappa, 2009). Transitional B cells can be distinguished from mature B cells by their cell surface marker expression (CD93, CD23, IgM, and CD21), their short half-life and the sensitivity to apoptosis induced by antibodies specific for IgM. Immature T1 B cells are subjected to negative selection in the spleen, which is crucial for the generation of mature B cells, which lack specificity for self-antigens. Expression of B cell activating factor (BAFF) a member of tumour necrosis family and its counterpart B cell activating factor receptor (BAFFR)

play crucial roles in the transitioning of transitional B cells through to mature B cells. The ligation of BAFF to its receptor mediates survival of B cells in the periphery by activating different signalling cascades including the NfκB pathway thus inducing pro survival functions (Eibel *et al*., 2014). After passage through the transitional stages, B cells become long-lived mature follicular (FO) (Follicular type I or Follicular type II) and marginal zone (MZ) B cells of the spleen or cortical cells in the lymph node. The decision of the B cells to become either FO or MZ B cells depends on the strength and nature of the BCR signal and also the subsequent expression of Notch2 (Pillai and Cariappa, 2009, Tobon *et al*., 2013). For example, differentiation to become MZ B cell would normally require a signal to be delivered through a Toll-like receptor as well as the BCR. It has been shown that immature B cells that are specific for phosphocholine, a constituent of the cell wall of encapsulated bacteria normally found in the gut flora, selectively develop into MZ B cells. Another mechanism of tolerance in the spleen involves anergy, and it is thought that T3 cells may either be anergic B cells (Merrell *et al*., 2006) or be a developmental intermediate for mature B cells; however, this is still under debate (Allman and Pillai, 2008, Hardy *et al*., 2007).

In the periphery, upon encounter with it's cognate antigen, B cells play a key role in driving the immune response by presenting antigens to the adaptive CD4+ T cells through presentation on MHC class II molecules. This in turn leads to the cross-linking of the BCR, and thus activation of the B cells. This type of B cell activation is called thymus dependent (TD) B cell activation. It also involves the binding of the CD40 ligand on the T- cell binding to the CD40 receptor on the B cell. The FO B cells plays the major role in TD immune responses (Allman and Pillai, 2008). B cells can also be activated independently of T cell and this is known as a thymus independent response (TI), this involves ligation of the toll Like Receptors (TLR) expressed on the surface of

B cells by antigens such as lipopolysaccharide (LPS), which is a component of the outer membrane of most Gram- bacteria. MZ B cells due to their unique location within the marginal zone of the spleen encounter TI antigens preferentially.

When B cells are activated via the CD40 ligand as well as the BCR, they form a germinal centre (GCs). GCs are distinct regions in the secondary lymphoid organs and contain proliferating B cells, follicular TfH cells, follicular dendritic cells (FDCs) and dark zone stromal cells (Eibel *et al*., 2014). The GC reaction is characterised by clonal expansion of activated cell, class switch recombination of the Ig locus, somatic hypermutation of the Vh genes and affinity maturation (LeBien and Tedder, 2008, Matthias and Rolink, 2005). Somatic hypermutation introduces point mutations in the V region of heavy and light chain genes which results in a subtly mutated BCR expressed on the mature B cells and this process is reliant on the expression of the enzyme that catalyses this process, activation induced deaminase (AID) (Eibel *et al*., 2014, Pieper *et al*., 2013). The GC reaction leads to the differentiation of B cells to become plasma cells and memory B cells, which can then reside in the periphery or migrate back to the bone marrow (Pieper *et al*., 2013). B cell development in the bone marrow and periphery of a mouse is illustrated in Figure 1.1.

1.1.3. Mature B cells

Peripheral B cells in mice can be divided into two major B cell subsets, B-1 and B-2 B cells that can be distinguished from one another based on their anatomical location, phenotype and function. B-2 B cells includes FO and MZ B cells (Hardy and Hayakawa, 2001).

1.1.3.1. B-1 Cells

B-1 cells are located in the peritoneal and pleural cavities, the gut lamina propria and a small proportion are located in the spleen and account for 5% of B cells in the mouse (Hardy *et al*., 2007). They are a unique subset of B cells further subdivided into B-1a cells, which are $CD5^+$ and B-1b, which are $CD5^-$ (Hardy and Hayakawa, 2001, Shapiro-Shelef and Calame, 2005, Berland and Wortis, 2002). B1 B cells derive mostly from fetal progenitors cells and express a BCR repertoire skewed towards TI antigens such as pathogen expressed molecules (Allman and Pillai, 2008, Berland and Wortis, 2002). B-1 B cells are considered innate immune cells and provide innate protection against bacterial infection in naïve hosts by producing antigens and spontaneously secreting natural IgM. Furthermore, B1 B cells can contribute to the production of IgA at mucosal sites. Additionally, B-1 B cells have self-renewing capacities (LeBien and Tedder, 2008, Shapiro-Shelef and Calame, 2005, Berland and Wortis, 2002, Allman and Pillai, 2008).

1.1.3.2. MZ B cells

MZ B cells are found mainly in the marginal zone of the spleen, which harbours a major antigen filtering and scavenging area. MZ B cells are uniquely positioned in the spleen to be in contact with blood borne pathogens (Hardy *et al*., 2007). MZ B cells carry a BCR that preferentially bind to blood borne antigens such as cell wall components of

bacteria and thus encounters mainly TI antigens. MZ B cells, upon activation rapidly differentiate into short-lived plasma cells (Eibel *et al.*, 2014, Allman and Pillai, 2008). MZ B cells and B1 B cells are crucial for the early humoral response.

1.1.3.3. FO B cells

FO B cells are the major contributors to TD immune responses. In the spleen, FO B cells are positioned in the follicles of the spleen where they can encounter T cells. Upon activation, these cells can either give rise to short lived plasma cells or form GCs, where proliferation and differentiation towards plasma and memory B cells may occur (Allman and Pillai, 2008).

1.1.3.4. Plasma Cells

Plasma cells are terminally differentiated B cells; they are essential for protective immunity. In plasma cells the production of immunoglobulin changes from a membrane bound form to a secreted form (Eibel *et al.*, 2014). Plasma cells are terminally differentiated, non-dividing cells and can be classified as either short-lived or long-lived cells. Short-lived IgM producing plasma cells can arise from FO B cells or MZ B cells. The BCRs expressed on these B cells do not go through somatic hypermutation and hence the affinity of these antibodies does not change over time. Long-lived plasma cells go through somatic hypermutation and can home back to the bone marrow where they continue secreting antibodies and become part of immunological memory (Eibel *et al.*, 2014). A range of transcription factors drives plasma cell development and differentiation including X box binding protein (XBP-1) and B cell induced maturation protein (Blimp-1). Blimp-1 initiates a plasma cell specific transcription program by inducing the transcriptional repression of Pax5 and Bcl6. Additionally; expression of

XBP-1, a stress response gene is up-regulated as part of the cell adaptation process required for sustained antibody secretion. Other transcription factors such as IRF-6, IRF-4 and IL-21 also drive plasma cell differentiation (Eibel *et al*., 2014, Matthias and Rolink, 2005, Shapiro-Shelef and Calame, 2005).

1.1.3.5. Memory B cells

B cells can differentiate to become non- antibody secreting memory B cells during the GC reaction, which can persist independently of antigenic stimulation (Kurosaki *et al*., 2015). Memory B cells have the ability to differentiate into plasma cells upon second re-encounter with antigen being responsible for the rapid secretion of high affinity, class switched antibody which is typical of the secondary immune response (Kurosaki *et al*., 2015).

1.1.3.6. Regulatory B cells

B cells also have the ability to negatively regulate the immune response giving rise to a recently described subset of B cells termed regulatory B cells. Regulatory B cells (Bregs) are a subset of B cells that are described to play a pivotal role in the prevention of pathogenesis of several autoimmune diseases as well as allograft tolerance. This unique cell population has been found to inhibit the function of other cells of the immune system by production of the anti-inflammatory cytokine Interleukin 10 (IL-10) (Goode *et al*., 2014).

1.2. Morphogens

1.2.1. Morphogens

Morphogens are long range signalling molecules that determine cell fate and organogenesis. They emanate from a restricted region of the tissue and diffuse across tissues from a localised source forming a concentration gradient with the high concentration close to the source and the lower concentration further away from the source (Rogers and Schier, 2011, Muller *et al*., 2013). The dose of morphogen that a cell perceives will depend on where the cell sits within this concentration gradient (Figure 1.2). The graded activity of morphogens in the tissue leads to differential target gene expression between cells, which are dependent on the dose of morphogen the individual cell encounters. Thus, cells exposed to a high concentration gradient activate different transcriptional genes and have different fates compared to those cells exposed to a lower morphogen concentration (Figure 1.2). Thus, the graded activity of the morphogen differentiates tissues into different cell types that are arranged as a function of their distance from the source (Ingham and McMahon, 2001, Stathopoulos and Iber, 2013, Rogers and Schier, 2011). Morphogens act in developing tissues to control spatial arrangement of cellular differentiation and it has been shown that not only does the concentration of the morphogen affect the cell fate but also the length of exposure to the morphogen as well as the state of the cell (Rogers and Schier, 2011).

Morphogens have been implicated in the development of many developmental processes in *Drosophila* including that of the wing, the vertebrate limb bud and the neural tube (Mehlen *et al*., 2005, Tabata and Takei, 2004). Morphogens play an essential role in the patterning of tissues during embryogenesis and are expressed constitutively during this process but also play a role in the control of the homeostasis

of adult tissues and are tightly regulated by numerous regulatory proteins during this later process (Tabata and Takei, 2004).

Several morphogens have been identified in vertebrates such as humans, mice and chick embryo and they include hedgehog protein (Hh), wingless/int (Wnt) family of proteins, the notch proteins and the transforming growth factor (TGF-β) family, which includes bone morphogenic protein (BMP) (Bijlsma *et al*., 2006, Rogers and Schier, 2011).

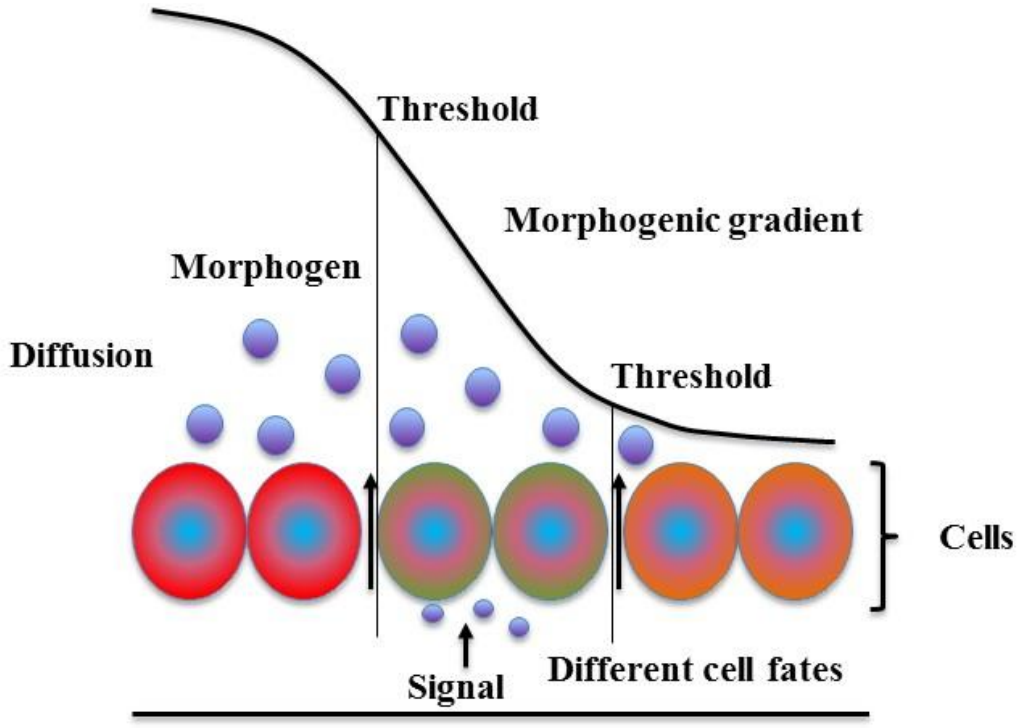


Figure 1.2. Morphogen concentration gradient

A diagram illustrating morphogen concentration gradient. The concentration gradient of the morphogen determines the cell fate. The cells secrete morphogens and they diffuse forming a concentration gradient. There is a higher concentration of morphogen produced in the source (red cells), and farther away is a low concentration of the gradient (orange cells). At different threshold of the morphogen, the cells have different cell fates (Adapted from Eldar *et al*., 2002).

1.2.2. Morphogens in B cell development

Several morphogens including hedgehog protein, BMP and notch proteins have been shown to play a role in B cell development. In peripheral B cell development, signalling via Notch protein, Notch2, have been shown to be required for the development of MZ B cells. In the spleen, Notch2 can interact with its ligand, Delta-Like-1 (DL-1). This leads to signalling cascade that can provoke a transcriptional complex thus inducing B cell commitment towards MZ B cells (Pillai and Carriappa, 2009), hence indicating a role for this morphogen in B cell development. Additionally, BMP have been shown to inhibit B cell lymphopoiesis in the bone marrow (Kersten *et al*., 2005), inhibit plasmablasts differentiation and proliferation (Huse *et al*., 2011) thus suggesting a role of this morphogen in B cells.

Hedgehog proten (Hh), has also been shown to play a role in B cell development. In this thesis, the role of Hh signalling in B cell development has been investigated.

1.3. Hedgehog protein

1.3.1. Hedgehog protein: discovery and function

The hedgehog proteins (Hh) form a family of morphogens that are all secreted signalling molecules that were first discovered in *Drosophila melanogaster* in 1980 (Nusslein-Volhard and Wieschaus, 1980). While carrying out a large scale screening for mutations that impair or change the development of the fruit fly larvae body plan, gene mutations were identified, that resulted in disruption to the dorsal ventral body plan of the larval. The disruption resulted in abnormal distribution of hair like projections on the ventral surface denticles thus resembling a hedgehog. So, this gene product was termed hedgehog protein (Nusslein-Volhard and Wieschaus, 1980).

Several homologues of Hh have been identified in all vertebrates including humans, frogs, fish, mouse and birds (Ingham, 2012, Ingham and McMahon, 2001). In the human embryo, Hh was first described as being expressed in the notochord, floor plate of the neural tube, the zone of polarising activity (ZPA), the region of the developing limb bud that specifies digit type and number (Briscoe and Rohatgi, 2012) during gut and developing limbs (Ingham and McMahon, 2001). Whilst in *Droshophila* only one Hh gene has been shown to be expressed to date, there are three homologues of Hh described to be expressed in vertebrates, each showing a different spatial and temporal distribution pattern. These include, Desert hedgehog (Dhh), Indian hedgehog (Ihh) and Sonic hedgehog (Shh) protein with the latter being the most extensively studied and broadly expressed in mammals and has been shown to be essential for the development of many tissues (Burglin, 2008). During embryogenesis, Shh is first expressed in the notochord and later in the floor plate of the neural tube, where it produces a gradient of activity in the ventral neural tube. It is also expressed in the zone of polarising activity of the limb and digit formation (Burglin, 2008). Shh and Ihh are also essential for gut development and are expressed by the epithelial cells in the stomach and the colon. They are both also important for cardiovascular development. Ihh has a function in bone formation and in the cartilage (Burglin, 2008, Varjosalo and Taipale, 2007). Dhh expression is largely restricted to gonads including the sertoli cells of the testis and granulosa cells of the ovaries (Varjosalo and Taipale, 2008). Dhh is required for testes formation, schwann cell function, erythrocyte development and proper recovery from stress–erythropoiesis (Lau *et al*., 2012). In mouse development, Shh and Ihh are essential for development of the mouse as demonstrated by the lethal phenotype of the knockout mutants (Chiang *et al*., 1996), however, mice deficient in Dhh are normal and healthy albeit males are infertile (Bitgood *et al*., 1996).

Hh signalling has also been shown to play a role in the development of a number of haematopoietic cells such as thymocytes (Outram *et al*., 2000), HSC (Bhardwaj *et al*., 2001, Trowbridge *et al*., 2006) and erythroid cells (Lau *et al*., 2012) T cells (Chan *et al*., 2006, Furmanski *et al*., 2013, Rowbotham *et al*., 2007) and B cells (Sacedon *et al*., 2005). The Hh signalling pathway is interconnected other morphogen and growth factor signalling pathways including, Wnt (β -cantenin) pathway, TGF-β BMP pathway, notch pathway and fibroblast growth factor (FGF) pathway. The correct integration of these different signalling pathways plays a critical role in morphogenic processes including tissue morphogenesis, homoeostasis, organogenesis and stem cell renewal in adults (Heretsch *et al*., 2010, Bijlsma *et al*., 2006).

1.3.2. Hedgehog protein: structure, synthesis and signalling

Hh proteins are synthesised inside the cell as precursor proteins of approximately 45kDa and require multiple processing events to take place in order to form a functional protein. The precursors are approximately 400- 460 amino acids and contain a series of motifs and domains, which include a signal peptide, a secreted amino-terminal (19kDa) that has signalling abilities and a carboxyl terminal (25kDa), which has autocatalytic activity (Burglin, 2008, Varjosalo and Taipale, 2007, Varjosalo and Taipale, 2008).

The signal peptide targets the newly synthesised precursor protein to the endoplasmic reticulum where the intramolecular auto-processing reaction of the protein occurs. The precursor protein first undergoes cleavage of the signal sequence. The autocatalytic C – terminal mediates the auto-processing reaction of the Hh precursor protein. There are two sequential stages of nucleophilic displacements that are involved. The first involves the rearrangement to replace the main chain peptide linkage between the glycine and

cysteine residues with a thioester involving the Cys side chain. The second step of the auto-processing reaction involves attack upon the carbonyl by a second nucleophile thus displacing the sulphur and severing the link between Hh-N and Hh-C (Mann and Beachy, 2004, Varjosalo and Taipale, 2008). The addition of cholesterol causes the Hh protein to be linked to the membrane and it is essential for the proper functioning of the protein including the long range signalling of the protein (Varjosalo and Taipale, 2007, Varjosalo and Taipale, 2008). The final step of processing involves the addition of a palmitol moiety, which is added to a cysteine residue via an amide bond of the N-terminus of the Hh protein by a transmembrane acyltransferase termed skinny Hedgehog and hedgehog acyltransferase in *Drosophila* and mammals respectively (Heretsch *et al*., 2010, Varjosalo and Taipale, 2008). The gene Rasp encodes the enzyme likely located within the endoplasmic reticulum required for the Hh acylation and the production of active Hh (Gupta *et al.*, 2010). Thus, this process generates a fully active lipidated Hh protein that can form multimeric complexes and interact with lipoproteins (Burglin, 2008, Varjosalo and Taipale, 2008).

The lipid modifications of the protein allows it to adhere to the membrane of the Hh synthesising cells, enabling short range signalling to neighbouring cells. After processing of the protein, the Hh protein is released from the cell via a 12 multi-pass transmembrane protein called Dispatched (Disp) which has a sterol-sensing domain (Burglin, 2008, Heretsch *et al*., 2010). Disp, may promote the Hh protein to assemble into multimeric micelle-like complexes or its incorporation into lipoprotein particles (Briscoe and Therond, 2013). Once out of the cell, Hh protein interacts with extracellular proteins, which include heparan sulphate proteoglycans (Hsps), dally-like (Dlp), iHog (in *Drosophila*) and growth arrest-specific 1 (GAS-1), which are positive regulators of Hh signalling and aid in long-range signalling activities. Hsps play a key

role in Hh transport and it is required for stable retention of the Hh on the cell surface (Tabata and Takei, 2004). Hh also interacts with hedgehog interacting protein (HIP), which is a negative regulator that acts by sequestering the modified Hh-N (Burglin, 2008, van den Brink, 2007).

All Hh proteins signal via a common pathway. The membrane receptors to which they bind to are called patched (Ptch) and smoothened (Smo) (Varjosalo and Taipale, 2007, Varjosalo and Taipale, 2008).

Ptch is a 12-pass transmembrane receptor with two hydrophilic extracellular loops with a sterol sensing domain, which in the absence of Hh localises in the primary cilium of an Hh responsive cell and it functions as a platform for Hh intracellular signal transduction. Ptch is a core receptor for Hh signalling, binding Hh ligands with an affinity in the low nanomolar range (Robbins *et al*., 2012, Varjosalo and Taipale, 2007). In addition to Ptch, other molecules that bind Hh and promote signalling have been identified. They include CAM related/ down-regulated by oncogenes (CDO), brother of CDO (BOC) (iHog and Boi in *Drosophila*) and growth-arrest specific 1(Gas1) (Briscoe and Therond, 2013). Smoothend (Smo) is a member of the frizzled (FZD) class of G-protein coupled receptors and functions as the key transducer in the Hh signalling pathway. It is an integral membrane protein containing seven membrane spanning alpha helices, a long N-terminal extracellular peptide and a C-terminal intracellular peptide. Smo also contains a membrane integrated heptahelical domain (Wang and McMahon, 2013). Ligand binding to Smo is essential for its regulation because mutations in the heptahelical domain render the protein either inactive or constitutively active. Smo is negatively regulated by pro-vitamin D3 and it is positively and indirectly regulated in an

allosteric manner by naturally occurring compounds produced through the oxidation of cholesterol called oxysterols (Wang and McMahon, 2013).

A schematic diagram, demonstrating Hh signalling in vertebrates is shown in Figure 1.3. In the absence of Hh, Ptch receptor is located in the basal cilium where it represses the activity of Smo, in a poorly understood mechanism, and thus represses the Smo-dependent downstream signal in to the cell (Ingham, 2012, Varjosalo and Taipale, 2007). In the presence of Hh, the Hh binds to Ptch and a number of accessory proteins, Cdo/Boc, which are integral membrane proteins and this binding results in a conformational change that allows the translocation of Smo from the endocytic vessel to the cilium and thus activation of the signalling pathway (Ingham, 2012, Heretsch *et al*., 2010, Varjosalo and Taipale, 2007, Varjosalo and Taipale, 2008) The ligand binding of Hh to Ptch also results in the translocation of Ptch out of the primary cilium whereupon it is degraded by lysosmes (Heretsch *et al*., 2010) As a result, Smo signalling may now take place in the primary cilium and a signal is transmitted into the cell. This leads to the activation of a family of oncogenic transcription factors termed the Glioma (Gli) five zinc finger transcription factors (Cubitus Interruptus (Ci) in *Drosophila*) (Varjosalo and Taipale, 2008, Briscoe and Therond, 2013). The Gli transcription factors enter the nucleus where they induce the transcription of Hh signalling target genes. Smo is critical for Hh signalling, as most Hh antagonist targets the Smo protein, cycloplamine, a naturally alkaloid is an inhibitor of Hh protein, works by inhibiting Smo signalling (Porro *et al*., 2009, Briscoe and Rohatgi, 2012). However, Hh signalling can occur independent of Smo signalling, as evidences suggests that Ptch can induce apoptosis in cells independently (Brennan *et al*., 2012).

Gli are bi-functional transcriptional regulators inhibiting transcription in the absence of Hh signalling but activate transcription after Hh signalling (Briscoe and Rohatgi, 2012). Three closely related Gli transcription factors are involved in the Hh signalling pathway with distinct temporal and spatial expression patterns; these include Gli1, which has a strong positive effect on expression of Hh target genes, and is itself a transcriptional target of the Hh signalling pathway. Gli2 and Gli3 function by exhibiting both repressing and activating signals for expression of Hh target genes. Gli2 functions primarily as a transcriptional activator downstream of Hh signalling and it is crucial to initiate the first transcriptional changes induced by the Hh signal. The main downstream functions of the Gli transcription factors signalling include leading cells to enter the cell cycle, inhibition of apoptosis, regulation of stem cell differentiation, angiogenesis, axonal guiduance and modulation of tissue polarity (Porro *et al*., 2009, Ditzel, 2011).

Target genes which Hh signalling has been described to activate, include genes involved in cell survival and apoptosis like *Bcl2*, other morphogens such as *Bmp4* and also genes involved in the control of the cell cycle including cyclin D, Myc, VEGF and Snail (Porro *et al*., 2009, Gupta *et al*., 2010).

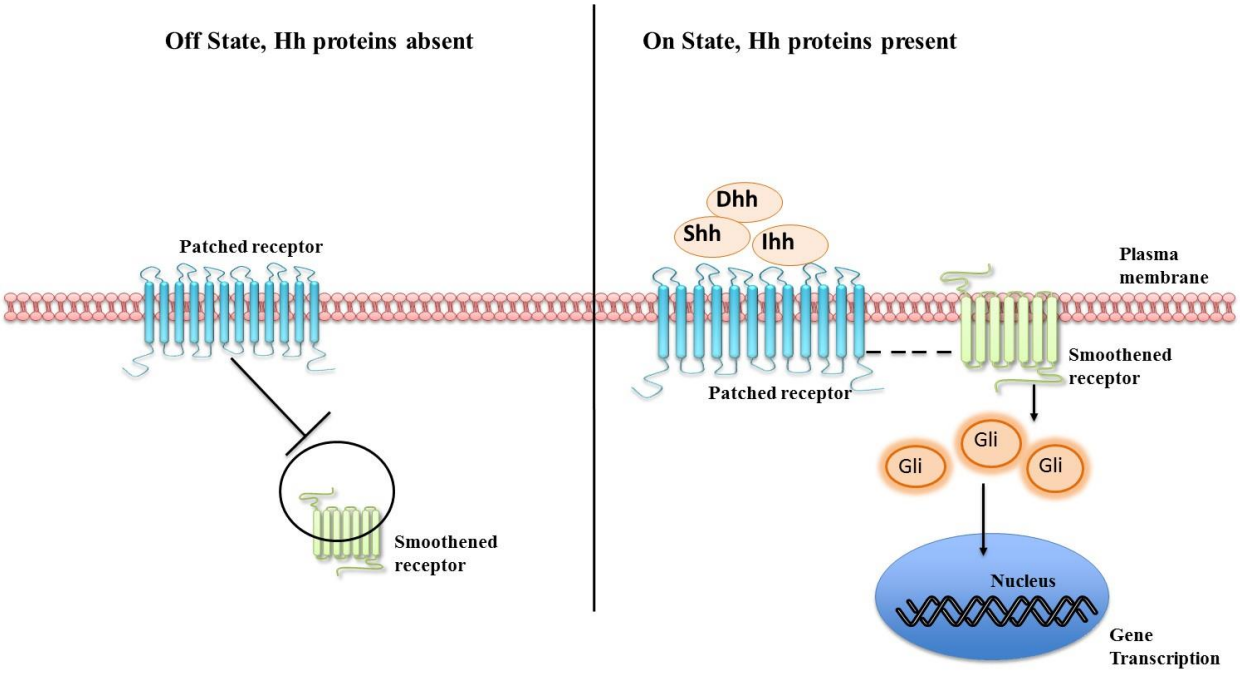


Figure 1.3. Hh Signalling

Ptch represses Smo in the absence of Hh and thus there is no transcription of target genes (left). Upon activation of Ptch by a member of the Hh family, Smo is relieved from inhibition and it signals to activate Gli transcription factors that enters the nucleus to induce gene transcription of target gene (right). Adapted from (Pasca di Magliano and Hebrok, 2003).

1.3.3. Hh signalling in B cells and B cell lymphomas

Hh signalling has previously been implicated in the development and differentiation of cells of the haematopoietic lineage including haematopoietic stem cells (HSc) (Bhardwaj *et al*., 2001, Trowbridge *et al*., 2006), T cells (Outram *et al*., 2000, Chan *et al*., 2006, Rowbotham *et al*., 2007, Furmanski *et al*., 2013), dendritic cells (Varas *et al*., 2008), NK cells (Zhu *et al* 2012) and macrophages (Schumacher *et al*., 2012). In contrast, the role of Hh signalling in immune cells such as B cells is still to be fully explored. Subsets of B cells in the early stages of B cell development in the bone marrow including pro-B cells and pre-B cells have been shown to express Ptch, Smo and Gli-1, which are all components of the Hh signalling pathway. The expression of

Ptch and Smo decreased as the B cells became more mature (Cooper *et al*., 2012) suggesting Hh proteins may play a role in early B cell development. Additionally, a role for Indian Hedgehog (Ihh) protein in early B cell development has recently been demonstrated (Kobune *et al*., 2008).

Ihh, a member of the Hh family, was first described to be expressed in chondrocytes of early cartilaginous skeletal elements (Bitgood and McMahon, 1995). It is involved in chrondocyte hypertrophy and bone formation in the developing skeletal system (Mak *et al*., 2008). Ihh has previously been shown to be involved early T cell development by controlling homeostasis in the thymus (Outram *et al*., 2009) and is capable of mediating an interaction between hematopoietic progenitor/ stem cells and bone marrow stromal cells thus leading to proliferation of the hematopoietic progenitor/stem cells *in vitro*. Using a single stranded adenoviral vector, which caused high level of ectopic Ihh expression, Kobune *et al*., (2008) showed that Ihh expression causes an increase in the number of immature splenic B cells and a decrease in the number of IgM+ mature splenic B-lymphocytes. This suggests Ihh inhibits the maturation of B cells (Kobune *et al*., 2008). These findings were further supported by studies using murine B-cell progenitors (BM) co-cultured with the Smo antagonist, cyclopamine, or Hh neutralising antibody, 5E1. Inhibition of Hh signalling prevented the proliferation of pro- B cells suggesting a key role in the maintenance of early B cell haematopoiesis (Cooper *et al*., 2012). Hh signalling has also been shown to take place in the stromal cells of the bone marrow promoting B cell lymphoid differentiation (Cooper *et al*., 2012). Taken together, these data, suggest Hh signalling has a key role in modulating early B cell maturation and development.

The role that Hh signalling plays in the later developmental stages of B cells in the periphery has not been fully explored. Sacedon *et al*., (2005), showed that FDCs contained within the GC secrete Shh. Additionally, they showed that GC B cells express the receptors for Shh signalling. They also showed that all the B cells in the GC express the Ptch receptor and of that population, approximately 30% co-expressed Smo protein. This indicates that although GC B cells express the Shh receptor, Ptch, only 30% of the cells are capable of transducing this signal via Smo into the cell. Smo expression was up-regulated to approximately 50% upon activation of GC B cells by anti-CD40 and anti-IgM thus indicating that GC B cells are themselves targets for Shh produced by FDCs, indicating that Hh signalling maybe required to play a role in directing a B cell mediated immune response in the periphery. The authors also showed *in vitro* that there was increased GC B cell apoptosis with inhibition of Shh and that the GC B cells were rescued from Fas mediated apoptosis upon addition of exogenous Shh. Thus, indicating that Shh is one of the biological conditions required for the survival of GC B cells. Furthermore, they reported that Shh could increase antibody production and proliferation of GC B cells (Sacedon *et al*., 2005).

Conversely, aberrant Hh signalling has been linked to the development of lymphoid tumours such as B cell lymphoma and multiple myeloma (MM). In the case of B cell chronic lymphocytic leukaemia, (B-CLL), a disease characterised by accumulated neoplastic B cells in the bone marrow, blood and peripheral lymphoid tissues, stromally induced Hh has been shown to play a role in the survival and proliferation of the tumour cells (Hegde *et al*., 2008). In diffuse large B cell lymphoma, Shh proteins have been shown to be over-expressed and the presence of Shh promotes proliferation and survival in the lymphoma cells (Kim *et al*., 2009, Singh *et al*., 2010).

In multiple myeloma (MM), a plasma cell malignancy of the bone marrow, which is characterised by abnormal proliferation of plasma cells known as Myeloma cells, these myeloma cells have been shown to secrete Shh themselves and this in turn, enhanced the proliferation of nearby MM plasma cells and protected them against stress-induced apoptosis and chemotherapy-induced apoptosis (Liu *et al*., 2014). Additionally, it has been shown that inhibition of Smo prevented the growth of the MM, thus suggesting that Hh signalling is required for the maintenance of the proliferative and clonally differentiated state of the MM plasma cells (Peacock *et al*., 2007).

Taken together, these findings indicate a role for Hh signalling in promoting the survival and proliferation of both normal and neoplastic B cells (Porro *et al*., 2009). Thus, understanding the mechanism by which Hh signalling regulates B cell development might lead to the development of novel strategies for modulating B cell function or treatment of B cell lymphomas.

1.4. Aims and objectives of research

1.4.1. Overall aim

Although the role of Hh signalling in T cell development is widely documented, the role of Hh signalling in peripheral B cell development is much less well understood. Initial investigations describe a positive regulatory effect of Hh signalling in driving peripheral GC B cell development (Sacedon *et al*., 2005). However, in T cells, Hh signalling can drive both a positive (Chan *et al*., 2006) and a negative (Rowbotham *et al*., 2007) regulatory effect to T cell development in the periphery and therefore, it may be conjectured that something similar may happen during B cell development. Thus, the major aim of the research presented in this thesis is to understand more precisely the

role of Hh signalling in B cell development in the periphery by gaining a better understanding of the cellular and molecular events that occur in the presence of exogenous Hh recombinant protein as well as in the absence of Hh signalling.

1.4.2. Objectives

- To determine the effects of rShh treatment on B cell survival and activation in a developmental time course.
- To determine the effects of Hh signalling on B cell function, including cytokine secretion, antibody production and B cell differentiation.
- To test the effect of the loss of Hh signalling on peripheral B cell development.
- To identify the difference between the transcriptome profiles in B cells isolated from Dhh knockout mice and wild-type mice.
- To identify target genes in B cells responding to Hh treatment *in vitro*.

1.4.3. Research hypothesis

Hh signalling can regulate B cell development and immune response in the periphery displaying both a positive and negative regulatory effect. These differential effects can explain, at least partially, the development of B cell lymphomas or autoimmune diseases in which Hh signalling is involved in its pathogenesis.

1.4.4. Original contribution to knowledge

Up until now, there has been no detailed report on the effect of Hh signalling on the development of B cells in the periphery at the molecular level. The original contribution of this research is the demonstration that Hh signalling could not only accelerate the rate of peripheral B cell development as previously published but also play a role in dampening down the immune response at a later point. This provides a mechanism whereby Hh signalling might serve to up-regulate the B cell immune response on first seeing antigen but also have the capacity to down-regulate the immune response when it is no longer needed thereby controlling the homeostasis of the immune response. Hh signalling was also demonstrated to regulate the expression of a number of target genes that can in turn regulate B cell development. The data collected during this research project could be used to inform future research into how the Hh signalling pathway may be down-regulated in neoplastic B cells or during autoimmunity, thus ameliorating the pathology. In particular, target genes that have been identified may be used as novel therapeutic targets in the treatment of B cell lymphomas and also in autoimmune diseases such as systemic lupus erythematous.

Chapter 2: Methods and Materials

2.1. Mice

Female BALB/c mice were purchased from Harlan Ltd U.K and maintained in the lab at University of East London, London. Female C57BL/6 wild-type and Desert knockout (Bitgood *et al*., 1996) mice were obtained from Professor Tessa Crompton at University College London. Mice were used when they were 4-12 weeks old and at this time spleens were isolated and single cell suspensions were prepared by crushing the spleen between a pair of frosted slides in AIM-V (Life Technologies, USA) medium containing, 10% Fetal Calf Serum and 100µM β-mercaptoethanol (β-ME) (Sigma-Aldrich, USA).

2.2. Cell culture

Splenic B cells were cultured at 3 x 10^6 to 5 x 10^6 cells/ml in AIM-V medium (Life Technologies, USA) per well in a 96 micro-titre plate (Thermo Fisher Scientific, UK). B cells were stimulated using 5µg/ml anti-mouse CD40 functional grade purified (anti-CD40) (e-Bioscience, CA, USA) and F(ab)2 anti-mouse IgM µ chain functional grade purified (anti-IgM) (e-Bioscience, CA, USA). Recombinant mouse sonic hedgehog protein (rShh) (e-Bioscience, CA, USA) was added at varying doses ranging from 0.005-5µg/ml (Shah *et al*., 2009). In some experiments, recombinant mouse desert hedgehog (rDhh) (R&D systems, Minneapolis, USA) or recombinant mouse Bone morphogenic protein 4 (rBMP4) (R&D Systems, USA) was added in varying concentrations ranging from 0.005-5µg/ml. For BMP interplay experiments, recombinant Noggin (e-Bioscience, USA) was added at a concentration of 5µg/ml (Kersten *et al*., 2005). For B cell RNA extraction, cells were cultured in a 24 well plate

or 48 well plate (Thermo Fisher Scientific, UK). B cells were cultured at 37^{o}C and 5% CO_2.

2.3. B cell purification

Single cell suspensions obtained from spleen of mice were subjected to purification using the Easy Sep Mouse B cell enrichment kit (Stem Cell Technologies, U.K.). Purified B cells were isolated by using negative selection using biotonylated magnetic beads and the method was performed according to the manufacturer's instructions. Briefly, spleen homogenate is suspended in 1X Phosphate buffered saline (PBS) (Sigma-Aldrich, USA) containing 2% Fetal Calf Serum (FCS) (Lonza, Switzerland) before Rat serum and B cell enrichment cocktail is added for 15mins at 4^{o}C. Then, Biotin selection reagent is added for 15mins at 4^{o}C before magnetic beads are added to bind to non- B cells. The eluate containing the B cells is removed and the non B cells bound to magnetic beads are retained within the magnet. Efficiency of purification ranged from 97% to 99% and purity was assessed using mouse B cell surface marker B220 and CD19 on the Accuri C6 Flow cytometer (BD, Bioscience, UK). Purified B cells were cultured as described above in Section 2.2.

2.4. Antibodies and flow cytometry

2.4.1. Cell surface staining

Activation and differentiation analysis on B cells was carried out by staining splenocytes with a combination of fluorescently conjugated antibodies supplied by e-bioscience (CA, USA) unless otherwise indicated. In other to avoid non-specific Ig trapping by the antibodies to FC receptors, cells were first blocked with CD16/CD32 (e-Bioscience, CA, USA). Cells were stained using fluorescently labelled anti-mouse markers. The following markers were used including anti–B220$^{Pe\text{-}cy5}$ anti-CD23^{PE}, anti-

CD25^{FITC} (BD-Bioscience, CA, USA), anti-CD27^{FITC}, and anti-CD80^{APC}. Characterisation of B cell subsets in the spleen was carried out using IgM^{PE}, IgD^{FITC}, anti-CD21^{APC} (Biolegend, UK), anti-CD23-^{PeCy5}, and anti-CD24^{PE}. Combinations of antibodies were mixed in staining buffer (Sterile PBS supplemented with 2% FCS (Lonza, Switzerland)). Staining of cells were carried out on ice and incubated for 30 to 60mins. After incubation, cells were washed with FACS washing buffer (Sterile PBS supplemented with 2% FCS (Lonza, Switzerland)). The mixture was then centrifuged at 1200rpm, for 5mins at 5^{o}C before analysis by the Accuri C6 Flow cytometer (BD-Bioscience, UK).

2.4.2. Annexin-V apoptosis staining assay

Annexin-V- staining was carried out using an Annexin-V-FITC apoptosis detection kit (e-Bioscience, CA, USA) according to manufacturer's instruction. Briefly, cells were stained as described above in Section 2.4.1, after which they were washed with Annexin-V binding buffer. The cells were then stained with Annexin-V-FITC (e-Bioscience, CA, USA) for 15mins at room temperature in the dark before collecting data using Accuri C6 Flow cytometer (BD-Bioscience, UK).

2.4.3. Carboxyfluoroescein diacetate succinimidyl ester (CFSE) dye proliferation assay

CFSE (e-Bioscience, CA, USA) staining was carried out to assess proliferation. Purified B Cells were washed with 1X PBS and then re-suspended in 1X PBS before staining with 10µM of CFSE (e-Bioscience, CA, USA) in the dark at 37^{o}C for 15mins. Labelled cells are then washed with 1X PBS containing 5% FCS (Lonza, Switzerland) to stop the reaction before washing with AIM-V medium (Life technologies, USA). Cells were cultured in AIM-V medium with 5µg/ml anti-CD40/anti-IgM with or without 0.005µg/ml and 0.05µg/ml rShh before addition of 5µg/ml Interleukin-4 (IL-4) (e-

Bioscience, USA) and incubated at 37ºC and 5% CO_2 for 72hours and 96hours. Cultured cells were stained with anti-$CD23^{PE}$ and anti-$CD25^{PeCy5.5}$ (BD,Pharmigen, San diego, CA) as described above in Section 2.4.1 and analysed by flow cytometry on the Accuri C6 (BD, Bioscience).

2.4.4. Intracellular staining for IL-10

IL-10 intracellular expression was detected using the BD cytofix kit (BD-Bioscience, CA, USA). For IL-10 expression, cells are stimulated with cell stimulation cocktail plus transport inhibitor (e-Bioscience, CA, USA) for the last 5hours of culture. Intracellular staining for IL-10 was carried out using anti-mouse $IL\text{-}10^{FITC}$ (e-Bioscience, CA, USA) or mouse $IgG2b^{FITC}$ (BD Pharmigen) as isotype control, cells were stained with a combination of $CD23^{PE}$ and $CD25^{APC}$. Cells were washed twice with FACS buffer. The cells were then fixed using the cytofix permeabilized using the BD cytofix/cytoperm fixation / permeabilization solution with BD golgi stop (B.D. Bioscience, CA, USA) for 30mins in the dark. Following this, cells were washed twice with BD perm wash before staining with $IL\text{-}10^{FITC}$ in permeabilization buffer (e-Bioscience, CA, USA). After staining, samples are washed with FACS buffer and analysed on Accuri C6 cytometer (BD-Bioscience, UK).

2.4.5. Functional sorting assays

Purified B cells were cultured for 40hours as described above in Section 2.2, the cells were treated with unlabelled CD16/32 before staining with the appropriate antibodies, $CD23^{FITC}$, $CD25^{PE}$ (e-bioscience CA, USA). The cells were washed and maintained on ice during the entire procedure. The cells were then sorted using the FACSAria III (BD-Bioscience) at the Institute of Child Health (ICH) Unit (University College London) into three populations, CD23+CD25-, CD23+CD25+ and CD23-CD25+. The sorted

populations were reactivated using anti-CD40/anti-IgM and cultured overnight before analysis for cell surface markers using the Accuri C6 Flow cytometer (BD-Bioscience, UK). The morphology of activated sorted B cell subsets was captured using the EVOS® line of Cell Image System (Advanced Microscopy Group, Washington, USA). Supernatant was collected and analysed for secretion of antibody, IL-6 and IL-10 using ELISA (e-Bioscience). Characterisation of sorted B cell subsets was done as described in Section 2.4.1.

2.4.6. Gating strategy

Cells were stained and collected using the Accuri C6 flow cytometer (BD-bioscience, UK). To distinguish between viable and dead cells, a side scatter (SSC-A) and forward scatter (FSC-A) plot was plotted. Higher granularity indicates higher SSC-A and thus dying cells. Peripheral B cell subsets were identified by following the protocol published by Carsetti, 2004 and Pillia and Carriappa, 2009 (Carsetti 2004, Pillia and Cariappa, 2009).

2.5. Enzyme linked immunoabsorbent assay (ELISA)

2.5.1. Antibody ELISA

Supernatants obtained from purified B cells cultured with or without rShh at 18 and 40hours were analysed for the presence of different isotopes of immunoglobulins using the Mouse Ig isotypes detection ELISA kit (e-Bioscience, CA, USA). The procedure was followed as per instructions of the kit. Briefly, Coastar 96 well plate was coated overnight at 4^{o}C with capture antibody. It is then blocked for 24hours at 4^{o}C. Plates were washed using 1X PBS containing 0.05% Tween-20 (Sigma-Aldrich, UK) before 50µl of supernatant samples were added accordingly for a 1hour incubation. Plate was washed and a detection antibody linked to horseradish peroxidase (HRP) was added.

The substrate was added before the addition of the stop solution (1N H_2SO_4). An internal positive control was used and the negative control used was cell culture medium. The plates were read at λ= 450nm and λ= 570nm and values were subtracted to get actual values using the Thermo Multiskan plate reader (Thermo,USA).

2.5.2. Cytokine ELISA

For IL-10 secretion and IL-6 secretion, supernatants was analysed using the mouse IL-10 ELISA ready set go kit (e-Bioscience, CA, USA) and Mouse IL-6 ELISA ready set go kit (e-Bioscience, CA,USA) respectively. The procedure was followed as per instructions of the kit. Briefly, Coastar 96 well plate was coated overnight at 4^{o}C with capture antibody IL-10 or IL-6. After washing with ELISA wash buffer (1X PBS/0.05% Tween-20) to remove unbound antibody, it was then blocked for 2hours at room temperature. The plate was washed before adding 100µl of supernatant samples along with a serial dilution of recombinant protein accordingly before an overnight incubation was carried out. The plate was washed and a detection antibody for each individual cytokine was added. The plate was washed again before addition of secondary antibody conjugated to HRP for 30mins. After washing of the plate, the substrate was added and colour was allowed to develop for 15mins. Then a stop solution was added to the plate (1N H_2SO_4). The plates were read at λ= 450nm and λ= 570nm and values were subtracted to get actual values using the Thermo Multiskan plate reader (Thermo,USA). A standard curve was plotted from the recombinant protein in order to get the concentration of the unknown values.

2.6. RNA extraction and complimentary DNA (cDNA) synthesis

Cultured B cell suspensions were pelleted and re-suspended in appropriate amounts of lysis buffer containing β-ME (Sigma-Aldrich). RNA was extracted according to the

protocol of the PureLink® RNA Mini Kit (Life technologies, UK) including a DNase digestion step using PureLink® DNase set (Life technologies, UK). The quality and quantity of RNA was assessed using the Nanodrop ND-1000 spectrophotometer (Nanodrop technologies, Wilmington, US). Samples with a λ= 260/280nm ratio between 1.8 and 2.1 were used in experiments. cDNA was synthesised from this RNA using the iScript^{TM} Select cDNA Synthesis Kit (Bio-Rad, CA, USA). The reaction was made up of 300-400ng RNA and the components provided in the kit including reverse transcriptase, Oligo(dt) primers according to the manufacturer's protocol. A total volume of 20µl was made and incubated using the Biorad T100^{TM} Thermal Cycler (Bio-Rad, Inc., US) at 42^{o}C for 65mins to allow elongation and it was heated to 85^{o}C for 5mins to inactivate the reverse transcriptase and terminate the reaction. RNA was stored at -80^{o}C and cDNA at 4^{o}C or -20^{o}C.

2.7. Quantitative real- time reverse polymerase chain reaction (qRT-PCR)

Quantitative real time polymerase chain reaction (qRT-PCR) was performed in triplicate on cDNA samples synthesised from cultured B cells RNA using Mx 3000p^{TM} Stratgene Real-Time PCR system (Stratagene, US) using the iTaq^{TM} Universal SYBR® Green Supermix (Bio-Rad, USA) following the manufacturer's instruction. The house-keeping gene Hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used to allow quantification of template and normalisation of each gene. Amplification of *Hprt* was quantified using a dilution series of neat cDNA, 1:10, 1:100, 1:1000 and 1:10000 prepared from embryo head RNA or control stimulated B cells RNA and relative standard curve for the gene of interest was generated by a 1:10 serial dilution of RNA prepared from cultured B cells. The efficiency of the qRT-PCR was determined for each gene with the slope of a linear regression model (Pfaffl, 2001). The efficiencies (E) were calculated according to the equation: $E= (10^{(-1/slope-1)} \times 100)$ (Fraga *et al*., 2008).

The primers used for the reaction are listed below in Table 2.1. All primers were purchased from Qiagen (Quantitect primer assay) and the primer sequences are protected (Qiagen, Inc, US). Each reaction contained approximately 100ng of cDNA, 300nM of forward and reverse primers, 10µl of SYBR green super-mix (containing, iTaq DNA polymerase, dNTPs, $MgCl_2$). The reaction was made up to 20µl using nuclease free water (Qiagen Inc., US) in a 96 well plate (Thermo Scientific, U.K).

qRT-PCR reaction was performed under these conditions for each primer used according to the manufacturer's protocol: initial denaturation at 95^{o}C, for 30s followed by 40 cycles of denaturation at 95^{o}C for 30s and annealing at 60^{o}C for 30s. In each experiment, a melting curve was obtained according to the manufacturer's programme in order to check the melting temperatures of the products so as to avoid contamination with primer-dimer and confirm the product size was as expected (Appendix 2.1).

Table 2.1. List of Quantitect primers (Qiagen, US)

Primer	Annealing Temperature	Primer Sequence	Catalogue Number
Hypoxanthine guanine phosphoribsyl transferase (*Hprt*)	60°C	Primer sequence protected (Quantitect Primer Assay) (Qiagen Inc, U.S)	**QT00166768**
Tumour Necrosis receptor associated factors 2 (*Traf2*)			**QT00103082**
Bone Morphogenic Protein 2 (*Bmp2*)			**QT01054277**
Bone Morphogenic Protein 4 (*Bmp4*)			**QT00111174**
Bruton's Tyrosine Kinase (*Btk*)			**QT00102179**
Nuclear Factor of activated T Cells c1 (*Nfatc1*)			**QT00167692**
BCL2/ adenovirus E1B interacting protein (*Bnip3*)			**QT00100233**

2.8. Microarray and data analysis

Purified B cells isolated from Dhh-/- and Dhh+/+ were cultured for 18hours and 40hours with or without 0.05μg/ml rShh and cells were lysed. RNA was purified using the PureLink® RNA Mini kit (Life Technologies, USA) as described in Section 2.6. An additional ethanol precipitation step was carried out in order to improve the quality of RNA. Purity of the RNA was assessed using a Thermostat Nanodrop spectrometer and Bioanalyser 2100 (Agilent, UK) University college London genomics, ICH, processed total RNA and RNA was hybridised to an Affimetrix Mouse Gene 1.0 ST platform. Data analysis was carried out as described in Furmanski *et al*., 2013. Briefly, CEL files produced were normalised using Robust Multi Array (RMA) algorithm. Differentially expressed genes were identified using the eBaye's method t-test by $p \leq 0.05$ considering a false discovery rate by Limma (Bioconductor). Principal component analysis (PCA) was performed using the CRAN package (Bioconductor, U.S). Genes identified from PCA was verified using qRT-PCR as described in Section 2.7.

2.9. Data analysis

Statistical analysis were performed using GraphPad Prism Software (Version 5.0; GraphPad Software Inc., USA) or Microsoft Excel. Error bars represent standard error of the mean (S.E.M). Dot plots and histograms were generated by Accuri C-flow software (BD- Bioscience, UK). P- Values were generated using unpaired Student *t* test and denoted as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. P values less than or equal to 0.05 were considered significant. Student *t* test was used because we are comparing the treated B cells with the control, and the samples have unequal variance.

For qRT-PCR data analysis, relative expression data were obtained using the relative standard curve method (Bolha *et al*., 2001). To do this, relative standard curves were

generated for the target gene and the housekeeping gene by plotting the Ct value against the logarithmic value of the cDNA concentrations. Ct is defined as the threshold cycle where fluorescent signal of the reporter dye increases significantly. The numerical value of Ct is inversely related to the amount of amplicon in the reaction (Schimittgen and Livak, 2008).

After obtaining the standard curves for both the target gene and housekeeping gene, gene expression in the samples was calculated using the equation of the standard curve. To calculate relative expression of the gene of interest, the data was normalised to the house-keeping gene, *Hprt* as follows (gene expression in target gene/ gene expression in housekeeping gene) (Appendix 2.2). Statistical analysis for RT-PCR result was carried out as described above.

Chapter 3: The role of Hh and BMP signalling in B cell activation and survival in a mixed splenocyte population.

3.1. Introduction

3.1.1. Hh signalling in the spleen microenvironment

The spleen is a unique lymphoid organ that is important for the maintenance of immune homeostasis. The spleen combines the innate and the adaptive immune system in a bgcompartments including marginal zone, red pulp and white pulp with each area having different functions (Cesta, 2006, Mebius and Kraal, 2005, Rosado *et al*., 2014). The spleen contains a variety of cells including macrophages, erythoid progenitors, T cells, B cells and dendritic cells that each have discrete functions. The majority of the B cells produced in the bone marrow enter the secondary lymphoid tissue, most commonly the spleen, which is the major site for early peripheral development and exposure of these immune cells to foreign antigen (Su *et al*., 2004, Carsetti *et al*., 2004). The spleen contains cells and factors that can support this maturation of B cell development in the periphery (Su *et al*., 2004).

Stromal cells of the spleen have been previously shown to secrete Shh (Dierks *et al*., 2007, Perry *et al*., 2009) and Dhh (Lau *et al*., 2012) as well as the receptor components of the Hh signalling pathway (Lau *et al*., 2012). Sacedon *et al*., 2005 showed that Shh is secreted by FDCs in the spleen and furthermore, the GC B cells of the spleen express the receptors of the Hh signalling pathway, Ptch and Smo, thus indicating that they can respond to this signalling pathway (Sacedon *et al*., 2005). In 2002, Lowrey *et al*., had previously shown that activated and resting CD4+ T cells in the spleen of the mouse

express the components of the Hh signalling pathway including Smo, Shh, Ptch and Gli-1 (Lowrey *et al*., 2002) and that these T cells were capable of responding to Shh thus indicating a role for Hh signalling in driving T cell maturation in the spleen. Additionally, Hh produced in the spleen has been shown to have an effect on splenic cell development in terms of inducing immune cells themselves to secrete further morphogens in response to Hh signalling. Dierks *et al*., 2007, showed that Hh produced in the spleen by the stromal cells could support the proliferation and survival of B cell lymphoma (Dierks *et al*., 2007). Perry *et al*., (2009), showed that secretion of Hh by the splenic stroma could induce BMP-4 expression in bone marrow cells, which in turn has an effect on the development of erythoid precursors in the spleen in response to stress (Perry *et al*., 2009).

Taken together, these data indicate that Hh signalling plays a number of distinct roles in the development and maturation of different haematopoietic lineages in the spleen.

3.1.2. Bone morphogenic protein and its role in B cell function.

Bone morphogenic proteins (BMP) are glycoslyated secreted signalling molecules that belong to the Transforming Growth Factor beta family (TGF-β) of cytokines. They control cellular processes such as proliferation, apoptosis, migration and differentiation of many cell and tissues (Ali and Brazil, 2014, Brazil *et al*., 2015). There are more than 20 members of the BMP protein family, which have been sub-grouped according to their sequence homology, structure and function (Nohe, 2004, Miyazono *et al*., 2010). BMPs play a role in embryogenesis and are potent stimulators of bone formation in adults, hence their name. They are also involved in the regulation of the hematopoietic stem cell (Huse *et al*., 2011, Bhatia *et al*., 1999).

BMP proteins are synthesised as precursor proteins with a hydrophobic stretch of approximately 50-100 amino acids which must be proteolytically removed from the carboxy-terminus to form functional protein (Ali and Brazil, 2014). BMP proteins signal via two types of serine-threonine kinase receptors, type I (BMPR-I) and type II (BMPR-II) to which they bind to with different affinities (Huse *et al*., 2011, Miyazono *et al*., 2010). Activation of type II receptors by a ligand leads to the activation of the type 1 receptor thus leading to the dimerization of the complexes. The activated type I receptor complex phosphorylates the intracellular R-Smads (receptor regulated Smads), which include Smad1, 5, 8. (Guo and Wang, 2009, Huse *et al*., 2011, Kersten *et al*., 2005). Activated R-Smad interact with Smad4 to form a complex that translocates to the nucleus where it regulates the expression of specific target genes through the interaction with a range of DNA binding proteins and transcriptional co-activators and repressors (Ali and Brazil, 2014, Brazil *et al*., 2015, Guo and Wang, 2009). A schematic diagram illustrating BMP signalling pathway is shown in Figure 3.1.

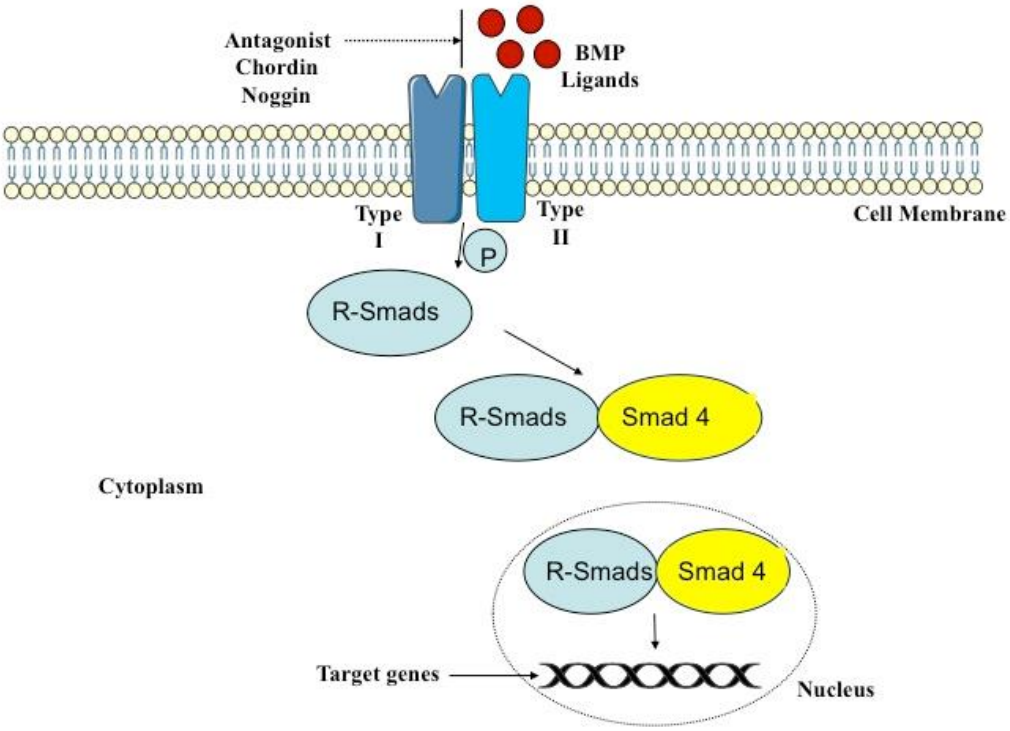


Figure 3.1. BMP signalling pathway

A scheme illustrating the BMP signalling pathway. Binding of BMP ligand to type II receptor, leads to type I phosphorylating R-Smads, which pairs with Smad-4 to form a complex. This complex translocates to the nucleus where it acts on target genes. Antagonists of the BMP pathway include Noggin and Chordin, which acts to prevent the binding of the BMP ligands to the receptors. Adapted from (Lo *et al*., 2014).

In immune cells, BMP signalling controls a myriad of events that includes apoptosis, cell proliferation, differentiation and effector functions (Miyazono *et al*., 2010, Yamashita *et al*., 1996). In lymphocytes, BMP signalling has been shown to inhibit T cell lymphopoiesis (Varas *et a*l., 2009, Yoshioka *et al*., 2012) and B cell lymphopoiesis (Kersten *et al*., 2005). In B cells, Huse *et al*., (2011) using a system in which purified human CD19+ B cells co-cultured with CD40L/IL-21 induced differentiation of plasmablasts. They showed that upon addition of BMP 2, 4, 6 or 7 to the cultures, there was an arrest in the differentiation towards plasmablasts. Co-culture with BMPs was

also shown to reduce Ig production by purified B cells and down-regulate proliferation of naïve and memory B cells (Huse *et al*., 2011). The results obtained show that BMPs can provide a negative regulatory signal to B cell development. Kertsen *et al*., 2005, have reported that BMP-6 can inhibit B cell lymphopoiesis in the bone marrow by impeding B cell proliferation via the upregulation of transcription factors inhibitors of differentiation (Id) 1 and 3. Id1 and Id3 are important target genes of BMP signalling (Kersten *et al*., 2005, Miyazono *et al*., 2005) and are negative regulators of B cell maturation and function (Sugai *et al*., 2004, Tsalavos *et al*., 2011) and other cells including neoplastic cells such as prostate cancer cells (Strong *et al*., 2013).

Twisted gastrulation (TWIGS1) a regulator of BMP signalling, which is secreted by activated B cells but not naïve B cells, can display both an agonist and antagonist effect on BMP function. TWIGS1 has also been shown to synergistically interact with chordin (Figure 3.1) to regulate BMP activity (Tsalavos *et al*., 2011). Using TWIGS1 knockout mice, Tsalavos *et al*., (2011) highlighted the negative regulatory effects of BMP signalling in B cell function showing KO cells were hyperresponsive to T independent antigen such as LPS, resulting in decreased apoptosis, increased plasma cell production and a corresponding increase in antibody secretion (Tsalavos *et al*., 2011).

3.1.3. Objectives

In previous studies by Sacedon *et al*., 2005, Hh signalling was shown to enhance GC B cell survival and proliferation. Hh has also been implicated in a variety of B cell cancers including B cell lymphoma (Dierks *et al*., 2007) and MM (Peacock *et al*., 2007). However, the role of Hh signalling in B cells is still unclear and thus requires further research. The aim of the work presented within this chapter is to test the role Hh

signalling plays in its activation and survival of B cells in a mixed splenocyte population during a developmental time-course. The specific role of BMP signalling in mediating the effects of Hh signalling and activities of the BMP inhibitor Noggin in B cell activation and survival are also explored within this chapter.

3.2. Results

3.2.1. The positive and negative regulatory effect of treatment of exogenous rShh on B cell activation and survival in a mixed splenocyte cell population.

Spleens from BALB/C mice were isolated and homogenised before seeding into culture dishes. B cells within these cultures were activated using 5µg/ml anti-CD40/IgM (Sacedon *et al*., 2005). Anti-CD40 and anti-IgM mimics the *in vivo* activation of B cells, which involves the CD40 ligand (CD40L) on T cells binding to the CD40 receptor on an antigen-presenting cell such as B cell thus mimicking T-dependent B cell activation. Anti-IgM antibody binds to the B cell sIgM thus leading BCR crosslinking and providing a polyclonal B cell activation signal mimicking T independent B cell activation (Wortis *et al*., 1995, Sacedon *et al*., 2005). The role Hh signalling plays in B cell activation and development within these cultures was investigated by the addition of recombinant Shh (rShh) at varying concentrations ranging from 0.005-5µg/ml (Shah *et al*., 2004). Shh was used because it is the most broadly expressed homologue of Hh protein (Varsiojo and Tiapale, 2008). Cells were harvested and analysed using flow cytometry for activation and survival status using B cell activation surface marker CD23 (Bonnefoy *et al*., 1995) and apoptosis marker annexin–V (van Engeland *et al*., 1998) after 18 and 40hours of culturing. B cells in the cultures were characterized from other cell types by co-staining with the murine B cell lineage marker B220. B220 is an isoform of CD45, which is generated by alternative splicing and differs from the other

isoforms of CD45 by having a higher molecular mass (Nikolic *et al*., 2002). Combinations of B220-PE-Cy5, CD23-PE and Annexin-V-FITC were used for the staining. The 18 and 40hours post-activation time points were chosen in order to assess any effects Hh signalling might have on early B cell activation response and late B cell activation responses.

The activation marker CD23, a 45kD surface membrane glycoprotein expressed primarily by B cells and monocytes; is the low-affinity receptor for the immunoglobulin E (IgE). It is a type II integral membrane protein with a single transmembrane region. In B cells, CD23 is up-regulated after B cells are activated and it is proposed to play a role in B cell auto-stimulation and regulation of IgE synthesis (Bonnefoy *et al*., 1995). CD23 is also involved in priming B cells for antibody production and promoting the survival of GC B cells (Corominas *et al*., 1993, Olteanu *et al*., 2011).

Apoptosis is programmed cell death; it is a normal physiological procedure that is necessary for the removal of damaged and infected cells and it plays an important role in the maintenance of tissue homeostasis in multi-cellular organisms (Ditzel, 2011, Fukuda *et al*., 2006, van Engeland *et al*., 1998). Annexin-V is a Ca^{2+} lectin phospholipid, which binds to an early marker of apoptosing cells, phosphatidylserine (PS), which is translocated, from the inner side of the cell membrane to the outer side. (van Engeland *et al*., 1998).

Figure 3.2 and 3.3, shows dot-plots from representative experiments showing dual-staining for expression of CD23 and annexin-V on B220+ cells treated with rShh at 18 and 40hours post-activation stimulus respectively. It was observed that treatment of B cells with rShh can affect B cell activation and survival. At 18hours post-activation

stimulus (Figure 3.2), the data shows that the percentage of CD23+Annexin-V- cells has increased from 72.1% in the untreated control to 80.9% in B cells treated with 0.005µg/ml rShh and this is associated with a reduction in Annexin-V+ CD23+ cells from 11.8 to 5.7% respectively, indicating a pro-survival signal delivered to B cells treated with rShh at 18hours post-activation stimulus.

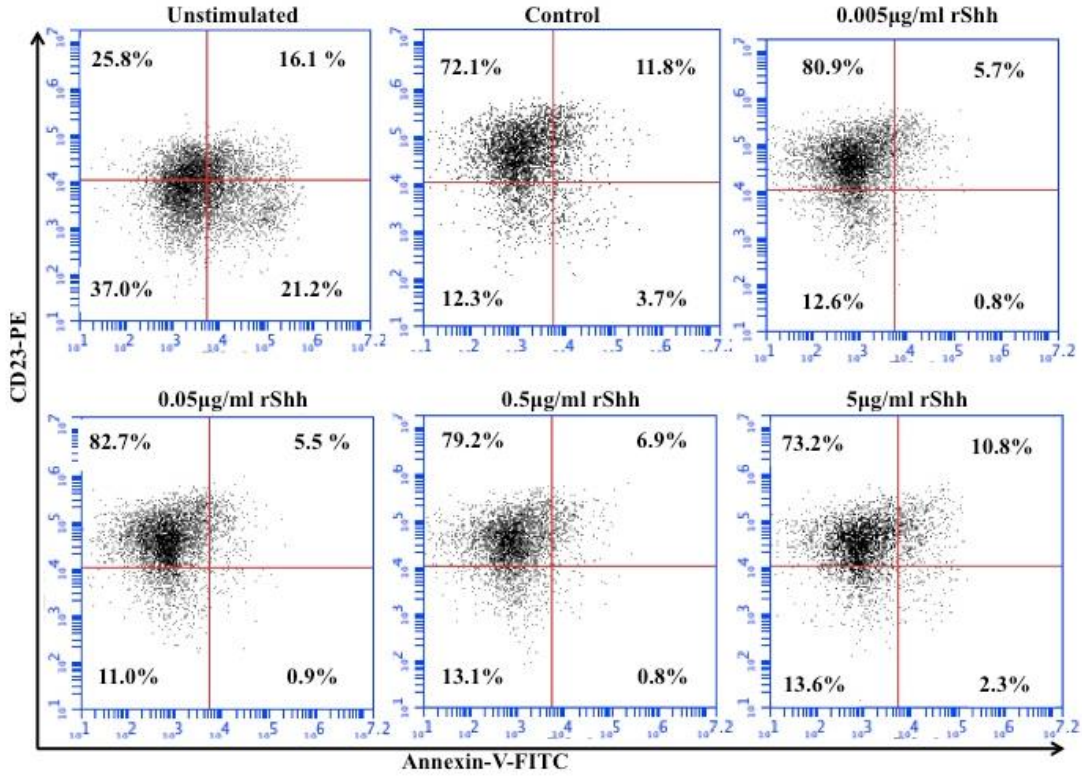


Figure 3.2. The activation and survival status of B cells at 18hours post-treatment with rShh

The dot-plots shown in the figure show the result of one experiment representative of four independent experiments. The activation and survival status of B-cells treated with varying levels of rShh at 18hours post-treatment is shown. Live cells were gated for their expression of B cell lineage marker B220 and positive cells analysed for the expression of CD23 and Annexin-V.

At 40hours post-activation stimulus, (Figure 3.3), 77.6% of B cells were CD23+Annexin-V- in the untreated control compared to 58.0% CD23+Annexin-V- B cells treated with 0.005µg/ml rShh. This was associated with a dramatic increase in death of the CD23+ cells as indicated by the presence of only 5.9% CD23+Annexin-V+

cells in the untreated control (Figure 3.3) compared with 16.7% CD23+Annexin-V+ cells in B cells treated with 0.005µg/ml rShh (Figure 3.3). Interestingly, at 40hours post-activation stimulus, a decrease in activation as judged by reduced CD23 expression was observed (Figure 3.3).

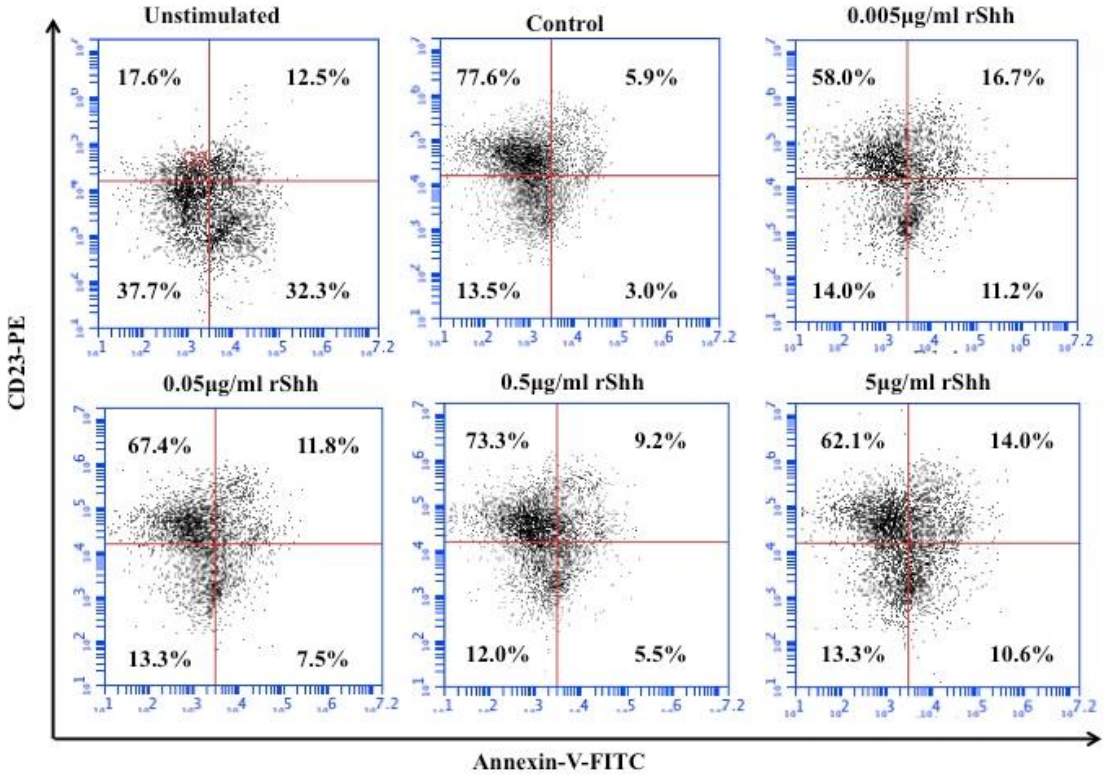


Figure 3.3. The activation and survival status of B cells after 40hours post-treatment with rShh

The dot-plots shown in the figure show the result of one experiment representative of three independent experiments. The activation and survival status of B-cells treated with varying levels of rShh at 40hours post-treatment is shown. Live cells were gated for their expression of B cell lineage marker B220 and positive cells analysed for the expression of CD23 and annexin-V. At 40hours, the expression of CD23 is decreased in rShh treated cells. A similar pattern was seen with the annexin-V staining with rShh treatment increasing the number of apoptotic cells in rShh treated cells.

To further quantify this data, the percentage of CD23+ and annexin-V + B cells was analysed independently. Figure 3.4, shows bar charts representing the statistical significance of activation and survival of B cells treated with varying concentrations of rShh in the mixed splenocyte cell population at 18 and 40hours post-activation stimulus.

B cells were identified, and gated on, using expression of the B220 surface marker before CD23 and annexin-V was analysed in this sub-population of cells.

At 18hours post-activation stimulus (Figure 3.4A), there was a small increase in the number of activated B cell as judged by CD23 expression in the presence of 0.005-0.5µg/ml of rShh. For example, there was a significant 10% increase in activation of B cells as judged by the up-regulation in expression of B220+CD23+ in the presence of 0.005 and 0.5µg/ml of rShh compared to the untreated control, thus suggesting a positive regulatory effect of Hh signalling on B cell activation at 18hours post-activation stimulus as judged by CD23 expression.

Additionally, there was a significant increase in survival of B cells in relation to untreated control in treated B cells at 18hours post-activation stimulus (Figure 3.4C) in all the doses of rShh. For example, there was approximately 40% decrease in the number of apoptotic B cells after treatment with 0.005µg/ml rShh as compared to the untreated control (Figure 3.4C). Interestingly, by 40hours post-activation stimulus, treatment with rShh induced a corresponding increase in the number apoptotic B cells in the culture (Figure 3.4D) and decrease in activation (Figure 3.4B). Additionally, when rShh was added to unstimulated B cells (result not shown) at 18hours and 40hours post-activation stimulus, there was no effect observed on B cell activation and survival, thus suggesting that the rShh effect observed in the B cells is dependent on B cell activation.

This first result obtained at 18hours post-activation stimulus is consistent with the results obtained by Sacedon *et al*. (2005), which showed that rShh, inhibited Fas mediated apoptosis in B cells in the GC (Sacedon *et al*., 2005), the latter result obtained at 40hours is entirely novel as exogenous rShh has not previously been shown to induce apoptosis in B cells and therefore requires further investigation. .

This negative effect of rShh on B cell activation and survival has not been observed before. However, treatment with rShh has been shown previously to inhibit peripheral T cell development and activation (Rowbotham *et al*., 2007).

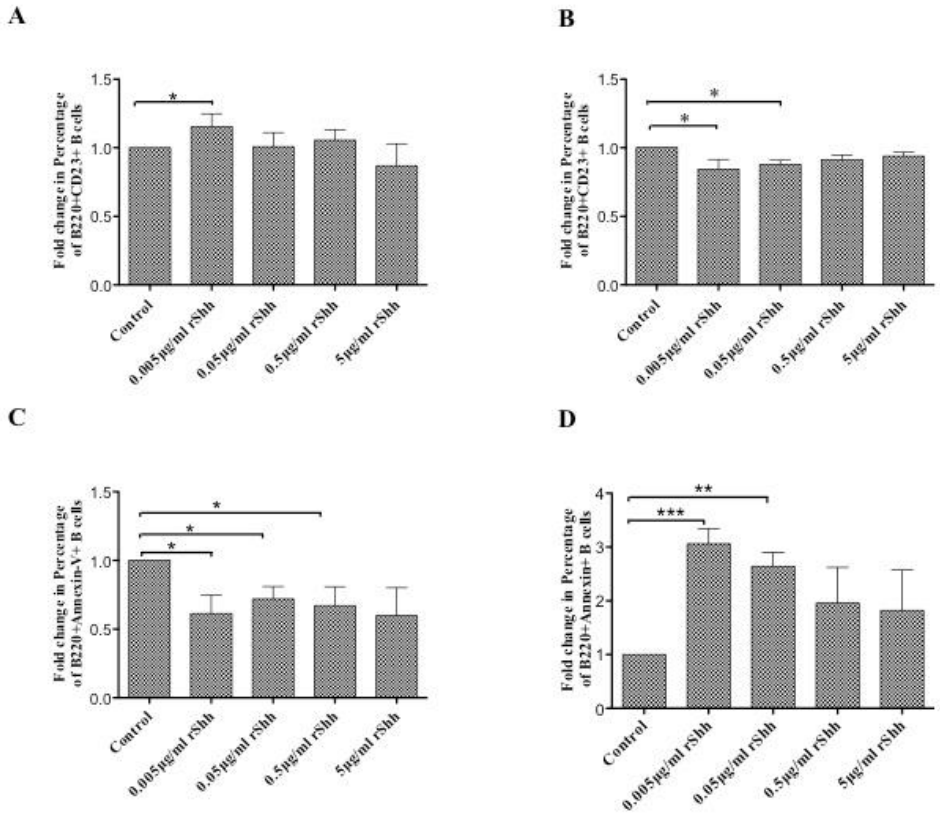


Figure 3.4. The activation and survival of B cells in a mixed splenocyte population after treatment with Shh

B cells were activated with 5µg/ml anti-CD40/IgM and were treated with rShh (0.005-5µg/ml). Cells were collected and analysed at 18hours (**A**) and 40hours (**B**) post-stimulation for B cell activation using early activation marker anti-CD23. The results shown in the bar chart are representative of three independent experiments. The significance of the differences observed in fold changes in CD23+ cells was assessed by student *t* test (*p≤0.05). B cell survival was analysed at 18hours (**C**) and 40hours (**D**) post-activation stimulus. The results shown in the bar chart are representative of three independent experiments. The significance of the differences observed in fold changes in annexin-V+ cells was assessed by student *t* test (*p≤0.05, **p≤0.01, ***p≤0.001).

3.2.2. Co-culture with BMP-4 promotes B cell death in a mixed splenocyte population

Hh signalling has been shown previously to induce BMP-4 expression *in vitro* in chick cells (Roberts *et al*., 1995) and in erythroid cells during murine stress erythropoiesis in the spleen (Perry *et al*., 2009). Perry *et al*., (2009), showed that treatment of bone marrow cells with Shh induced them to become BMP-4 responsive cells. They showed that Hh dependent signalling induces BMP-4 expression and they both act together to promote the differentiation of bone marrow progenitor cells to stress (Perry *et al*., 2009). Fukuda *et al*., 2006, showed that in response to BMP-4 signalling, mouse B cell hybridoma cells showed increased apoptosis through a p53 dependent pathway (Fukuda *et al*., 2006). In our mixed B cell cultures and with treatment with rShh, it was observed that at 40hours post-activation stimulus, there was an increased cell death in B cells (Figure 3.3D). It is possible that Hh signalling in the B cells, is leading to the induction of expression of one of its target genes, BMP-4, which itself has been shown to have a negative effect in B cell development (Fukuda *et al*., 2006, Tsalavos *et al*., 2011). To test if rShh induced BMP-4 expression could be responsible for its effects on B cell activation and survival, B cells in mixed splenocytes cultures were treated r-BMP-4 and then analysing at 18 and 40hours post-stimulus as previously described in Section 3.2.1.

At 18hours post-activation stimulus (Figure 3.5A), there was a significant decrease in B cell activation in B cells treated with rBMP-4 as judged by CD23 expression. This is associated with a dramatic increase observed in B cell apoptosis at 18hours post-activation stimulus in the presence of all doses of rBMP-4 (Figure 3.5C). However at 40hours post-activation stimulus, there was a decrease in the number of activated B cells (Figure 3.5B) and an increase in the number of apoptotic B cells after treatment with rBMP-4 (Figure 3.5D). This may reflect the fact that these apoptotic cells observed

at 18hours post-activation stimulus have now undergone extensive apoptosis and no longer form part of the live gate and therefore not included in the gated cells that were being analysed for expression of both CD23 and annexin-V.

Taken together these results suggest that BMP-4 signalling delivers a negative regulatory signal to B cell reducing their activation and promoting early apoptosis.

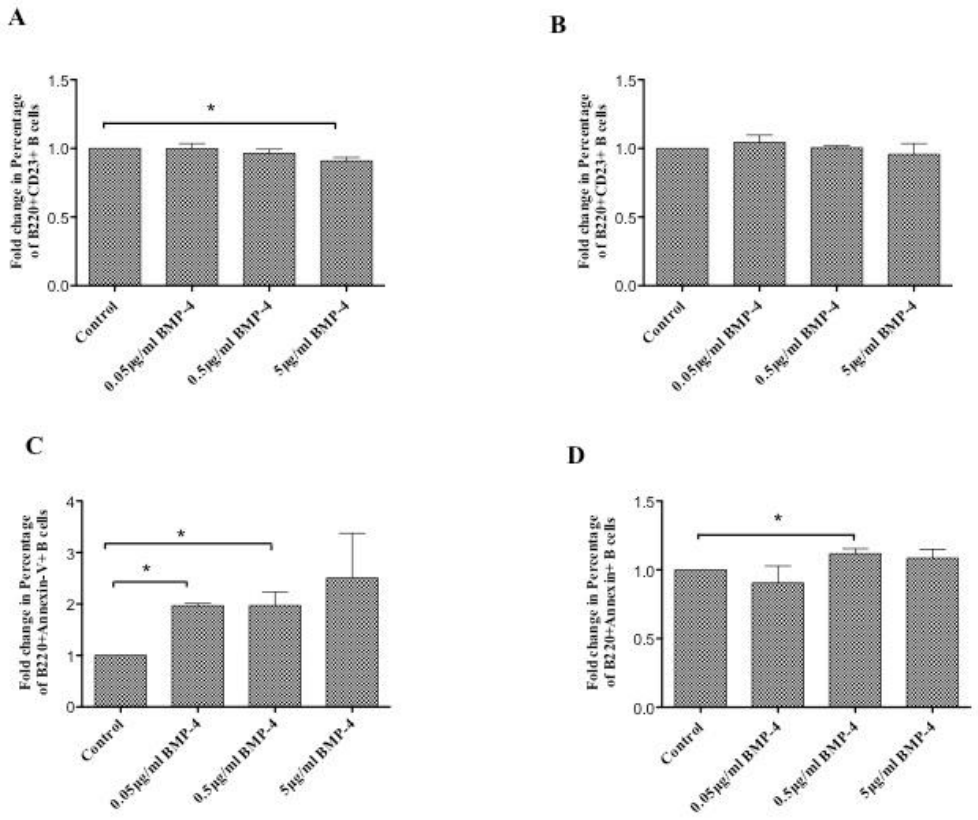


Figure 3.5. Treatment of B cells with rBMP-4 decreases B cell activation and survival

B cells were activated with 5µg/ml anti-CD40/IgM and were treated with rBMP-4. Cells were collected and analysed at 18hours (**A**) and 40hours (**B**) post-stimulation for activation B cells using anti-CD23, an early B cell activation marker and analysed for survival at 18hours (**C**) and 40hours (**D**) post-activation stimulus. The bar chart shown is representative of three independent experiments. The significance of the differences observed in fold changes in CD23+ and annexin-V+ cells was assessed by student *t* test (*$p \leq 0.05$).

3.2.3. Interplay between Hh signalling and BMP signalling.

After observing the negative regulatory effect of BMP-4 signalling on B cells activation and survival *in vitro* in a mixed splenocyte population, it became important to investigate if this BMP-4 mediated effect is also observed in the case of rShh treated B cells. To investigate this possibility, Noggin was also added to cultures. Noggin is a potent inhibitor of BMP signalling and mediates its effect by binding to several BMP proteins including BMP-2, BMP-4 and BMP-7 (Figure 3.1). By tightly binding to BMP, noggin blocks the sites on the protein required for their interaction with BMP receptors thus antagonizing BMP signalling (Figure 3.1) (La Rosa *et al*., 2011, McMahon *et al*., 1998).

Mouse splenocytes were isolated and the B cells were activated as previously described in Section 3.2.1 along with co-treatment with the BMP inhibitor, Noggin (5µg/ml) (Kersten *et al*., 2005). As this experiment was designed to analyse the negative regulatory effect of co-culture with rShh observed at 40hours post-stimulus, only these cultures were investigated. At 40hours post-activation stimulus, the splenocyte cells were collected and the B cells analysed by flow cytometry as previously described Section 3.2.1. Figure 3.6, shows dot-plots representing expression of CD23 and annexin-V on B cells at 40hours post-activation stimulus.

Co-culture of splenocytes with rShh results in a decreased level of B cell activation and increased B cell death compared to the untreated control following 40hours in culture. However, the effects of rShh are inhibited upon addition of Noggin. For example, Figure 3.6, CD23+Annexin-V- has decreased from 54.4% in the untreated control to 49.3% in B cells treated with 0.05µg/ml rShh and this result is associated with an increase in Annexin-V+CD23+ from 17.8% to 25.5%. Addition of Noggin to 0.05µg/ml

rShh treated B cells increased CD23+Annexin-V- to 55.1% and reduced annexin-V+CD23+ B cells to 18.7%. These data suggest that in our splenocyte experiments, Hh signalling was shown to significantly affect late B cell development at 40hours post-activation stimulus by decreasing levels of activation and promoting apoptosis. The effects of rShh could be partially recapitulated by addition of Noggin, a BMP inhibitor to the cultures indicating the effects of Hh signalling is due, in part, to downstream BMP signalling. Thus Hh and downstream BMP signalling may play a significant role in peripheral B cell development.

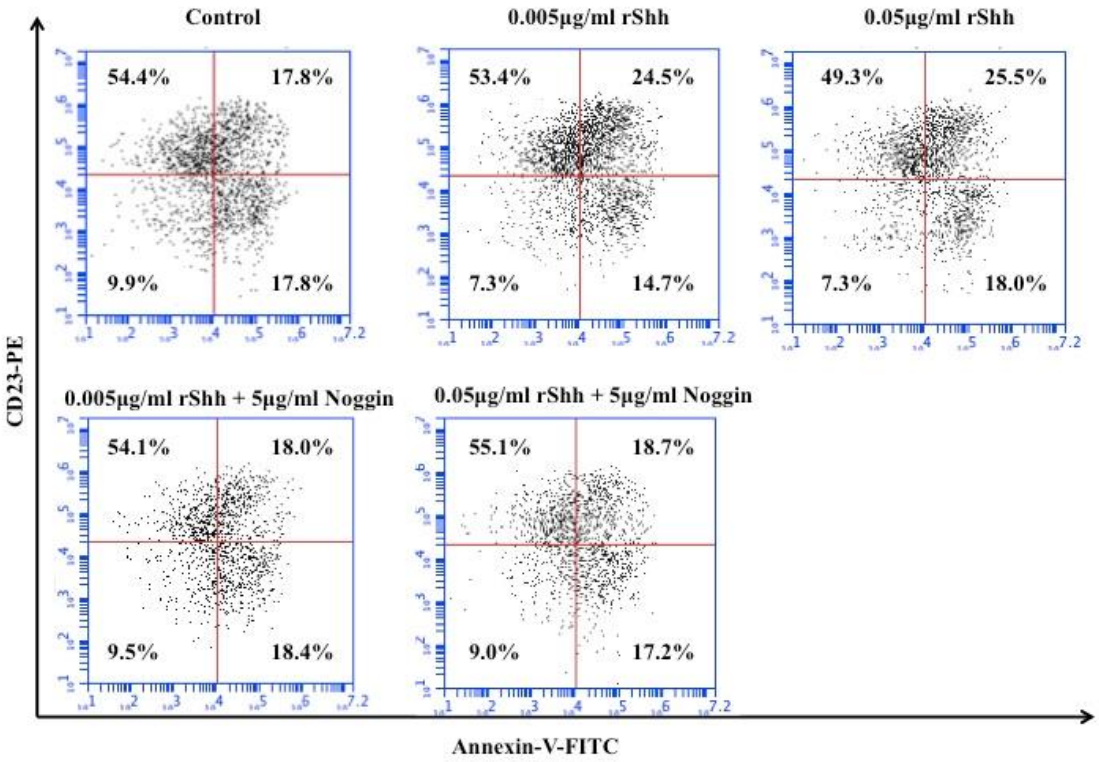


Figure 3.6. Noggin inhibits the effects of rShh treatment on B cells at 40hours post-activation stimulus

The dot-plots shown in the figure show the result of one experiment representative of three independent experiments. The activation and survival status of B-cells treated with varying levels of rShh and also in the presence of Noggin at 40hours post-treatment is shown. Live cells were gated for their expression of B cell lineage marker B220 and positive cells analysed for the expression of CD23 and annexin-V.

3.3. Discussion

It has been reported previously that Hh signalling can induce a positive regulatory effect on B cell function by regulating the apoptotic pathway thus increased B cell survival and increasing B cell antibody production (Sacedon *et al*., 2005). Similarly, Hh signalling has also been shown to be involved in the survival of neoplastic B cells such as B-CLL (Hedge *et al*., 2008) and MM (Peacock *et al*., 2007).

Activation of Hh signalling pathway can suppress the apoptotic pathway by up-regulating the expression of anti-apoptotic proteins. Hh signalling can promote P13K activation, which in turn degrades p53, which is a stimulator of apoptosis thus enhancing survival of target cells. Hh signalling can also induce other anti-apoptotic proteins such as *Bcl2* (Ditzel, 2011). However, Hh signalling can also induce the apoptotic pathway through different mechanisms involving the transcriptional up-regulation of potent inhibitors of both the extrinsic and intrinsic apoptotic pathways. Shh can induce apoptosis via the up-regulation of the Ptch receptor, Ptch is a tumour suppressor protein that when mutated is associated with neoplasia. Ptch can induce apoptosis via a caspase cleavage site at Asq 1392 and it also promotes DNA fragmentation (Ditzel, 2011,Mille *et al*., 2009). Ptch has been shown to be pro-apoptotic in a number of different systems, In the neural tube, Ptch induces apoptosis by binding to capase and this is independent of expression of the Smo receptor and Gli activity (Atwood *et al*., 2013, Mille *et al*., 2009).

Based on this idea, we hypothesised that Hh signalling can positively regulate B cell function by increasing B cell activation and survival and also negatively regulate B cell function by decreasing B cell activation and survival at different time points. The data presented in this chapter showed that Hh signalling could induce a positive signal at

18hours possibly by strengthen the BCR signal and inducing target genes that are anti-apoptotic such as *Bcl2* thus increased B cell activation and survival. At 40hours, a negative regulatory effect on B cell activation and survival was observed in the presence of Hh signalling. The negative regulatory effect observed at 40hours might also be as a result of the delayed induction of expression of another Hh target gene such as the secreted morphogen, BMP-4 (Ditzel, 2011). Hh signalling would appear to be able to fine tune the BMP and TGF-B signalling cascades through the regulation of expression of multiple ligands and inhibitors (Katoh and Katoh, 2009) thus allowing constant negative feedback in order to regulate the signalling effects mediated by these morphogens. BMP-4 has not only been shown to be a Hh target gene but has also been shown to promote death of B cells (Fukuda *et al*., 2006). The data showed in this chapter suggests that Hh signalling and BMP signalling interplay to regulate B cell development in the periphery.

Taken together, these data are consistent with the repeated findings that immune responses must first be activated by the presence of antigen but they must subsequently be down-regulated in order to prevent the onset of pathology caused by inappropriate activation of the immune response. In B cell lymphomas such as B-CLL (Hedge *et al*., 2010) and MM (Peacock *et al*., 2007), where Hh signalling has been shown to be involved in their pathogenesis, it is possible that the Hh signalling pathway is defective. The ability to induce the transcriptional up-regulation of pro-apoptotic proteins such as BMP proteins is possibly silenced in these cancerous cells thus increased survival of these cells. Hh signalling induction of down-stream BMP signalling could be investigated in B cell lymphomas.

Having identified these regulatory effects on B cell development, it will be important to repeat these studies on purified B cells in order to determine whether this effect was

intrinsic to B cells or required the presence of another cellular subset provided by the spleen.

3.4.Conclusion

In this chapter, rShh was shown to induce a positive regulatory signal soon after the onset of antigen mediated signalling events that can drive enhanced B cell activation and survival at 18hours post-activation stimulus as judged by levels of CD23 and annexin-V expression. Also, Hh signalling could deliver a negative signal to B cell activation and survival at 40hours post-activation stimulus. The negative regulatory effect observed at 40hours post-stimulus, was thought to possibly involve BMP expression. Consistent with this idea, the addition of BMP-4 to the cultures delivered a negative regulatory signal to B cell activation and survival at 18 and 40hours post-stimulus. Co-treatment of cultures with Noggin, was shown to reverse the pro-apoptotic effects mediated by the presence of rShh at 40hours post-stimulus, suggesting that these later pro-apoptotic events were indeed mediated by BMP signalling. The next chapter extends these findings of this chapter by investigating the effect of Hh signalling in purified B cells.

Chapter 4: The effects of Hh signalling in purified B cells.

4.1. Introduction

4.1.1. B cell maturation in the spleen

B cells undergo early maturation in the bone marrow and then once they express a functional antigen receptor (BCR) that does not recognise self-antigen, they leave the bone marrow and travel to the peripheral organs such as the spleen. They enter the spleen as transitional B cells, T1, T2 or T3. B cell maturation in the spleen is illustrated in Figure 1.1. Depending on the type and strength of the B cell receptor (BCR) signal, transitional B cells can then differentiate to become follicular (FO) or marginal zone B cells (MZ) (Pillai and Cariappa, 2009). It has been reported, that during peripheral B cell development, T1 B cells normally differentiate to become T2 B cells. Upon tonic BCR engagement, T2 B cells have been shown to differentiate towards FO B cells. However, TLR ligand activation in addition to BCR signalling in T2 B cell drives the differentiation of the B cells towards MZ B cell development (Su *et al*., 2004). FO B cells also have the ability to differentiate to Transitional type 2- Marginal zone Precursor B cells (T2-MZP). T2-MZP B cells form a reservoir for MZ B cells during depletion and have suppressive functions. Follicular B cells populate the B cells follicles of the spleen associating closely with follicular dendritic cells (FDCs). FDCs are stromal cells in the germinal centre and they interact with B cells thus making them a crucial component for humoral immune responses (Park and Choi, 2005).

Upon encounter of antigen, and with T cell help via CD40 ligand, FO B cells, also referred to as conventional or naïve B cells are stimulated to proliferate and thus form a germinal centre (GC). In the GC, B cells undergo affinity maturation, somatic hypermutation and isotype switching thus driving them to differentiate into effector

cells such as plasma cells capable of producing high affinity antibodies of different isotypes and memory B cells that are programmed to recognise and respond to antigens on repeat exposure (Natkunam, 2007, Su *et al*., 2004, Luu *et al*., 2014). Antibody production can be divided into T cell dependent (TD) or T cell independent (TI). TD antibody production requires activation of the B cells via a co-stimulation through the CD40, which is provided by CD40L on activated T cells. B cell activation, which does not require T cell help, is termed TI response and are mediated by antigens such as LPS (Tsalavos *et al*., 2011).

4.1.2. The production of cytokine and B cell maturation and function

Cytokines are small non-structural proteins with molecular weight ranging from 8-40kDa. Nearly all nucleated cells have the ability to synthesise cytokines and also respond to them. Cytokines can have a variety of functions, however they are primarily involved in host responses to disease (such as infection), the maintenance of cellular homeostasis, and are often secreted by immune cells in response to various stimuli. In the context of immune system, they can mediate both pro-inflammatory and anti-inflammatory responses (Dinarello, 2000, Vazquez *et al*., 2015). Cytokines can also function as growth and/or differentiation factors that act via an autocrine or paracrine manner. Many cytokines share similar functions through binding of specific cell surface receptors thus initiating signal transduction (Moens and Tangye, 2014).

B cells produce a variety of cytokines and cytokines play a major role in B cell development and differentiation (Lund, 2008). B cell differentiation is influenced by many cytokines including interleukin (IL) -2, IL-10, IL-6, IL-21, IL-13, IL-15, transforming growth factor beta (TGF-β) and IL-21 (Berglund *et al*., 2013, Luu *et al*.,

2014). In early B cell development, the cytokine, interleukin 7 (IL-7), is involved in B cell development in the bone marrow, inducing the development of pro-B cells to pre B cells (Vazquez *et al*., 2015). In the peripheral organ such as the spleen, Lymphotoxin and TNF-α, produced by B cells have been shown to control the development of follicular dendritic cells. The development of B cells in the follicles and the development of specialized stromal cell subsets in the spleen is also regulated by lymphotoxin and TNF-α (Lund, 2008).

B cells can make cytokines that amplify immune responses such as IL-6 and IL-4 but they also have the ability to make cytokines that can supress the immune system such as IL-10 and TGF-β (Vazquez *et al*., 2015). IL-10 has been shown to negatively regulate B cells and suppress harmful immune responses, regulating the balance between the different peripheral T cell responses such as Th1 and Th2 (Saraiva and O'Garra, 2010).

4.1.3. Objectives

The data presented in Chapter 3 suggests that Hh signalling can exert an apparent positive and negative regulatory effect on B cell function in a mixed splenocyte population with regards to activation and survival. However, other cell types in the spleen can also respond to Hh signalling and thus may influence the effect observed on B cells. The aim of this chapter is to investigate the effect of Hh signalling in purified B cells. The objectives of this chapter are:

- To determine the effect of Hh signalling in purified B cells, with emphasis on activation, survival, and differentiation.
- To investigate the effect mediated by Hh signalling on antibody production and cytokine secretion in B cells as well as the effects of Hh signalling on the proliferation of B cells in the periphery.

4.2. Results

4.2.1. The effect of exogenous rShh on B cell activation and survival in a purified B cell population

After examining the effect of exogenous rShh on B cells in a mixed splenoctye population at 18 and 40hours post-stimulus with respect to activation and survival, it was important to look at the effect of exogenous rShh on purified B cell populations. This is because it is possible that the effect observed in mixed cultures was due to a factor secreted by other cellular subset, contained within the spleen. For example, it was recently shown that Hh signalling is capable of polarising splenic T cells to become Th2 cells in certain pathological situations and Th2 cells in turn drive B cells to secrete IgE (Furmanski *et al*., 2013). Also, the effects of dendritic cells (DCs), or monocytes in the spleen might have an effect on the B cell function in response to Hh signalling. DCs are specialised antigen presenting cells that are involved in primary T cell response and it has been shown that human thymic DCs (Varas *et al*., 2008) and mouse FDCs (Sacedon *et al*., 2005) express the components of the Hh signalling pathway including Ptch and Smo. Varas *et al*., 2008, showed that the human thymic DCs secrete Shh which acts in an autocrine manner promoting the survival of the cells as well as influencing DC activation and allostimulatory capacity (Varas *et al*., 2008).

One factor complicating the results presented in Chapter 3 is that the marker B220, used to differentiate the B cells from other cells in the mixed splenocyte cultures can also sometimes be expressed by natural killer cells (NK) (Nikolic *et al.*, 2002), T cells (Oka *et al*., 2000) and DCs (Segura *et al*., 2009). Oka *et al*., (2000), reported that B220 is constitutively expressed in a cytoplasmic form in T cells which may translocate to the cell membrane during apoptosis (Oka *et al*., 2000). These other potential B220+ cellular subsets might have an effect on the results observed using the mixed splenocyte culture.

In order to exclude this possibility, it became important to analyse the role of Hh signalling in B cells purified by negative selection rather than on the basis of B220 expression. To this end, purified B cells were obtained using negative selection (Stem Cell Technology) and the role of Hh signalling was analysed in B cell activation, survival, proliferation and differentiation as before.

Spleens were isolated from BALB/C mice, and cell suspensions were derived by homogenization. The spleen cells that were not required are then removed out from the mixed cell suspension using magnetic beads leaving only the desired population of B cells by the process of negative selection. The resultant B cells were enriched with a purity of 97-99% as confirmed using CD19 and B220 expression in flow cytometry. Purified B cells were then activated with 5µg/ml anti-CD40/IgM and then treated with varying concentration of rShh ranging from 0.005-5µg/ml (Shah *et al*., 2009). At 18 and 40hours post-activation stimulus, cells were analysed for their degree of activation and survival. A combination of B220-$^{PE\text{-}Cy5}$, CD23-^{PE}, Annexin-^{FITC} was used to analyse activation and survival.

Figure 4.1 shows data on B cell activation and survival. As observed with the mixed splenocyte B cell culture, there was a significant increase in CD23 expression and decrease in apoptosis as judged by loss of annexin-V staining in B cells treated with rShh compared to the untreated control at 18hours post-activation stimulus (Figure 4.1A and Figure 4.1C respectively). At 40hours post-activation stimulus, there was a significant decrease in CD23 expression and an increase in apoptosis of B cells treated with rShh (Figure 4.1B and 4.1D respectively). For example, there was a significant increase of approximately 1.5 fold in CD23 expression in B cells treated with 0.005µg/ml rShh at 18hours post-activation stimulus (Figure 4.1A) and a significant decrease in annexin-V staining of 60% (Figure 4.1C). This data indicated that B cell

survival was greatly enhanced in B cells treated with 0.005µg/ml rShh at 18hours post-activation stimulus. This is followed by a decrease in CD23 expression at 40hours (Figure 4.1B) and a significant increase in death of approximately 1.5 fold as judged by annexin-V expression in B cells treated with 0.005µg/ml rShh (Figure 4.1D). These results confirm that, the results obtained using purified B cells mirrored the results obtained using mixed splenocyte cultures, it would appear that the positive and negative regulatory effect of rShh on B cell activation and survival at 18 and 40hours post-activation stimulus respectively was intrinsic to B cells and not due to any other cell type present in the mixed splenocyte cultures.

Interestingly, the effects of addition of rShh to the purified B cell cultures with respect to CD23 expression and survival were enhanced when using purified B cells in comparison to the data obtained using mixed splenocyte cultures (Chapter 3). This would indicate that the other cell types contained within the mixed cell cultures had the effect of 'dampening down' the regulation of the B cell response in the presence of Hh signalling.

We have previously used CD23 expression (in Chapter 3) to assess the status of B cell activation as it is reported that CD23 expression goes up on B cells upon activation (Bonnefoy, 1995). However, expression of CD23 along with other surface markers may also be used to characterise the developmental status of B cells in the spleen, as different subsets in the spleen, namely T1, T2, T2-MZP and follicular B cells all express varying amounts of CD23 on their cell surface (Pillai and Cariappa, 2009). For this reason, we chose to analyse expression of another cell surface marker associated both with B cell activation and B cell differentiation, CD25.

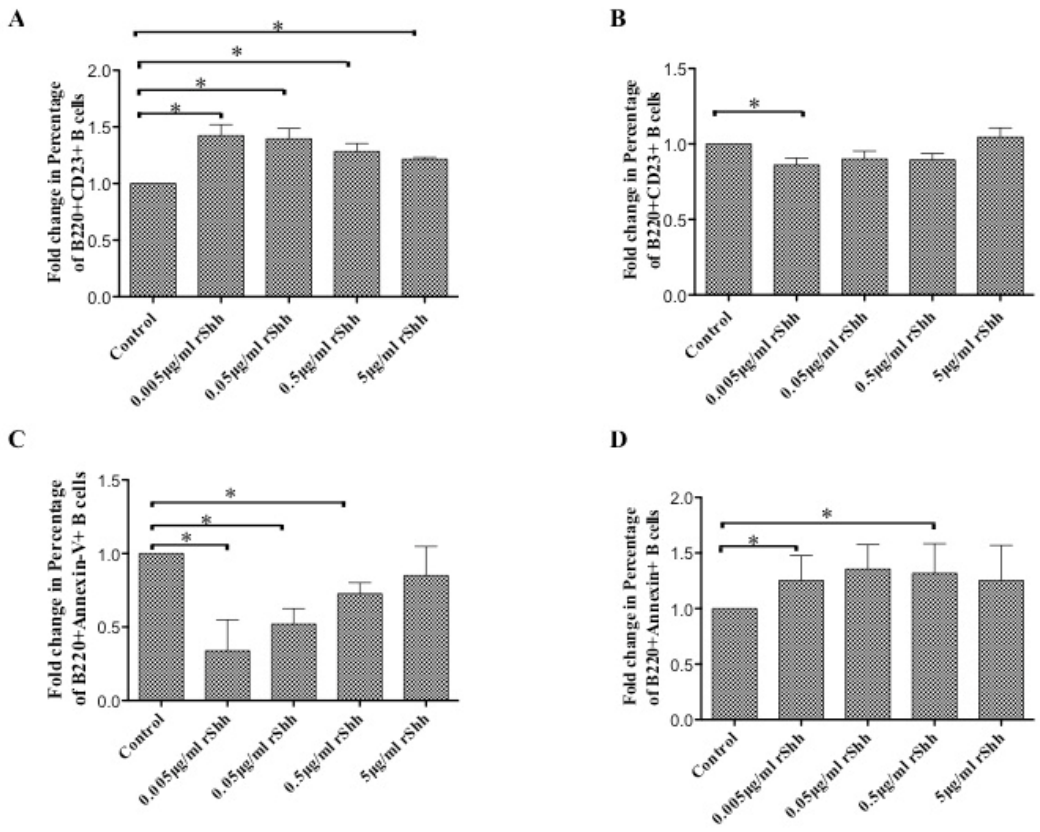


Figure 4.1. Treatment of purified B cells with rShh can affect B cell activation and survival at 18hours and post-activation stimulus

Purified B cells were activated with 5µg/ml anti-CD40/IgM and were treated with varying concentrations of exogenous rShh (0.005-5µg/ml). Cells were collected and analysed at 18hours (**A**) and 40hours (**B**) post-activation stimulus for B cell activation using anti-CD23, an early B cell activation marker. Cells were also analysed at 18hours (**C**) and 40hours (**D**) post-activation stimulus for apoptosis using annexin-V, an apoptotic marker. The results shown in the bar chart are representative of three independent experiments. The significance of the differences observed in fold changes in CD23+ cells and Annexin-V+ cells was assessed by Student *t* test (*p≤0.05, **p≤0.01).

4.2.2. CD25 expression in purified B cells treated with rShh

CD25 (IL-2Rα) is a 55kDa polypeptide with an extracellular domain, transmembrane domain and a cytoplasmic domain. The gene encoding this protein is located on chromosome 10 in humans and 2 in mice (Anderson *et al*., 1995). The CD25 protein, which forms part of the IL-2 receptor (Burlinson *et al*., 1996), is a molecule normally found on particular subsets of B cells; the main function of CD25+ B cells is still unclear. CD25 expression on B cells have been characterised in activated B cells,

memory B cells and also in regulatory B cells (Amu *et al*., 2010, Brisslert *et al*., 2006). In mice, CD25 expressing B cells from secondary lymphoid organs showed a mature and activated phenotype with high expression of the co-stimulatory molecule CD80 and CD86 (Amu *et al*., 2010). Thus, after observing the effect of Hh signalling on CD23 expression in B cell activation, we also wanted to investigate the role of Hh signalling in B cell activation using expression of CD25 as a marker of late activation.

Purified B cells were activated and then treated with or without rShh as previously described in Section 4.2.1. At 18 and 40hours post-activation stimulus, B cells were stained with a combination of CD25-^{FITC}, B220-$^{Pe\text{-}CY5}$ and CD23-^{PE}. Initially, activated B cells were analysed for their expression of CD25 alone at 18 and 40hours post-activation stimulus. Figure 4.2, shows CD25 expression in purified B cells treated with or without varying concentrations of rShh. At 18hours post-activation stimulus, relative to the untreated control, similar low levels of CD25 were expressed, indicating that this marker had not yet been expressed on the activated B cells to any significant degree. Thus, there was no significant difference in expression of CD25 in the untreated control B cells and B cells treated with rShh.

However, at 40hours post-activation stimulus, there was a very significant increase in the expression of CD25 expression on B cells treated with 0.005µg/ml rShh. Interestingly, an increase in CD25 expression at 40hours in the presence of all doses of rShh was observed with the highest increase in expression of 75% observed in the presence of the lowest dose of rShh (Figure 4.2).

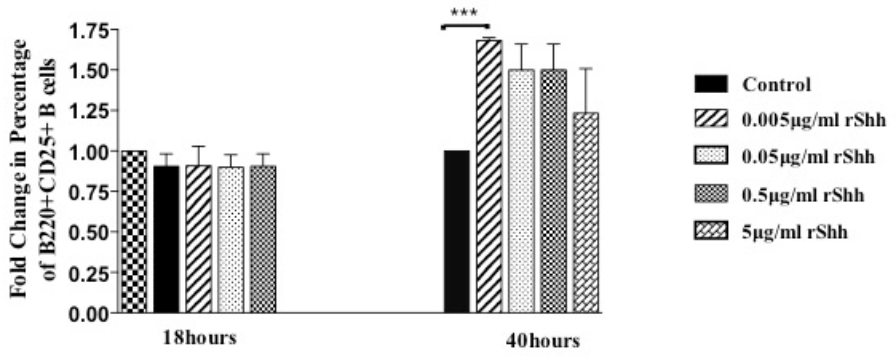


Figure 4.2. Treatment of purified B cells with rShh increases CD25 expression at 40hours post-activation stimulus

Purified B cells were activated with 5µg/ml anti-CD40/IgM and were treated with varying concentrations of exogenous rShh (0.005-5µg/ml). Cells were collected and analysed at 18 and 40hours post-activation stimulus. B220+ cells were analysed for anti-CD25, a late B cell activation marker. The results shown in the bar chart are representative of three independent experiments. The significance of the differences observed in fold changes in CD25+ cells was assessed by Student *t* test (*$p \leq 0.05$).

We then analysed the co-expression of CD23 and CD25 on the purified B cells following co-culture with the activating antibodies and treatment without or with rShh. Figure 4.3 and 4.4, shows dot-plots of B cells positive for activation marker CD23 and late activation marker CD25 at 18 and 40hours post-activation stimulus respectively. Similar low levels of CD25 expression was observed in B cells at 18hours post-activation (Figure 4.3) indicating that CD25+ B cells do not appear at this early time point in our system. At 18hours post-activation stimulus, the percentage of cells that are CD23+CD25- was higher in B cells treated with rShh when compared with the untreated control.

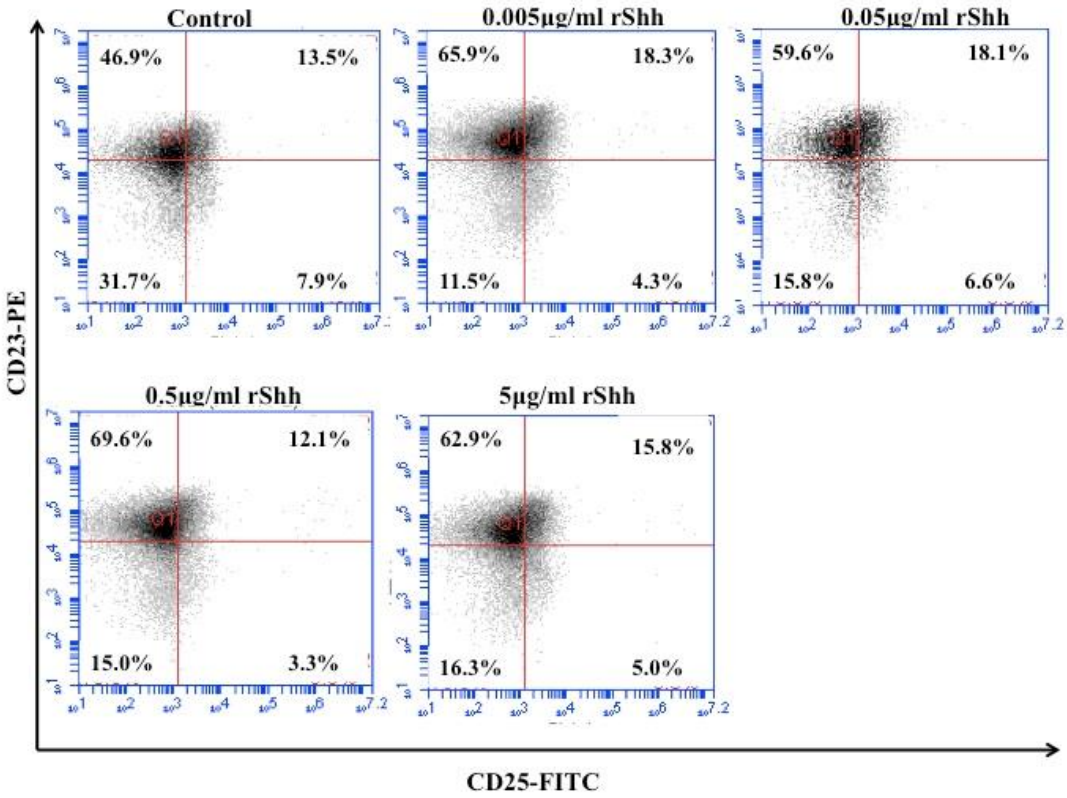


Figure 4.3. CD23 and CD25 co-expression in rShh treated B cells 18hours after activation

The dot-plots shown in the figure show the result of one experiment representative of three independent experiments. The co-expression of CD23 and CD25 of purified B cells treated with varying concentrations of rShh (0.005-5µg/ml) at 18hours post-activation stimulus is shown where it was observed that CD23+CD25- B cells increases with rShh treatment.

Surprisingly, at 40hours post-activation stimulus (Figure 4.4), it was observed that there are three populations of B cells, these include, CD23+CD25-, CD23+CD25+ and CD23-CD25+ B cells. It was observed that there was an increase in the percentage of CD23-CD25+ B cells treated with rShh. For example, as shown in Figure 4.4, in the untreated control, the percentage of CD23-CD25+ B cells was 13.7%, which increased approximately two-fold to 28.2% in B cells treated with 0.005µg/ml rShh. As observed with the previous data, the lowest dose of rShh gave the most dramatic effect. At the same time as the appearance of this new subset of CD23-CD25+ cells, there is a decrease in the presence of CD23+CD25- cells indicating that as one subset might

appears the other disappears. It is interesting to note that the very high dose of rShh (5µg/ml) had little effect on the purified B cell subsets at 40hours post-activation stimulus.

Taken together this data, it suggests that treatment of B cells with rShh, can increase the expression of CD23-CD25+ B cells at 40hours post-activation stimulus, thus indicating that Hh signalling can increase B cell differentiation. It would be important to characterise these cells further, as they may play a role in the effects of Hh signalling observed on B cell activation and survival in our purified B cell culture and mixed B cell culture.

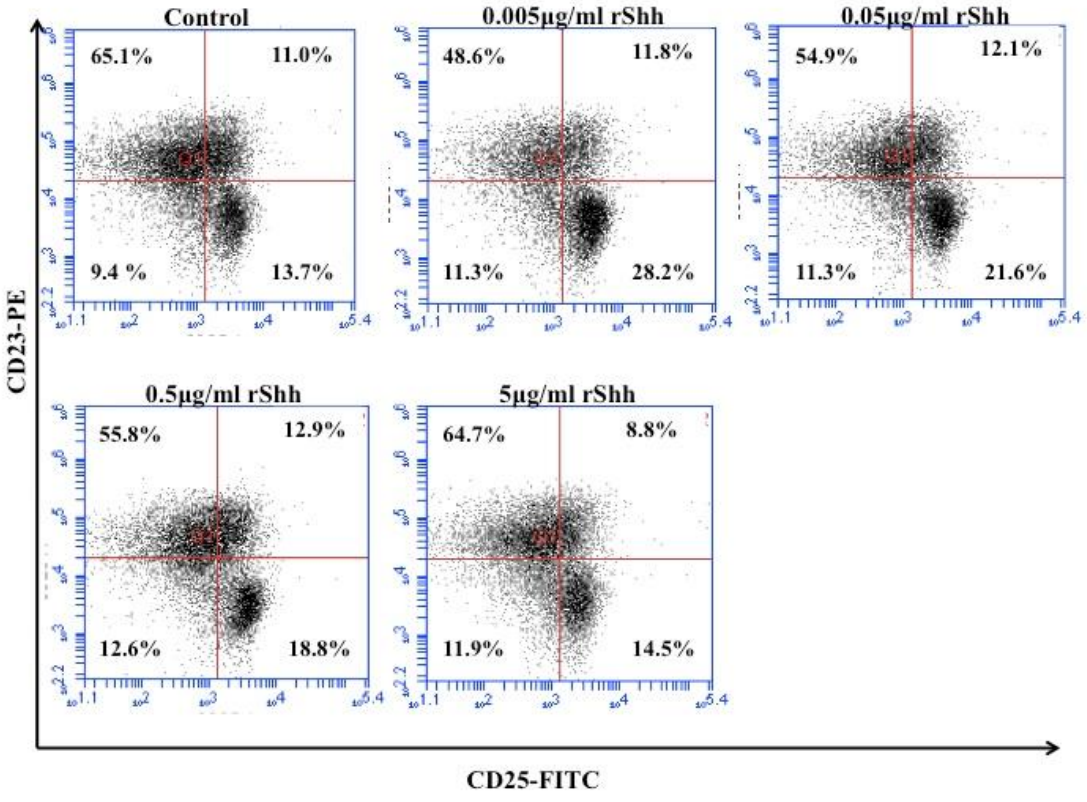


Figure 4.4. Treatment of purified B cells with exogenous rShh increases CD23-CD25+ B cells

The dot-plots shown in the figure show the result of one experiment representative of three independent experiments. The co-expression of early activation marker anti-CD23 and late activation marker anti-CD25 in purified B cells at 40hours post-activation stimulus is shown.

4.2.3. rShh treated B cells differentiate into different subsets.

Upon the observation that, in our system, rShh induces an increase in the representation of a subset of B cells that are CD23-CD25+- at 40hours post-activation stimulus, it was important to characterize these subsets further. CD25+ B cells have been described to play an important role in the immune system and may participate in the pathogenesis of inflammation (Amu *et al*., 2006). In mice deficient for CD25+ B cells, mice suffer from a range of auto-reactive diseases including inflammatory bowel disease and rheumatoid arthritis (RA) (Amu *et al*., 2010) thus indicating a regulatory role for CD25+ B cells. In T cells, CD25+ T cells have regulatory features and have been shown to supress effector T cell functions (van Amelsfort *et al*., 2004). In mice, Amu *et al*., 2010, showed that CD25+ B cells secrete IL-10 following CpG, LPS and Pam3Cy5 stimulation indicating that these B cells may be regulatory B cells (Bregs). They also showed that CD25+ B cells had the ability to secrete higher levels of class switched immunoglobulin including IgA, IgG thus also having the properties of memory B cells (Amu *et al*., 2010). Brisslert *et al*., 2006, showed that 65% of CD25+ B cells express the memory B cell marker CD27 compared to 20% CD25- B cells (Brisslert *et al*., 2006). Hence, CD25+ B cells may either be regulatory B cells (Bregs) as they have been shown to secrete IL-10 or memory B cells that secrete class switched immunoglobulins post-activation.

In our system, at 40hours post-activation stimulus, there was an increase in expression of CD25+ B cells in B cells treated with rShh (Figure 4.4). It is possible that the apparent down-regulation of activation and increased levels of apoptosis induced by rShh signaling in B cells at 40hours post-activation stimulus in our system might be due

to the increased numbers of CD25+ B cells that are capable of producing IL-10. Thus, in order to identify if these CD25+ B cells are Bregs or memory B cells, we analyzed expression of IL-10 (Mauri and Bosma, 2012) and CD27 (Agematsu *et al.*, 2000) as surrogate markers of Bregs and memory B cell respectively.

Based on previous findings using varying concentration of rShh in mixed B cell cultures and purified B cell cultures, we determined that the optimal concentrations of rShh that gave the clearest result were 0.05µg/ml and 0.005µg/ml based on the data on activation and survival (Figure 3.4A&C and Figure 4.1A&C) and thus for further experiments, these two concentrations would be used either together or alone.

Purified murine B cells were activated and at 40hours post-activation stimulus, cells were stained with a combination of anti-CD23-^{PE}, anti-CD25-$^{Pe\text{-}Cy5.5}$, anti-CD27-^{FITC} or IL-10-^{FITC}. At 18hours post-activation stimulus, neither IL-10+ B cells nor CD27+ B cells were observed in our culture. As previously described, at 40hours post-activation stimulus (Figure 4.7), we observed three distinct subsets of B cells and named them subset I (CD23+CD25-), subset II (CD23+CD25+) and subset III (CD23-CD25+). We then analyzed these subpopulations for their expression of CD27 and IL-10. This was done in order to identify if the CD23-CD25+ population observed in B cells treated with rShh at 40hours post-activation stimulus in purified B cells were either memory B cells or possibly Bregs.

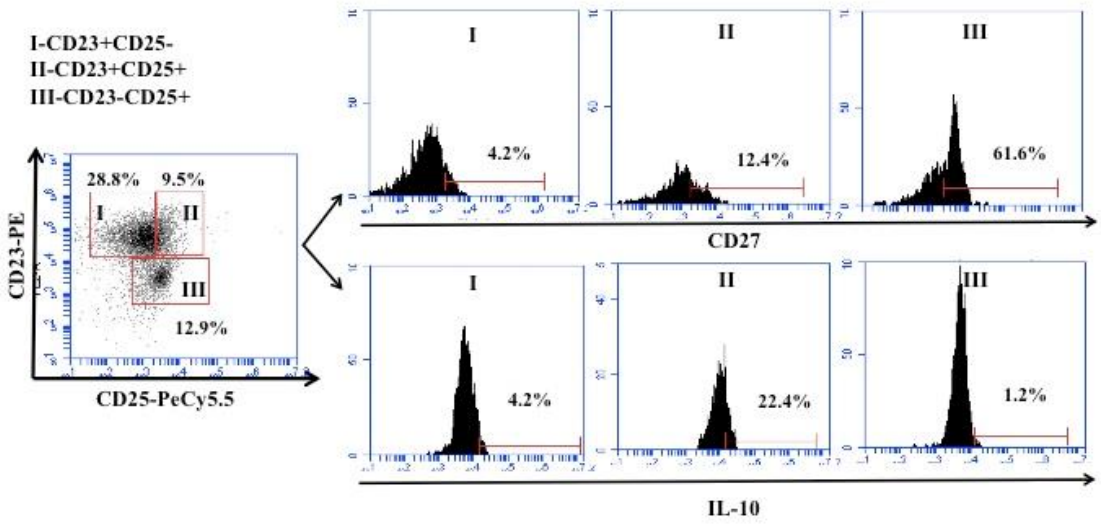


Figure 4.5. Three subsets observed at 40hours post-activation stimulus

Purified B cells were activated with 5µg/ml anti-CD40/IgM either with or without rShh. At 40hours post-activation stimulus, co-expression of CD23 and CD25 was analyzed. Three subsets, named subset I, II and III were identified and analyzed for their expression for CD27 and IL-10. The histogram shown is from one experiment representative of three independent experiments. It shows subset I, II, III and their expression of IL-10 and CD27.

At 40hours post-activation stimulus (Figure 4.5), we observed that B cells in subset I (CD23+CD25-) do not express IL-10 neither do they express CD27 suggesting that this subset of B cells is neither memory B cells nor regulatory B cells. Interestingly, we observed that subset II (CD23+CD25+) B cells were positive for IL-10 expression but not subset III (Figure 4.5). For example, subset II have 22.4% of B cells positive for IL-10 compared to the 4.2% and 1.2 % observed in subset I and III respectively (Figure 4.5).

When we analyzed CD27 expression, we observed that subset III (CD23-CD25+) B cells express the highest level of CD27 (Figure 4.5). This led to the conclusion that in our system, upon activation the B cells may develop to become at the minimum, one of three different populations. These populations include subset I, which we speculate

includes early activated B cells or FO B cells as they are high in the activation marker CD23, subset II, which we speculate include regulatory B cells as they express IL-10 and subset III which we suggest could be memory B cells as they are high in expression for the human memory B cell marker CD27.

4.2.4. Hh increases the expression and secretion of IL-10

The human and murine IL-10 cytokine is a non-covalent linked homodimer consisting of 160 amino acids. IL-10 is a potent anti-inflammatory cytokine expressed by cells of both the innate and adaptive immune system including B cells, T cells, and macrophages. It is a multifunctional cytokine with diverse effects, which include inhibition of inflammation via several mechanisms including down regulation of the production of pro-inflammatory cytokines such as IFN-α, IL-6, IL-12 (Couper *et al*., 2008) and prevention of autoimmune pathologies such as those observed in murine collagen induced arthritis models (Saraiva and Garra, 2010, Mauri *et al*., 2003). The term "regulatory B cells" was first presented by Mizoguchi *et al*., 2000, in which B cells were able to suppress auto-immune disease in a mouse inflammatory bowel disease model (Mizoguchi *et al*., 2000). Since this initial study, Bregs have also be shown to ameliorate the onset of experimental autoimmune encephalomyelitis (Evans *et al*., 2007). Bregs are distinguished from other B cell populations by their ability to secrete the anti-inflammatory cytokine IL-10. Bregs have been suggested to develop from the immature B cell pool, T2-MZP in the spleen following CD40 and TLR stimulation (Mauri and Bosma, 2012, Mauri and Blair, 2014, Kalampokis *et al*., 2013).

Having identified these different subsets of differentiating B cells in our culture at 40hours post-activation stimulus and showing that subset II (CD23+CD25+) were positive for IL-10 (Figure 4.5), we then further analyzed IL-10 production in subset II

(CD23+CD25+) in B cells treated with or without exogenous rShh. Intracellular IL-10 staining was carried out at 40hours post-activation stimulus; Figure 4.6A, shows histograms representing IL-10 expression at 40hours post-activation stimulus. The percentage of B cells expressing IL-10 has increased from 11.0% in the untreated control to 16.5% in B cells treated with 0.05μg/ml rShh (Figure 4.6A). This increase of IL-10+ cells was significant as seen in Figure 4.6B; there was approximately 1.5 fold increase in IL-10 expression in the treated B cells in the late 40hours time point as compared to the untreated control.

To confirm the increase detection of intracellular IL-10+ cells correlates with an increase in IL-10 secretion by B cells, the supernatants were analyzed for the presence of IL-10 protein in culture using an ELISA assay. Consistent with the results observed when analyzing intracellular IL-10 expression, there was also a significant increase in the levels of IL-10 being secreted by B cells treated with 0.05μg/ml rShh at 40hours post-activation stimulus (Figure 4.6C). For example, the mean of the untreated control B cells secreted was 22ng/ml of IL-10 and this significantly increased to 26ng/ml of IL-10 in B cells treated with 0.05μg/ml rShh. Addition of rShh has been previously shown to induce the expression of IL-10 via STAT-3 in pancreatic acinar cells suggesting that this phenomenon may not be exclusive to B cells. In 2012, Zhou *et al*, showed that the presence of rShh induces expression of the Hh target gene, IL-10 and this reduced the inflammatory activities in acute pancreatitis in a mouse model (Zhou *et al*., 2012).

Taken together, these results suggest that Hh signaling, can increases the differentiation to B cells that can secrete IL-10 at 40hours post-activation stimulus.

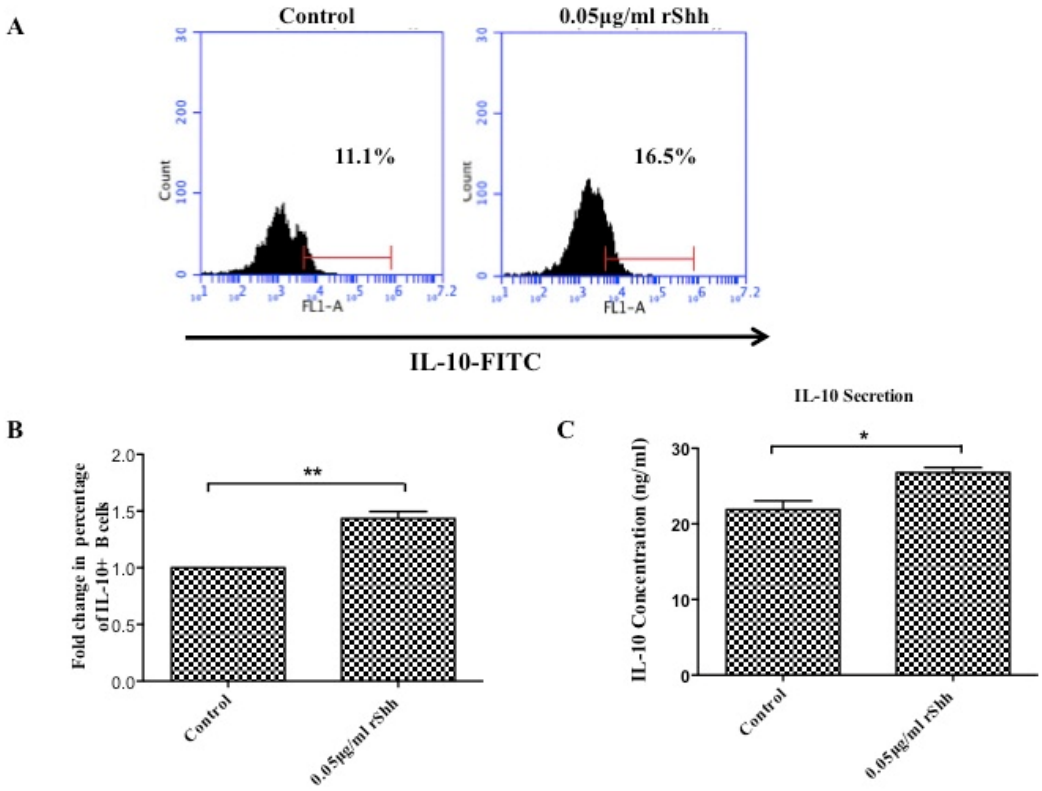


Figure 4.6. Treatment of B cells with exogenous rShh can increase IL-10+ B cells and IL-10 secretion

Purified B cells were activated and treated with 0.05µg/ml rShh. At 40hours post-activation stimulus, B cells were gated on CD23+CD25+ and analyzed for intracellular IL-10+ B cells. The histograms shown in (**A**) are from one experiment representative of three independent experiments and it shows IL-10+ B cells. A bar chart representing the IL-10+ B cells in (**B**). Supernatants from B cell cultured either with or without treatment with rShh were analyzed for IL-10 secretion and it is represented in a bar chart (**C**). The data presented in the bar chart is representative of three independent experiments. The significance of IL-10+ cells in fold change and IL-10 concentration was determined using Student *t* test (*p≤0.05, **p≤0.01).

4.2.5. Hh signaling up-regulates the expression of CD27

Memory B cells can either be formed in a T-dependent manner or T-independent manner (Kurosaki *et al.*, 2015). T-independent memory B cells are generated from the B1 B cell pool and in the peritoneal cavities of rodents. Memory B cells have the ability to respond, upon second reencounter with an antigen, in an accelerated fashion with

higher efficacy. They have the ability to rapidly differentiate into plasma cells secreting specific antibodies against their cognate antigen (Kurosaki *et al*., 2015). CD27 is a type 1 glycoprotein with unique cysteine rich motifs that is expressed on some B cells and majority of T cells and is a member of the tumor necrosis (TNF) receptor family. Expression of CD27 has previously been used to characterize memory B cells in humans (Amu *et al*., 2007). These CD27 expressing B cells have been shown to produce more IgE, IgA, IgM and IgG subclasses suggesting that they are class switched primed B cells and thus memory B cells (Agematsu *et al*., 2000). It was demonstrated previously that, CD27 is expressed in subset III (CD23-CD25+) (Figure 4.5). To investigate whether the expression of this memory B cell marker is modulated by Hh signaling, expression of CD27 was analyzed in the in B cells treated with rShh at 40hours post-activation stimulus in the subset III (CD23-CD25+).

Figure 4.7A, shows histograms representing CD27 expression in CD23-CD25+ B cells at 40hours post-activation stimulus. CD27 expression has increased from 22.2% in the control to 28.5% in B cells treated with 0.05μg/ml rShh. This increase in expression of CD27 was significant as illustrated on the bar chart (Figure 4.7B).

From these results, it may be concluded that Hh signaling is apparently capable of driving B cell development preferentially to become B cells that can secrete IL-10 and also B cells with a memory B cell like phenotype.

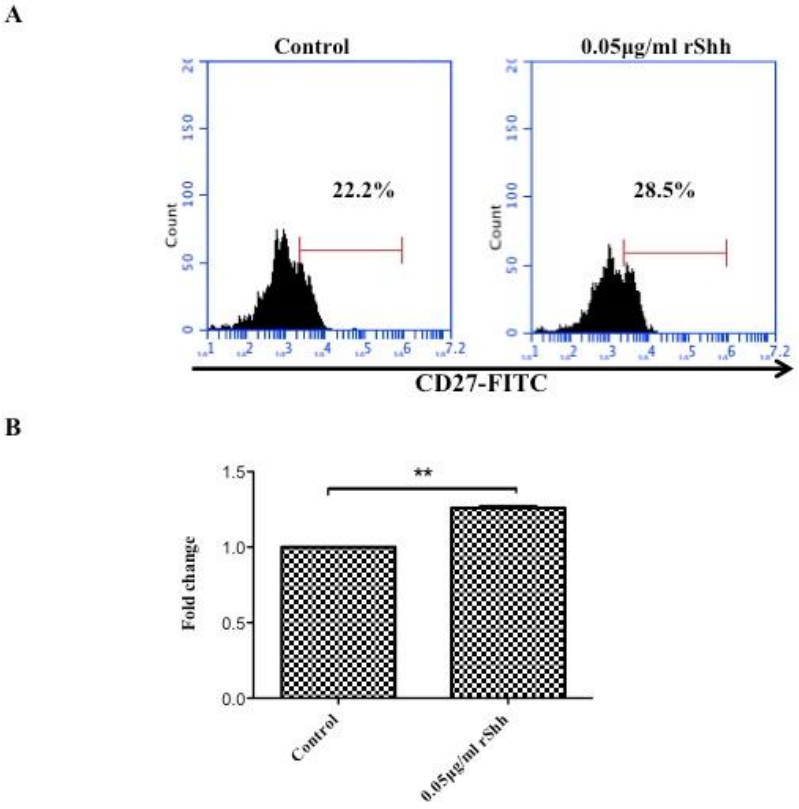


Figure 4.7. Treatment of purified B cells with rShh increases CD27+ B cells

Purified B cells were activated and treated with 0.05µg/ml rShh. At 40hours post-activation stimulus, CD27, a putative memory B cell marker was analysed. The histograms shown in (**A**) are from one experiment representative of three independent experiments and it shows CD27+ B cells that were analyzed in subset III. A bar chart representing the percentage of CD27+ B cells in (**B**). The data presented in the bar chart is representative of three independent experiments. The significance of CD27+ cells in fold change was determined using Student *t* test (**p≤0.01).

4.2.6. The effect of exogenous rShh on antibody production by purified B cells.

It has been reported that stromally produced Hh play a role in the proliferation and antibody production by GC B cells (Sacedon *et al*., 2005). Having looked at the effect of rShh on peripheral B cells with respect to activation, survival and differentiation, it was important to analyse the effect of rShh on the production of antibodies by B cells.

Purified B cells were activated either with or without treatment with 0.05µg/ml rShh. At 18 and 40hours post-activation stimulus, supernatants were collected and analysed for

production of the different isotypes of antibody using an ELISA assay. Six-mouse antibody isotype were analysed and these include IgG1, IgG2a, IgG2b, IgG3, IgA and IgM. IgG antibodies make up to 90% of the blood sera, they play a role in the front line of immunity. IgG1 is more induced relative to the other IgG subclasses and is associated with Th2 function together with IgE (Vidarsson *et al*., 2014). IgG2 is usually secreted in response to bacterial antigens. IgG3 is a potent pro-inflammatory antibody and has effector functions (Vidarsson *et al*., 2014). IgA is an antibody that plays a role at the muscosal surfaces and it is involved in fighting off infection in the gut. IgM is the main antibody secreted in a primary immune response and it functions mainly by opsonizing antigen for destruction and fixing complement (Schroeder and Cavacini, 2010).

Figure 4.8, shows secretion of different antibody isotypes by activated purified B cells after rShh treatment. At 18hours post-activation stimulus, there was an increase in antibody production of each isotype of immunoglobuin in B cells treated with 0.05µg/ml rShh when compared with the control (4.8A, B, C, D, E, and F). This finding is consistent with the fact that at 18hours post-activation stimulus, we are getting an apparent increased B cell activation with treatment with rShh, This is also consistent with results obtained by Sacedon *et al*., (2005) which showed that use of the Hh signalling antagonist, cylcopamine, reduced antibody production by GC B cells.

For example, at 18hours post-activation stimulus, the levels of secretion of IgG2a (Figure 4.8B) in B cells treated with 0.05µg/ml rShh was significantly increased (1.3 fold higher) when compared to untreated control B cells and this was true for all the isotypes analysed. Interestingly, although, there was an increase in antibody secretion by treated B cells at 18hours post-activation, at 40hours, a quite different effect was observed. At this time point, while the levels of the different antibody isotypes had

almost doubled from 18 to 40hours following activation in the untreated control supernatants, the levels of antibody production by B cells in the treated with rShh had not increased significantly between the 18 to 40hours time points. For example, with IgG1 (Figure 4.8A), the untreated control have significantly increased antibody production by approximately 1.7 fold change between 18 and 40hours compared to B cells treated with 0.05µg/ml rShh which have remained almost the same (Figure 4.8A).

These results suggest that treatment of B cells can increase antibody production at 18hours post-activation, which might be as a result of increased B cell activation. However, at 40hours post-activation stimulus, there is no further increase in antibody production, possibly as a result of the increased apoptosis observed at this time-point or as a result of an increased differentiation of B cells towards B cells secreting IL-10 or B cells that exhibit a memory B cell like phenotype.

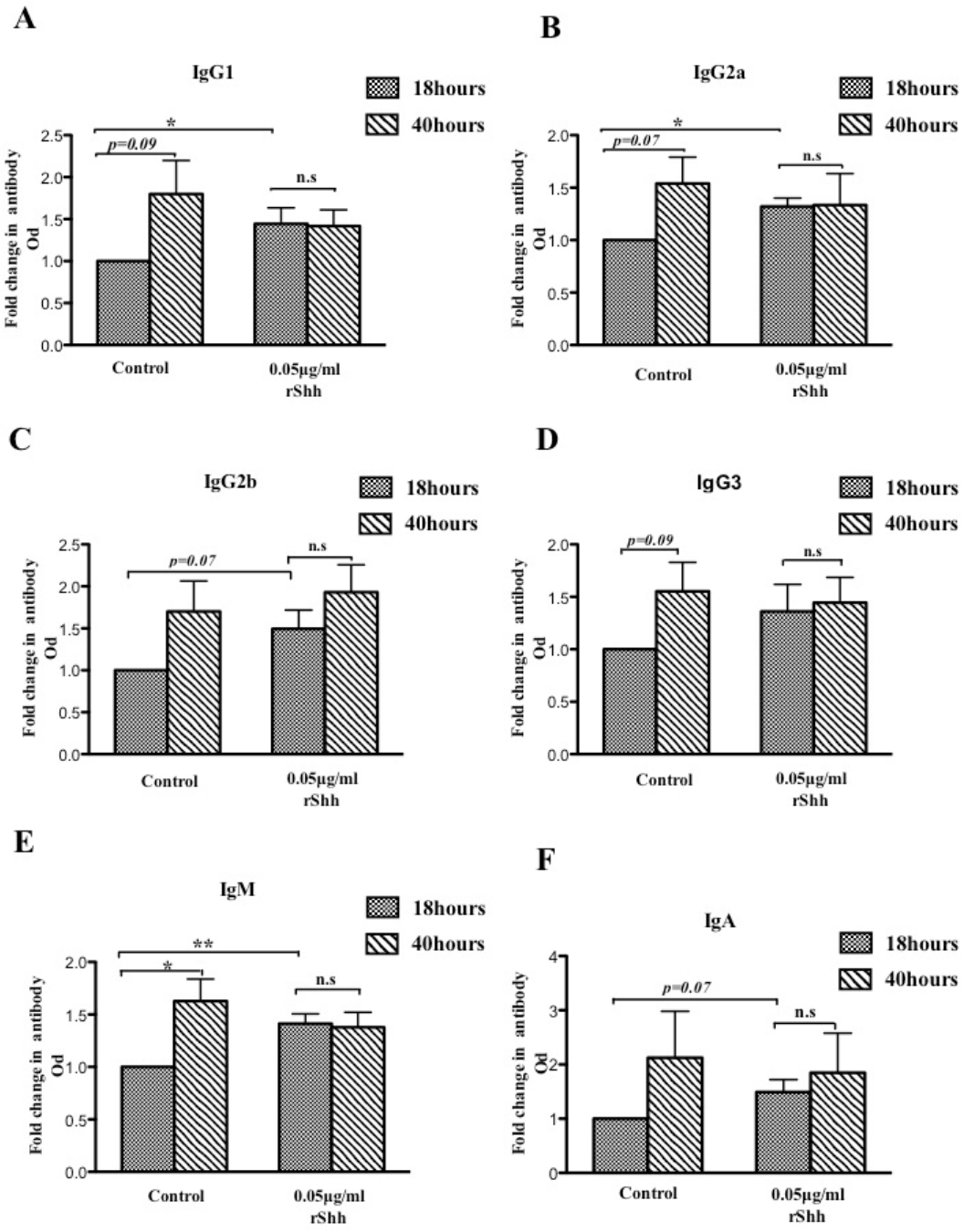


Figure 4.8. Treatment of B cells with exogenous rShh can affect antibody production by purified B cells

Purified B cells were activated with anti-CD40/IgM and treated either with or without treatment with 0.05µg/ml rShh. At 18 and 40hours post-activation stimulus, supernatants were collected and analyzed for the production of antibody isotypes including IgG1 (**A**), IgG2a (**B**), IgG2b (**C**), IgG3 (**D**), IgA (**E**) and IgM (**F**) using ELISA. The bar charts shown are representative of four independent experiments. The significance of antibody production in fold change was determined by Student *t* test (*$p \leq 0.05$, **$p \leq 0.01$).

4.2.7. IL-6 secretion is increased in the presence of rShh

Interleukin 6 (IL-6) is a pleiotropic cytokine that has been initially characterized as a cytokine that enhances differentiation of B cells into antibody producing B cells (Amu *et al*., 2009, Kishimoto, 2010). IL-6 is a pro-inflammatory cytokine produced by antigen presenting cells (APCs) including macrophages, dendritic cells and B cells. IL-6 binds to a receptor complex consisting of the specific IL-6Rα and the gp130 signal-transducing unit that activates the transcription factor STAT-3 among other signalling molecules. Over expression of IL-6 in mice causes plasmocytosis, indicating it can promote differentiation of B cells into plasma cells (Dienz *et al*., 2009, Kishimoto, 2010). IL-6 can also control B cell survival and proliferation as it regulates Blimp-1via STAT-3 which drives the production of immunoglobulin (Hunter and Jones, 2015). In multiple myeloma (MM), where Hh signalling has been shown to play a role in its pathogenesis, there is an observed increase in B cell proliferation (Peacock *et al*., 2007). Increased IL-6 production has also been shown to be correlated with increased inflammatory activity and may contribute to the onset of pathogenesis associated with chronic inflammatory disease and also Systemic Lupus Erythematosis (SLE) and RA (Urashima *et al*., 1996, Dayer and Choy, 2010). Conversely, IL-6 has also been shown to have anti-inflammatory properties via the induction of IL-10 and TGF-α (Scheller *et al*., 2011) indicating a complex role for this cytokine in regulating immune responses.

In our experiments, at 18hours post-activation stimulus, we observed that there is antibody production in B cells treated with rShh. As IL-6 plays an essential role in promoting B cell differentiation into IgG secreting B cells (Maeda *et al*., 2010), we hypothesised that this increase in Ig production may be correlated with IL-6 secretion, which is able to drive B cells to secrete antibody in our culture. Additionally, it has previously been shown by Wakelin *et al*., (2008), that the Hh signalling pathway has the

ability to up-regulate the production of IL-6, IL-8, MCP-1, IP-10, MIG and RANTES by macrophages (Wakelin *et al*., 2008). Furthermore, the downstream effects of the Hh signalling pathway in fibroblasts have been shown to be mediated via the transcription factor GLI-1, inducing it to bind to the IL-6 promoter thus increasing IL-6 expression in a paracrine manner (Mills *et al*., 2013)

Figure 4.9, shows the IL-6 secretion by B cells treated with or without 0.05μg/ml rShh at 18 and 40hours post-activation stimulus. At 18hours post-activation stimulus, there was a significant increase in secretion of IL-6 in B cells treated with rShh to approximately 0.23ng/ml compared to the level determined for the untreated control of 0.10ng/ml. This is consistent with the finding that at 18hours, there is an increase in antibody produced by B cells in the treated with rShh compared to the control. At 40hours post-activation stimulus, as observed for antibody secretion by B cells (Figure 4.9), the level of IL-6 secretion doubled in the activated untreated control whereas in treated B cells, there was no further increase in IL-6 secretion from 18 to 40hours in culture. Indeed, there was a slight but insignificant reduction in secretion of IL-6 (Figure 4.9).

The increase in IL-6 from 18 to 40hours in the untreated control is correlated to the increased antibody being produced at 40hours whereas the apparent arrest in further secretion of IL-6 at 40hours correlates with the arrest in antibody production observed at 40hours in the presence of rShh. Taken together these data would suggest that enhanced Hh signalling at 18hours post-stimulus may induce the secretion of IL-6 which in turn induces enhanced production of isotype switched antibodies. However, at 40hours, post-activation stimulus, there may be no further secretion of IL-6 in B cells treated with exogenous rShh, possibly as a result of increased apoptosis of B cells

secreting the cytokine, or as a result of increased differentiation of B cells towards B cells that no longer secrete IL-6.

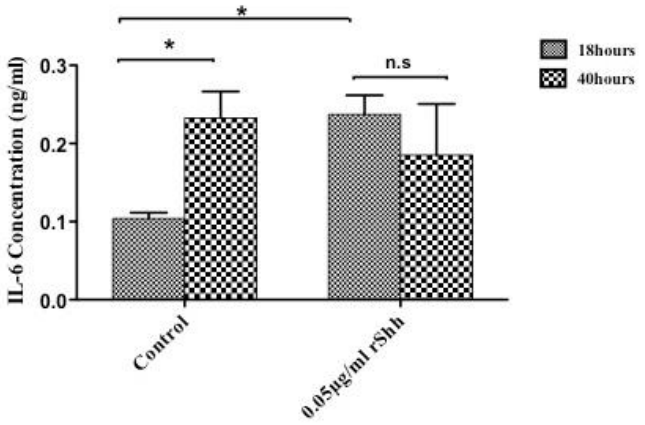


Figure 4.9. Treatment of purified B cells with rShh can affect IL-6 secretion

Purified B cells were activated and treated with or without 0.05µg/ml rShh. At 18 and 40hours post-activation stimulus, supernatants were collected and analysed for IL-6 secretion using ELISA. The bar chart shown is representative of four independent experiments. The significance of IL-6 concentration was determined using Student *t* test (*$p \leq 0.05$).

4.2.8. B cell proliferation in the presence of rShh

The combination of B cell receptor and CD40 ligation, along with certain other signals thought to be derived from direct T-cell contact such as IL-4, leads to B cell proliferation or clonal expansion of B cells specific for a particular antigen. Several rounds of proliferation need to occur prior to antibody production *in vivo*. It was important therefore, to investigate the effect of Hh signalling on B cell proliferation.

In order to analyse proliferation, we used CFSE conjugated to FITC. This is a cell proliferation dye, which enters the cell, where it crosslinks with certain intracellular proteins. Upon cell division, there is a successive halving of the fluorescence intensity

as the daughter cells share out the fluorescently labelled intracellular proteins (Hawkins *et al*., 2007).

Purified B cells were stained with 10µm CFSE before activating with 5µg/ml anti-CD40/IgM and IL-4 (5µg/ml). The cells are then cultured for 72hours and 96hours, these time points were chosen as there were few proliferating cells detected using CFSE at 18 and 40hours post-activation stimulus. At 72 and 96hours post-activation stimulus, the purified B cells were analysed for proliferation by flow cytometry. Proliferation was analysed by calculating the levels of CFSE staining remaining within the total population.

Figure 4.10, shows CFSE staining at 72hours post-activation stimulus. It was observed that there was a significant decrease in percentage of resting B cells in B cells treated with rShh indicating that Hh signalling was driving B cell proliferation. For example, the percentage of resting B cells have decreased from 27.9% in the control to 23.1% in B cells treated with 0.005µg/ml rShh and this was significant as shown in Figure 4.10B. Consistent with this, there was also an increase of B cells that had entered the 1st and 2nd cycle of division in the presence of both doses of rShh (Figure 4.10C and D).

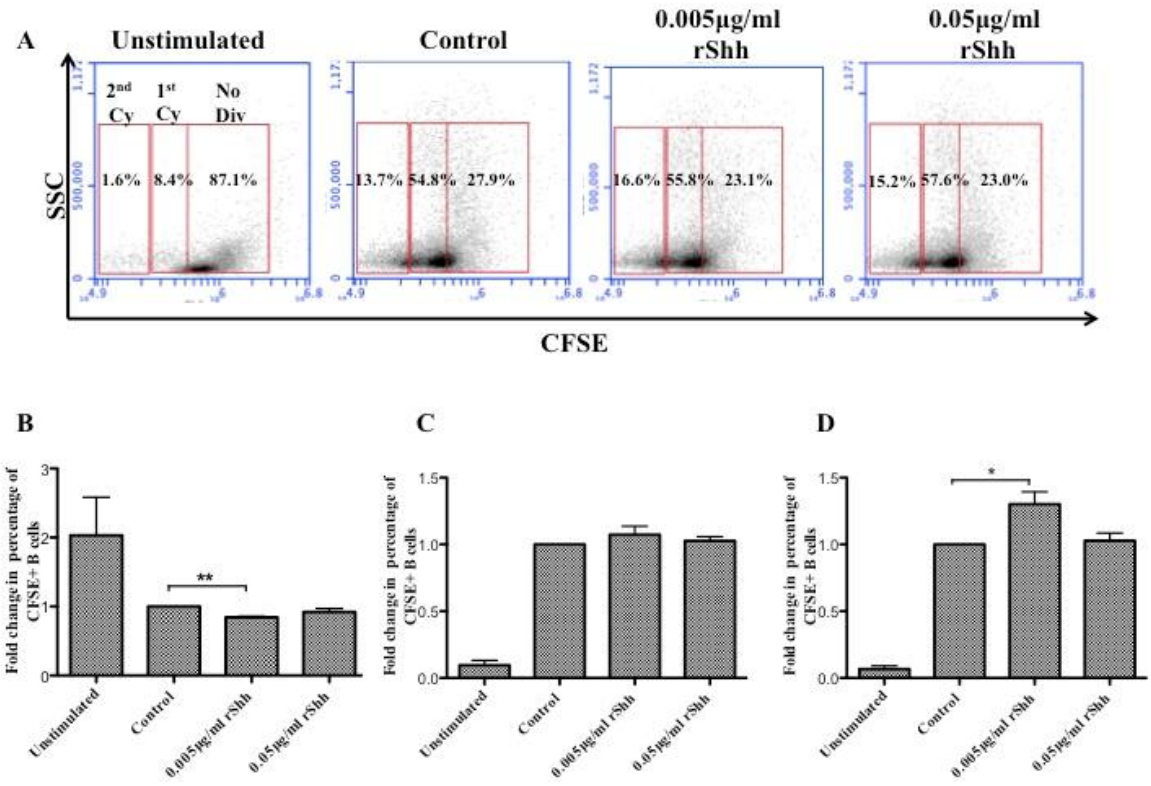


Figure 4.10. Treatment of purified B cells with rShh increases B cell proliferation at 72hours post-activation stimulus

Purified B cells were stained with CFSE, then activated and treated with rShh. At 72hours post-activation stimulus, B cells were measured for CFSE intensity. A representative dot-plot from one experiment representative of three experiments is shown in (**A**). It shows B cells that are non-dividing (resting), in the 1st cycle and 2nd cycle of division. Bar chart illustrating B cells that are resting (B), 1st cycle (C) and 2nd cycle (D). The data in the bar chart is representative of three independent experiments. The significance of the data in fold change was assessed using Student's *t* test (*p≤ 0.05, **p ≤ 0.01).

At 96hours (Figure 4.11), the percentage of B cells in the resting phase, 1st, 2nd and 3rd cycle of division was analysed. There was a significant increase in percentage of B cells that had entered the third cycle of division in the presence of 0.05µg/ml compared to the untreated control (Figure 4.11D). These results suggest that Hh signalling can promote B cell proliferation *in vitro.* These findings are consistent with the results obtained by Sacedon *et al*. (2005), using cyclopamine; an inhibitor of the Hh signalling pathway, they showed that inhibition of the Hh signalling pathway can inhibit B cell proliferation (Sacedon *et al*., 2005).

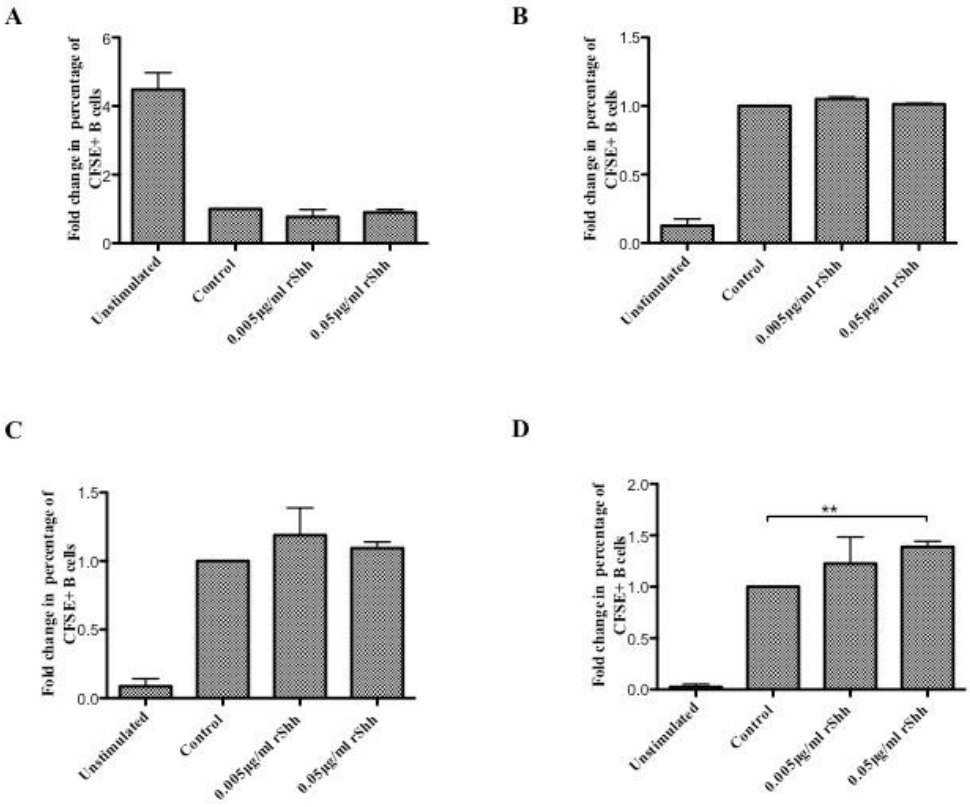


Figure 4.11. Treatment of purified B cells with rShh increases B cell proliferation at 96hours post-activation stimulus

Purified B cells were stained with CFSE, then activated and treated with rShh. At 96hours post-activation stimulus, B cells were measured for CFSE intensity. The percentages of B cells are resting (**A**), 1st cycle (**B**) 2nd cycle (**C**) and the (**D**) 3rd cycle is shown in the bar charts. The data in the bar chart is representative of three independent experiments. The significance of the data in fold change was assessed using Student's *t* test (*p≤ 0.05, **p ≤ 0.01).

4.3. Discussion

In the previous chapter (Chapter 3), data presented demonstrated that Hh signalling could have an effect on B cell activation and survival in B cells in a mixed splenocyte population. However, it was possible that the effect observed on B cells in the mixed splenocyte population was due to the presence of other cell types in the spleen or from soluble factors produced by these other cell types. Thus, the aim of this Chapter was to investigate the effect of exogenous rShh treatment on purified B cells.

The data presented in this chapter demonstrates that treatment of purified B cells with exogenous rShh can increase B cell activation (as judged using CD23) and survival (as judged using Annexin-V) at 18hours post-activation stimulus and at 40hours, there was a decrease in B cell activation and increased apoptosis. The findings demonstrate that the effect of exogenous rShh treatment on B cells with emphasis to activation and survival was intrinsic to B cells and not due to any other cellular subset present within the spleen. The effect observed was more prominent in the purified B cell experiments when compared to the mixed cultures (Chapter 3) thus suggesting that in the mixed cultures, the other cell types may influence the response of B cells to exogenous rShh.

Additionally, using late B cell activation marker, anti-CD25, it was observed that, treatment of B cells with rShh increases the expression of this late B cell activation marker at 40hours post-activation stimulus. Furthermore, it was demonstrated that at 40hours post-activation stimulus, three populations of B cells was observed (Figure 4.7) and these were characterised as subset I (CD23+CD25-), subset II (CD23+CD25+) and subset III (CD23-CD25+), with rShh treatment increasing the representation of subset III and decreasing the representation of CD23+CD25- B cells at this time point. As

CD25+ B cells have been characterised as either Bregs or memory B cells, these subsets were analysed further using Bregs marker, IL-10 and memory B cell marker CD27.

Co-analysis of IL-10 and CD27 showed that subset I, does not express either of these markers suggesting that this subset may represent B cells of an earlier, less mature lineage such as antibody secreting FO B cells. A proportion of subset II B cells express IL-10 and are potentially Bregs while the subset III were positive for CD27, suggesting they may be memory B cells (Figure 4.7). It was demonstrated that treatment of B cells with exogenous rShh increases IL-10+ and CD27+ B cells. Additionally, it is possible that the enhanced production of IL-10 in the presence of rShh by B cells (Figure 4.8 and 4.9) might be responsible for causing the observed down-regulation in B cell activation, antibody secretion and enhanced cell death observed at 40hours in our mixed splenocyte culture and purified B cell culture (Figure 4.2 and Figure 4.3). The increase of CD27 expression and IL-10 in our culture suggests that Hh signaling can regulate B cell development in the periphery, possibly accelerating the process of B cell development preferentially into certain lineages.

Furthermore, it was observed that treatment of B cells with rShh can increase antibody production and IL-6 secretion at 18hours post-activation stimulus and at 40hours post-activation stimulus, the levels has not increased much compared to the untreated control. It is possible, that at 18hours, treatment of B cells with exogenous rShh can increase the immune response by B cells but at 40hours, it is possible that the increased B cell death is correlated to B cells that secrete antibody and IL-6. Additionally, it is possible that at 40hours post-activation in our culture, Hh signalling induces the expression of another morphogen, which has the capacity to negatively regulate the production of antibodies such as BMP-4, which has been shown in Chapter 3 to exert a negative regulatory effect

on B cell activation and survival or alternatively, that the increased apoptosis, might represent the decrease in antibody secreting cells. Additionally, when B cell proliferation was analysed using CFSE at 72 and 96hours, Hh signalling was shown to increase levels of B cell proliferation thus suggesting that Hh may act to accelerate many aspects of B cell differentiation in the periphery.

In conclusion, the findings in this chapter demonstrate that Hh signaling can drive peripheral B cell differentiation, at 18hours, post-activation stimulus, Hh signaling appears to increase differentiation of B cells that secrete antibody and IL-6, possibly FO B cells. However, at 40hours post-activation stimulus, Hh signaling would also appear to be driving the B cells to differentiate into B cells that secrete IL-10 or B cells that display a memory like phenotype. This process of lineage commitment in B cells is not well understood and is no doubt driven by the expression of particular transcription factors that may indeed be transcriptional targets of Hh signaling. It is thus important to further characterize the subsets I-III in terms of the cellular subsets that would normally be observed in the mouse spleen in order to ascertain their true function *in vivo*. For this reason, sorting of the subsets was carried out so that the phenotype and function of each isolated subset could be observed *in vitro.* Having identified an effect caused by the presence of enhanced levels of exogenous Hh, it was also important to analyze the effects of the presence of reduced levels of Hh protein.

4.4. Conclusion

The outcomes of this chapter demonstrate that Hh signalling can increase activation of B cells as assessed by CD23 expression and survival at 18hours post-treatment, as was also observed in the mixed splenocyte culture thus confirming that the effect of Hh signalling in B cell development is intrinsic to B cells and not due to any other cell type in the spleen. We showed that Hh signalling modulates antibody production, IL-6 and IL-10 secretion in B cells at these different time points. We have also shown, that Hh signalling appears to drive B cell differentiation towards both memory B cells and Bregs at 40hours post-activation stimulus.

Taken together, these data suggest an important role for Hh signalling in driving development, survival and lineage commitment decisions in B cells following stimulation by a signal that mimics the presence of a TD antigen. The next chapter extends the findings of this chapter by carrying out a functional sorting assay in order to identify the true function of the individual subsets in isolation.

Chapter 5: Characterisation and sorting of peripheral B cell subsets

5.1. Introduction

5.1.1. Peripheral B cell subsets and surface marker expression

Newly generated antigen receptor bearing B cells in the bone marrow either mature in this site or migrate to the peripheral lymphoid organs such as the spleen. The naïve B cells leave the bone marrow as transitional B cells, where they are still functionally immature, have a short half-life and a tendency to undergo apoptosis rather than proliferate following BCR engagement (Allman and Pillai, 2008). Loder *et al*., (1999) first proposed that immature B cells or transitional B cells in the adult spleen may be subdivided into two distinct subsets (T1) and (T2) based on their expression of IgD and CD21 (Loder *et al*., 1999), and then subsequently a third transitional B cell was identified thought to be implicated in the development of autoimmunity, T3 (Allman *et al*., 2001). Immature B cells enter the spleen as T1 B cells that can develop further upon BCR stimulation to become T2 B cells, follicular B cells (FO), T2-marginal zone B cells precursor (T2-MZP) and marginal zone (MZ) B cells. Depending on the surface marker expression on B cells, these different subsets of B cells in the spleen can be identified (Figure 5.1). In the mouse, subsets of B cells can be most easily distinguished by their expression of some or all of the markers including IgM, IgD, CD21, CD1d, CD23, CD24, CD93 (Blair *et al*., 2008). The cell surface markers expressed on human B cells show a pattern of expression that differs markedly from the mouse.

Transitional B cell subsets express the B-lineage precursor marker CD93. They also express low to intermediate levels of CD21 and subsets are differentiated by levels of IgM and CD23 surface expression (Allman and Pillai, 2008). T1 B cells are

characterised as CD23-CD21-IgD-IgM^{hi}. T1 B cells can differentiate to become T2 B cells, which are characterised as CD23+, IgD^{hi}, IgM^{hi}. T2 B cells can develop into T3 B cells, which are CD23+IgD^{high}IgM^{low} (Allman and Pillai, 2008, Pillai and Cariappa, 2009, Carsetti *et al*., 2004). Depending on the type or strength of the signal received via the BCR, T2 B cells can give rise to mature B cells. This differentiation involves the activity of signalling pathways such as those mediated by BAFF, NF-κB, Notch2 and Bruton's tyrosine kinase (Btk) (Pillai and Cariappa, 2009).

T2 B cells can differentiate to become FO B cell type 1 (FO-I) characterised as CD23+IgD^{high}IgM^{low}CD21^{mid}, which requires strong BCR signalling triggered by high affinity antigens and Btk signalling or FO B cell type II (FO-II) characterised as CD23+IgD^{high}IgM^{high}CD21^{mid}, which is Btk independent (Pillai and Cariappa, 2009). More recently, it has been suggested that FO-II serves as an intermediate for T2 differentiation towards FO-I and MZ B cells (Figure 5.1) (Kleiman *et al*., 2015).

FO-I B cells specialize in T cell dependent immune responses while the function of FO-II B cells is unknown. However, FO-II B cells can potentially represent a reservoir from which to rapidly obtain MZ B cells, following depletion by blood borne pathogens by first differentiating to become T2-MZP and then MZ B cells (Pillai and Cariappa, 2009, Cariappa *et al*., 2007, Kleiman *et al*., 2015).

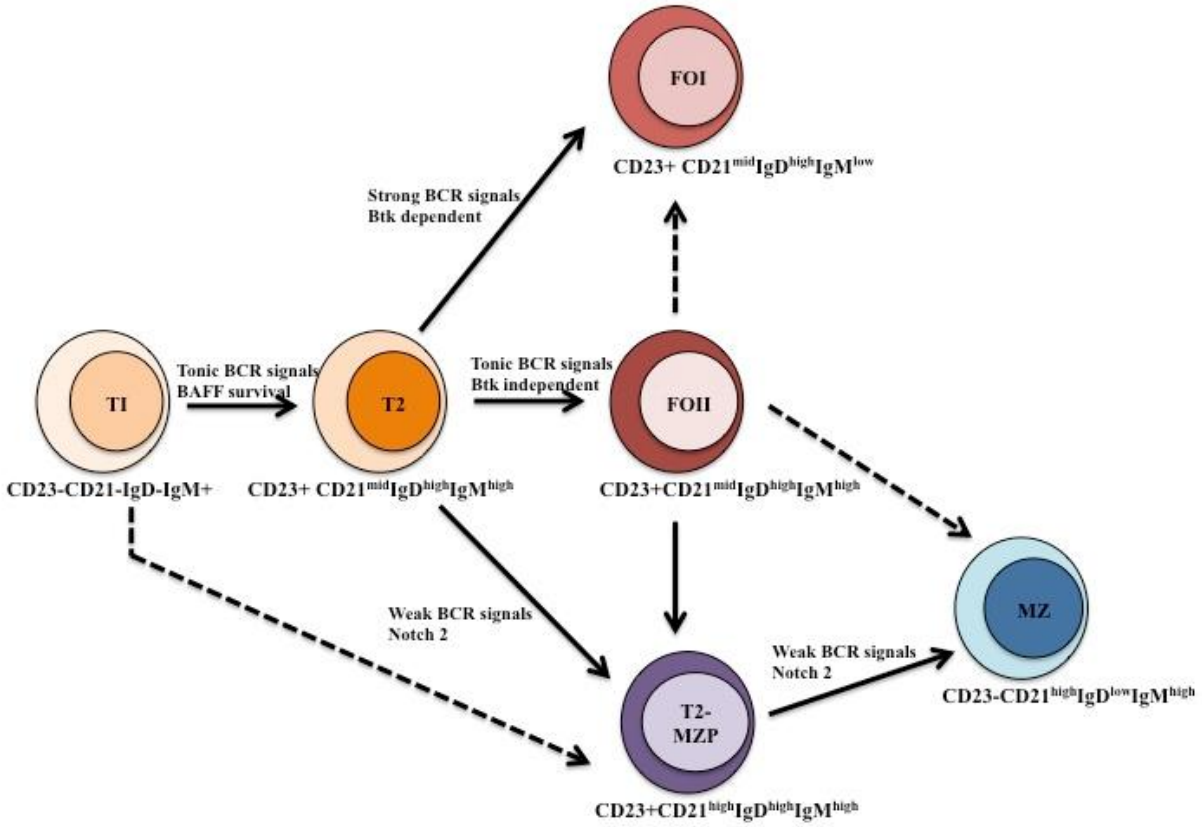


Figure 5.1. Peripheral B cell differentiation

A schematic diagram, illustrating peripheral B cell differentiation and their surface marker expression. Peripheral B cells differentiate according to a signal strength model. When antigens weakly triggers the BCR, it results in insufficient Btk, which leads to activation of Notch2, thus differentiation towards MZ B cells. Conversely, strong BCR signal lead to the activation of Btk which increases differentiation of B cells into FO-I. Adapted from (Kleiman *et al*., 2015, Pillai and Cariappa, 2009).

T2-MZP B cells can arise directly from the T1, T2 or FO-II B cell pool as a result of weak BCR signals and Notch2 signalling via Delta-like 1 (Kleiman *et al*., 2015, Pillai and Cariappa, 2009). T2-MZP B cells can be characterised as CD23+IgD^{high}IgM^{high}CD21^{high} (Blair *et al*., 2008) and can differentiate into MZ B cells which are CD23-CD21^{high}IgD+IgM^{high} (Pillai and Cariappa, 2009). The surface marker expression of peripheral B cell subsets is shown in Table 5.1.

Table 5.1. Markers used to characterise different B cell populations in the spleen

B cell phenotypes	IgD	IgM	CD23	CD21
T1	-	+	-	-
T2	+++	+++	+	++
FO-I	+++	+	+	++
FO-II	+++	+++	+	++
T2-MZP	+++	+++	+	+++
MZ	+	+++	-	+++

In the spleen, various B cell populations can be characterized using several surface markers. These markers can be used to distinguish between T1, T2, FO-I, FO-II and MZ B cells. +Low, ++mid, +++high (Allman and Pillai, 2008, Pillai and Cariappa, 2009, Su *et al*., 2004, Carsetti *et al*., 2004).

5.1.2. Objectives

In Chapter 4, three subsets of B cells were shown to emerge at 40hours post-activation stimulus characterised by differential expression of CD23 and CD25. The objectives of this chapter are:

- To isolate the different subset populations by cell sorting in order to assess the functional properties of each of these B cell subsets in isolation.
- To extensively characterize the B cell subsets in the periphery after treatment with rShh at 18hours and 40hours post-activation stimulus.

5.2. Results

5.2.1. Functional sorting assay

5.2.1.1.Morphological characteristics of sorted B cell population

In Chapter 4, three subsets of B cells were shown to emerge at 40hours post-activation in culture and these were called subset I (CD23+CD25-), subset II (CD23+CD25+) and subset III (CD23-CD25+). Subset II and III were shown to be positive for IL-10 and CD27 respectively, with treatment of rShh increasing the expression of these markers on B cells at this time point. Thus, it became important to ascertain, and to further characterize, the identity of these subsets and attempt to evaluate their individual contribution to the B cell immune response after treatment with rShh.

Purified mouse splenic B cells were activated with 5μg/ml anti-CD40/IgM and treated without (Control (C)) or with (Treated (T)) 0.05μg/ml rShh and cultured for 40hours. Following 40hour post-activation stimulus, B cells were stained with a combination of anti-CD23-^{FITC} and anti-CD25-^{PE} before sorting into three subsets (Figure 5.2B); CD23+CD25-, CD23+CD25+ and CD23-CD25+. The subsets sorted from the control (C) and treated B cells (T) were then cultured once again either with 0.05μg/ml rShh (-T) or without (-C) treatment overnight. A schematic illustration of the sorting experiment is shown in figure 5.2A. The nomenclature of the experimental conditions is shown in Table 5.2. Following overnight culture, sorted B cells were analysed morphologically, stained and analysed for cell surface marker expression. Supernatants were also collected and analysed for cytokine content and antibody production.

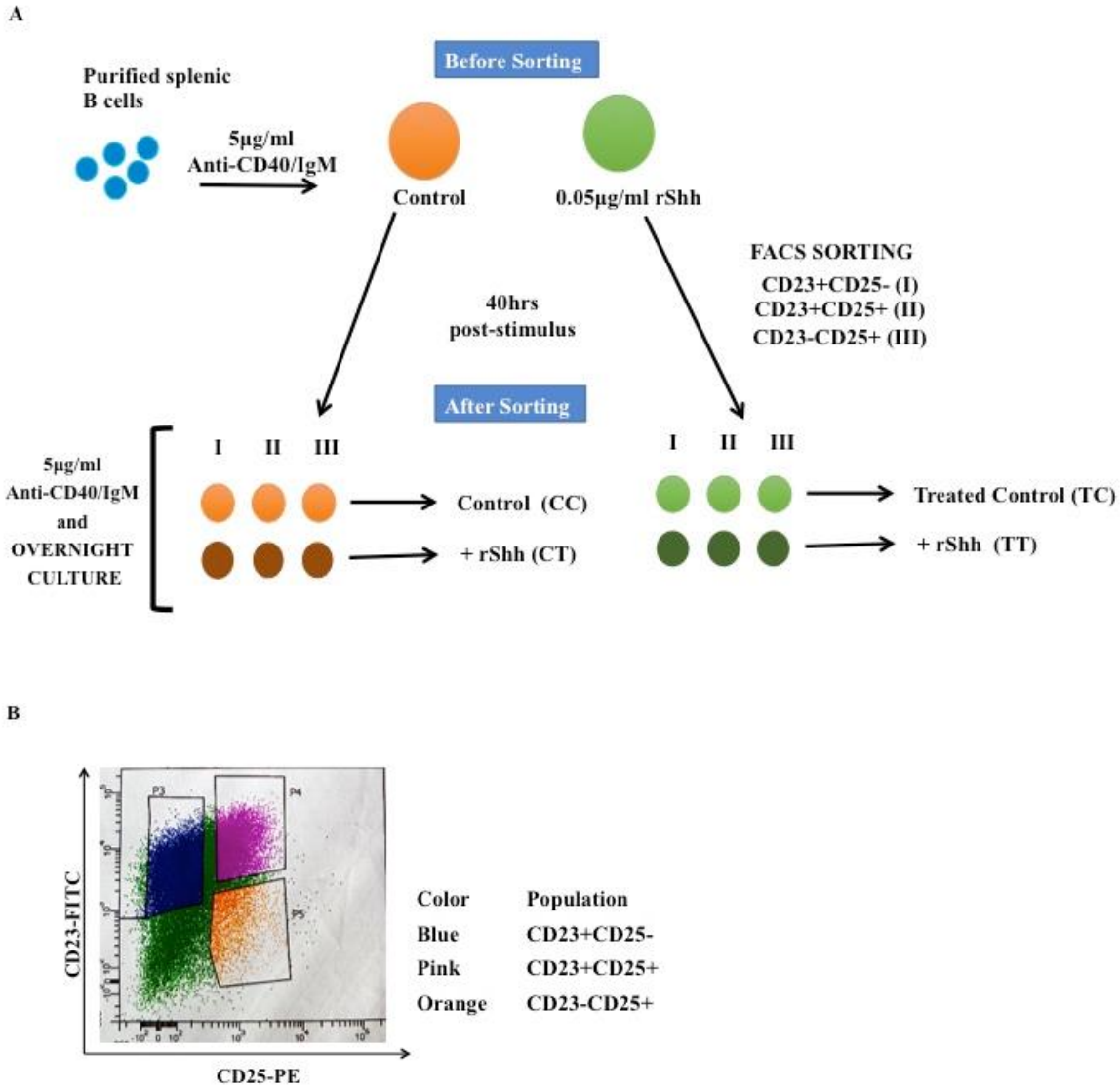


Figure 5.2. Sorting of B cell subsets

A schematic diagram illustrating the functional sorting experimental design is shown in (**A**). Purified B cells were activated with 5µg/ml anti-CD40/IgM. At 40hours post-activation stimulus, B cells were stained with anti-CD23-FITC and anti-CD25-PE before sorting into three populations shown in the dot plot (**B**) CD23+CD25- (Blue), CD23+CD25+ (Pink) and CD23-CD25+(Orange). After sorting, (**A**), B cell subsets from the control and rShh treated were activated and cultured overnight either with or without treatment with rShh. The dot plot shown in (**B**) is from one experiment representative of four independent experiments.

Table 5.2. Nomenclature of experimental conditions

Codes	Treated with rShh before sorting	Treated with rShh after sorting
CC	-	-
CT	-	+
TC	+	-
TT	+	+

Upon activation via the BCR, B cells form an immunological synapse, which is characterised by a cluster of key molecules at the interface between two 'communicating' cells such as integrins. The expression of integrins at the cell surface allows two or more B cells to adhere to one another thus forming clumps or 'micro-clusters' (Harwood and Batista, 2010, Yuseff *et al*., 2013). Hence, the extent of B cell activation can be visualised by determining the numbers and size of clumps or micro-clusters of B cells observed in the cultures when analysed under the microscope. Sorted populations were first analysed morphologically under the light microscope to access for the presence of clumps of activated cells, to observe whether any of the subsets had gross phenotypic differences.

In respect of the sorted CD23+CD25- (Subset I) B cells, clusters of B cells could clearly be seen in the control cultures (CC) within which many individual highly refractile cells could be observed (Figure 5.3A). This would be consistent with the idea that this subset contains within it mostly FO B cells, which are stable and able to respond readily to the presence of antigen. When treated with rShh subsequent to sorting (CT) these clusters could still be observed but now individual cells did not appear to be so refractile and the size of the cluster was reduced (Figure 5.3B). This may be attributable to the induction of apoptosis in individual cells within the clusters leading to a loss of cells within the clusters. Interestingly, when analysing for the presence of clusters in CD23+CD25- B

cells that had been treated with rShh prior to sorting, these pre-treated B cells (TC) did not appear to form clusters and did not look particularly refractile (Figure 5.3C). The same was the case for B cells treated with rShh prior to sorting and subsequent to sorting (TT) suggesting that treatment of these pre-treated cells with rShh after sorting had no consequence for these B cells in terms of activation and survival as apoptosis may already have been induced (Figure 5.3D). One possibility is that this induction of apoptosis in these cells is actually due to a strengthening of the BCR signal delivered to the B cells and thus induced cell death in these B cells (Metzler *et al*., 2015).

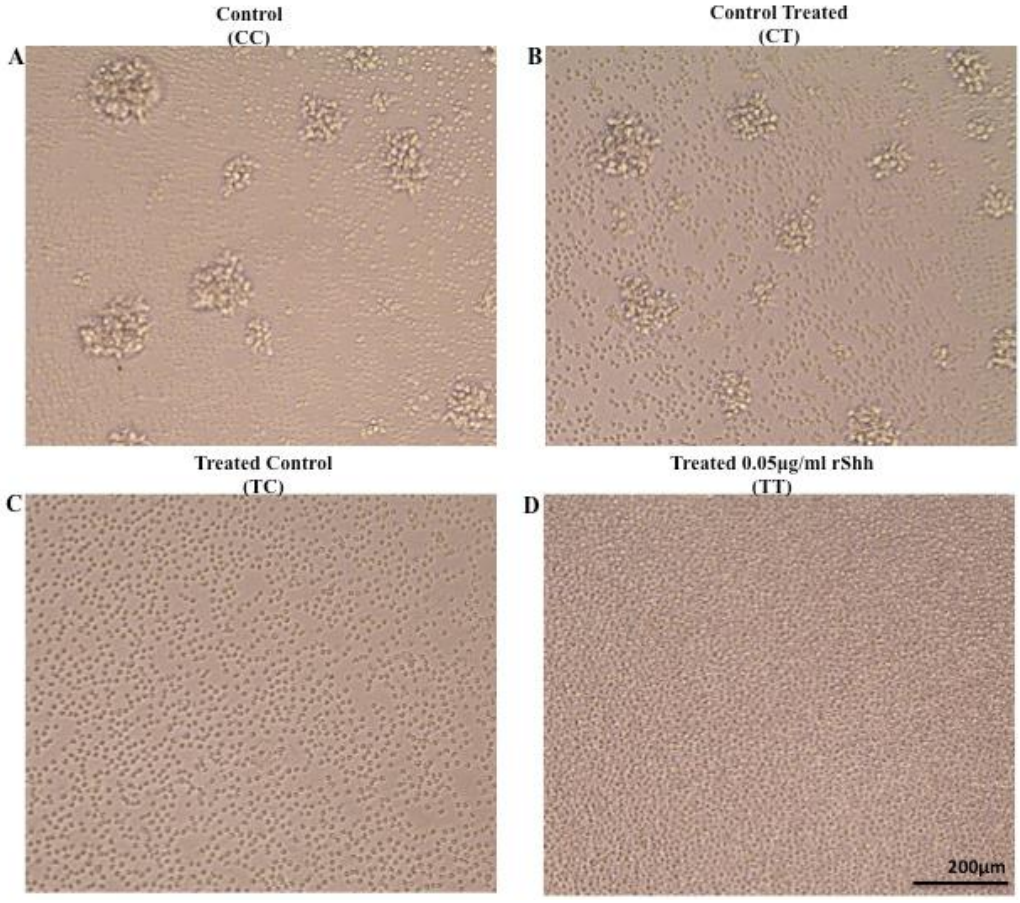


Figure 5.3. CD23+CD25- B cells did not form clumps of activation after rShh treatment

CD23+CD25- B cells were activated with 5µg/ml anti-CD40/IgM overnight with or without treatment with rShh. Pictures showing B cell morphology in (**A**) control (**B**) control B cells treated with 0.05µg/ml rShh, (**C**), treated B cells, (**D**), treated B cells retreated with 0.05µg/ml rShh. Representative images from three independent experiments are shown.

Trypan blue is a stain that permeates the nucleus of dead cells thus colouring them blue whilst leaving live cells uncoloured (Cooksey, 2014). In order to confirm the hypothesis that treatment of sorted CD23+CD25- B cells with rShh induces death in the cells, a trypan blue cell count was carried out and is shown in Figure 5.4. The percentage of live cells was counted in CD23+CD25- B cells after overnight culture. It was observed that CD23+CD25- B cells treated with rShh prior to sorting (TC) and also retreated (TT) had less live cells compared to the untreated control (CC). For example, the number of live CD23+CD25- B cells in the control (CC) was 11 x10^4cells/ml compared to 5 x 10^4cells/ml in CD23+CD25- B cells treated with rShh (TC) (Figure 5.4.) thus indicating that this population of B cells were dying after treatment with rShh thereby highlighting a negative regulatory effect for Hh signalling on B cell survival in this subset.

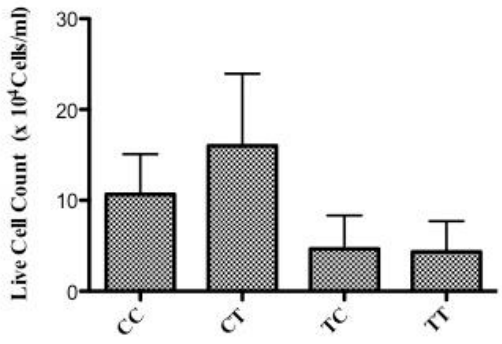


Figure 5.4. Decrease in live CD23+CD25- B cells after treatment with rShh

Live cells from activated CD23+CD25- B cells with or without treatment with rShh was counted using trypan blue. Data presented in the bar chart is represent of an average live cell count of three independent experiments.

After observing these effects in the CD23+CD25- subset, the morphology of CD23+CD25+ B cells was then investigated in order to investigate any morphological differences between the two subsets. It was observed that the apparently most active

subset of all three subsets is CD23+CD25+ as the clusters appeared to be more frequent and larger (Figure 5.5A) in the cultures of control cells (CC) compared to those cultures containing CD23+CD25- (Figure 5.3A) or CD23-CD25+ alone (Figure 5.6A), These findings suggest that this subset was more activated when compared to CD23+CD25- (Figure 5.3) and CD23-CD25+ subsets (Figure 5.6) perhaps indicating they have a greater potential to survive. While we did not formally quantify this, there were also bigger micro-clusters of activation observed in the cultures containing the untreated control B cells (CC) (Figure 5.5A) compared to the B cells treated with rShh (CT) (Figure 5.5B) suggesting that either less activation of B cells occurs when treated with rShh or a greater degree of apoptosis is induced.

When CD23+CD25+ B cells were treated with rShh prior to sorting (TC) (Figure 5.5C) a drastically reduced number of clusters was observed and clusters were much smaller and less refractile possibly indicating some degree of apoptosis or lessened ability to be activated. Surprisingly, these pre-treated cells, when activated subsequent to the sort in the presence of rShh (TT) (Figure 5.5D) did appear to form a small number of large clusters, unlike CD23+CD25- B cells in which no clusters were formed by these double treated cells (Figure 5.3D). This either indicates that these cells survive the double treatment far better than the CD23+CD25- subset or retain the ability to be activated, possibly attributable to more immature B cells being newly differentiated to FO B cells in these cultures and retaining the capacity to be activated.

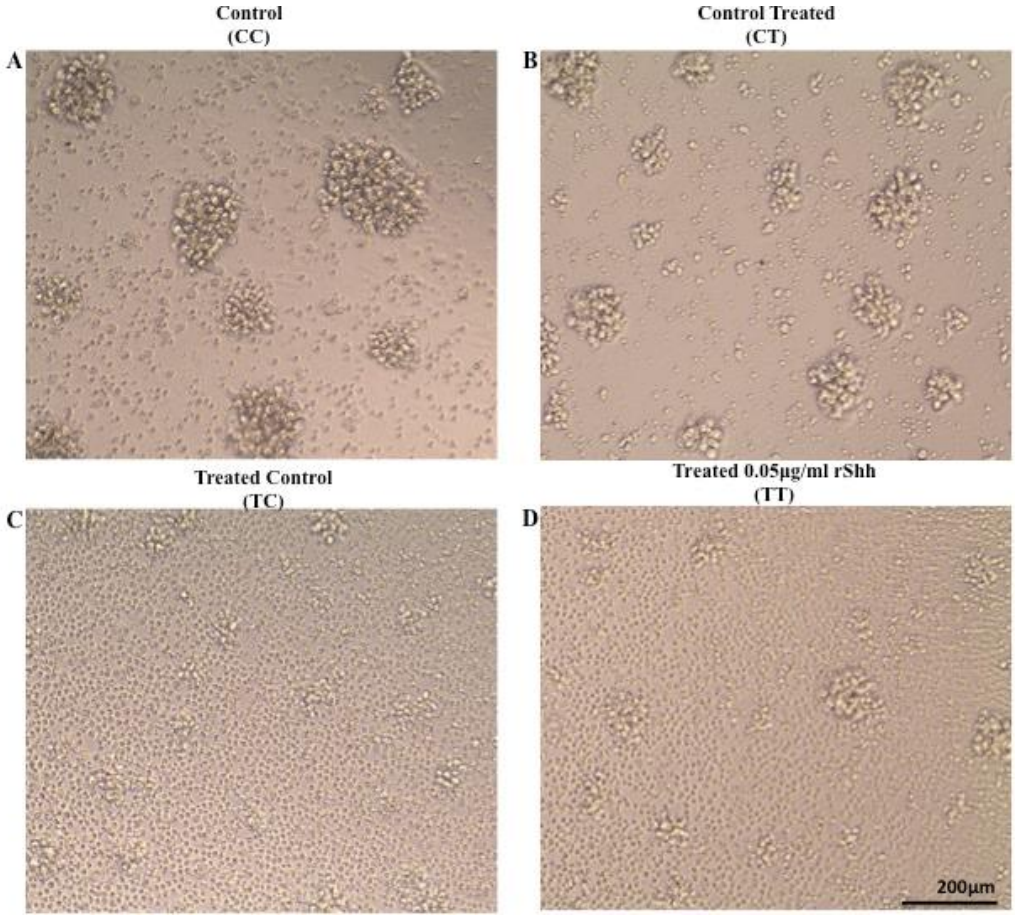


Figure 5.5. B cell activation decreases after treatment with rShh

CD23+CD25+ B cells were activated with 5µg/ml anti-CD40/IgM overnight with or without treatment with rShh. Pictures showing B cell morphology in (**A**) control (**B**) control B cells treated with 0.05µg/ml rShh, (**C**), treated B cells, (**D**), treated B cells retreated with 0.05µg/ml rShh. Representative images from three independent experiments are shown.

When investigating the morphology of CD23-CD25+, a new and different morphology was observed. Untreated control (CC) appeared to take on a more polymorphic appearance with some cells apparently becoming adherent and becoming polarised (Figure 5.6A). When treated with rShh (CT) cells lost this polymorphic appearance and appeared to form clusters of similar shaped cells (Figure 5.6B). When cells were treated with rShh prior to sorting (TC), there was an increase in the appearance of the adherent-type cells and few round lymphocyte-like cells were observed (Figure 5.6C). Upon

double treatment of these cells (TT) (Figure 5.6D) the cells took on a similar appearance to those observed previously (TC).

These data may suggest either the appearance of a non-lymphocytic cell contained within this CD23-CD25+ sorted population or that these cells may become differentiated after treatment with rShh to cells that no longer retains the typical lymphocytic appearance. Additionally, live cell imaging has shown that upon B cell activation, B cells initially bind antigen by forming cellular protrusions that extend from the B cell membrane before forming micro-clusters (Tolar *et al*., 2008). Therefore, there is a possibility that CD23-CD25+ B cells may be forming these cellular protrusions that would enable them attach to one another during B cell activation but because of the cell numbers in the well, these protrusions may become visible.

Taken together these results suggest that treatment of B cells with rShh can regulate B cell activation and survival differently in the different subsets. The differences of morphology between these subsets indicate that these subsets exhibit different phenotype, which result in their unique functions. It also suggests that the treatment of rShh can induce different response in these B cell subsets and this can alter the differentiation of B cells *in vitro*. Thus, it is important to introduce the use of more markers in order to characterize these individual B cell phenotypes to achieve a better understanding of the role of Shh in B cell differentiation. After observing the morphological differences of the sorted B cell subset, the expression of surface markers was then analysed.

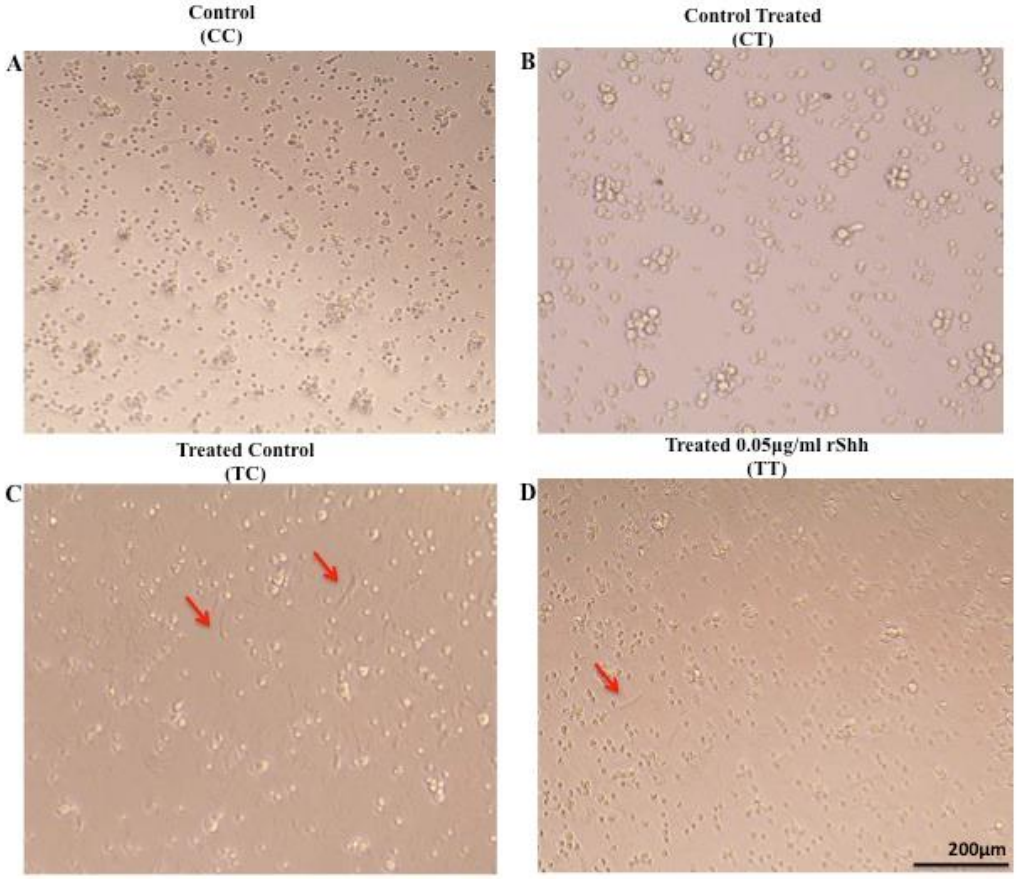


Figure 5.6. Morphology of CD23-CD25+ B cells

CD23-CD25+ B cells were activated with 5µg/ml anti-CD40/IgM overnight with or without treatment with rShh. Pictures showing B cell morphology in (**A**) control (**B**) control B cells treated with 0.05µg/ml rShh, (**C**), treated B cells, (**D**), treated B cells retreated with 0.05µg/ml rShh. Representative images from three independent experiments are shown.

5.2.1.2. The percentage of CD80+ B cells increases after treatment with rShh

In Chapter 4, it was reported that CD23-CD25+ B cells were positive for the human memory B cell marker CD27, which lead to the initial conclusion that this subset of B cells contained, amongst them, memory B cells. However, CD27 is a marker for memory B cells in humans and it is controversial as to whether or not it is also a marker for mouse memory B cells (Agematsu *et al.*, 2000) and thus further characterisation of

this subset was required using an alternative marker for mouse memory B cells, CD80, (Good-Jacobson *et al*., 2012, Zuccarino-Catania *et al*., 2014).

CD80, a B7 family member that can bind to CD28, CTLA-4 and PDL-2 is a marker that has been found to be up-regulated in human and murine memory B cells (Good-Jacobson *et al*., 2012, Zuccarino-Catania *et al*., 2014). For this reason, we used CD80 to further characterise the CD23-CD25+ B cells. The three sorted B cell subsets were stained using CD80-^{APC} and then analysed for their expression using flow cytometry.

Figure 5.7A, shows dot-plots representing the expression of CD23 and CD80 on the three populations of sorted cells. CD23+CD25- B cells are negative for CD80 as only 7.7% of B cells are CD80 positive suggesting that this subset does not contain memory B cells. There is a higher level of expression of CD80 in the CD23+CD25+ B cells with 18.9% of cells being positive for CD80. Finally, expression of CD80 is at its highest in the CD23-CD25+ B cells, as 39.5% of this subset are stained positive for CD80. This data confirms that CD23-CD25+, which was positive for CD27 (as shown in Chapter 4), and CD80^{high}, does indeed include a memory-like B cell population.

After observing that CD23-CD25+ contained cells that were positive for CD80, we asked whether the expression of this marker is modulated by Hh signaling. Indeed, CD80 expression was increased on cells contained within the CD23-CD25+ subset following treatment with 0.05μg/ml rShh. Figure 5.7B, shows data on B cells that were CD80+CD23-CD25+. There was a significant increase in CD80+ B cells that were treated twice with rShh of approximately 10% compared to the untreated control. It was previously observed in chapter 4, that rShh treatment increases CD27 expression on B cells at 40hours post-activation stimulus. The increase in percentage of CD80+ B cells

observed in the sorted CD23-CD25+ B cells after treatment with rShh further confirms that rShh drives the increased differentiation of B cells to become memory B cells or to a cell with a memory-like phenotype.

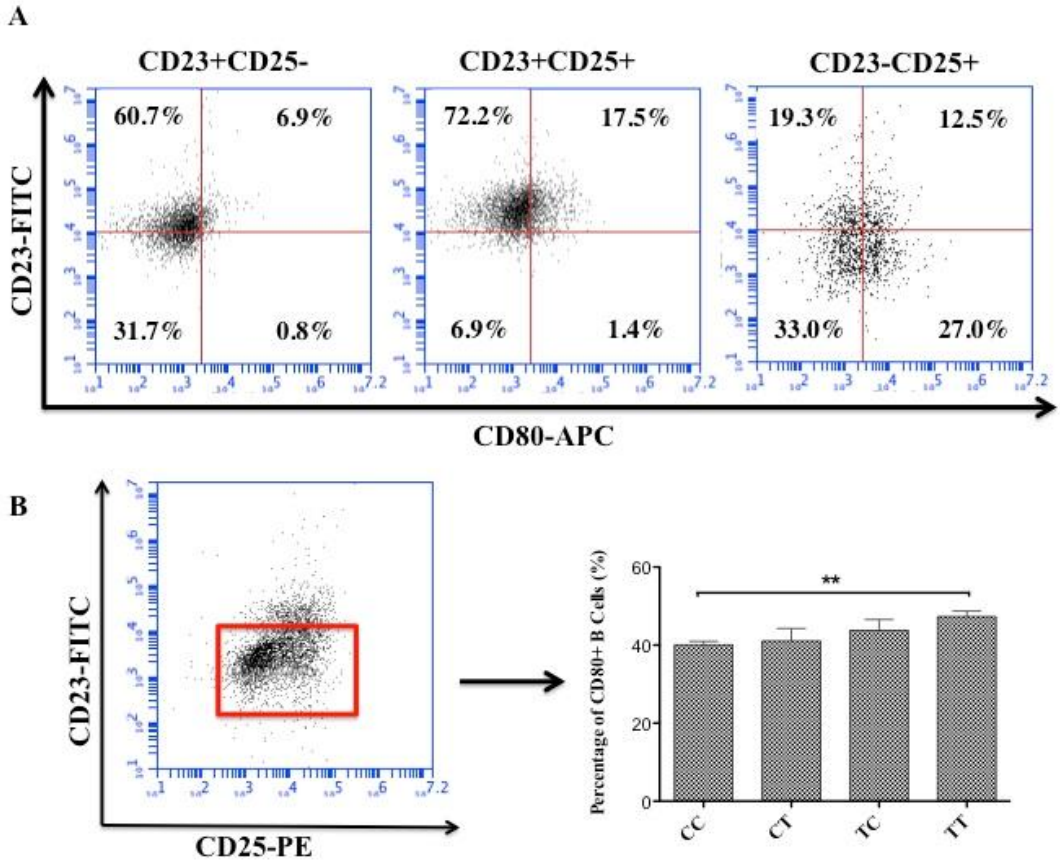


Figure 5.7. CD80+ B cells increases after treatment with rShh

The dot-plot shown in (**A**) is representative of one experiment from three independent experiments and it shows CD80 expression in the sorted B cell subsets. (**B**) CD80+ B cells in CD23-CD25+ B cells. The results shown in the bar chart are representative of three independent experiments. The significance of the difference observed in CD80+ B cells was assessed using Student *t* test **p≤0.01.

5.2.1.3. Cytokine and antibody secretion by the sorted B cell populations

In order to functionally characterise the sorted B cell population, we went on to investigate whether the different subsets could secrete lineage specific cytokines or antibody and whether the levels of secretion differed between the populations.

Figure 5.8A, shows data on IL-6 secretion by the sorted B cell subsets. From the assay, it can be seen that more IL-6 is secreted by CD23+CD25+ B cells under all conditions analysed. For example, there was a significant 2.8-fold increase in IL-6 secretion by CD23+CD25+ B cells compared to CD23+CD25- in the untreated control (CC). Addition of rShh to the overnight cultures (CT) reduced the IL-6 secreted by CD23+CD25+ whereas secretion of IL-6 by CD23+CD25- appeared to have increased slightly. There was a significant 3.5-fold increase in secretion of IL-6 by cells contained within the CD23+CD25+ subset in the treated cells (TC) in comparison to the untreated control (CC). However, this increase in IL-6 secretion was lost when cells were treated twice with rShh (TT) from an average of 0.55ng/ml to 0.25ng/ml. it was observed that CD23-CD25+ secreted the least amount of IL-6 of all the subsets. These results suggest that it is the CD23+CD25+ cells that secrete most of the IL-6 and levels of secretion are regulated by Hh signalling (Figure 5.6 A).

Figure 5.8B, shows data for IL-10 secretion by the sorted B cell subsets. Interestingly, all the sorted B cell subsets are capable of secreting IL-10 under all conditions. It was observed that the levels of IL-10 secreted by CD23+CD25- and CD23+CD25+ B cells were similar in B cells treated with rShh (TC) prior to sorting with a slight decrease in CD23-CD25+ B cells. Surprisingly, the IL-10 concentration decreased when these cells where treated twice with rShh (TT). Additionally, it was observed that CD23+CD25- cells secreted the most IL-10 of all the subsets in the control (CC). However, when control B cells were treated with rShh (CT) subsequent to sorting, IL-10 secretion decreased in B cell subsets with the exception of the CD23-CD25+ B cells, which showed a 1.5 fold increase in IL-10 secretion. Interestingly, CD23-CD25+ B cells secreted IL-10 in all conditions, this result was unexpected, as this subset has been characterised to contain memory-like B cells. However, memory B cells have been

described as having the capacity to secrete IL-10 after stimulation (Kalampokis *et al*., 2013). These results suggest that upon activation of B cell subsets, they can differentiate to become possibly T2-MZP that can secrete IL-10. However, the amount of IL-10 was very low and the differences between the amounts secreted by the different groups was not significant.

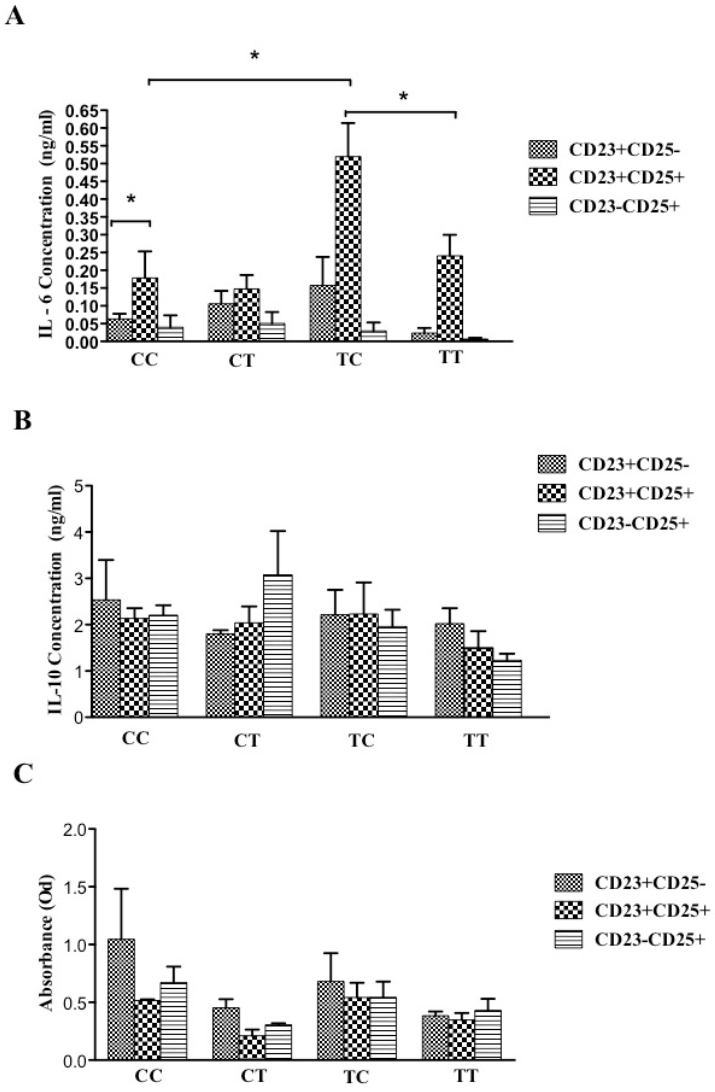


Figure 5.8. Treatment of B cells with rShh can affect cytokine secretion and antibody production in sorted B cells

Sorted B cell subsets were activated with 5µg/ml anti-CD40/IgM and were treated with rShh overnight. Supernatants were collected and analysed for IL-6 secretion (**A**), IL-10 secretion (**B**), and IgG1 production (**C**), using ELISA assay. The results shown in the bar chart are representative of three or two (C) independent experiments. The significance of the difference observed in IL-6 secretion was assessed using Student *t* test (*p≤0.05).

Figure 5.8C, shows IgG1 secretion by all the subsets either with or without treatment with 0.05μgml rShh. In all cases, CD23+CD25- B cells secreted the most IgG1 compared to the other subsets. Treatment with rShh reduced the amount of IgG1 secreted by all sorted B cell subsets. This data is consistent with the previous findings that by 40hours in culture, rShh appears to arrest the production of antibody by B cells.

From these cytokine assays, antibody secretion assay and cell surface characterisation a preliminary conclusion can be made about the different subsets as follows. CD23+CD25- B cells are most likely to be FO B cell as this subset showed the highest secretion of antibody of all the three subsets and FO B cells has been shown to be capable of secreting IL-6 following CD40 ligation, and also secrete antibody (Urashima *et al*., 1996). CD23+CD25+ B cells could secrete IL-6, IL-10 and IgG1 and are positive for CD80, which suggests they may be a heterogeneous population of cells containing FO and T2-MZP B cells (Blair *et al*., 2008). CD23-CD25+ B cells, were shown to be $CD80^{high}$ and had previously been shown to be CD27+ (Chapter 4). Thus, the CD23-CD25+ subset contains cells that exhibit a memory-like B cell phenotype (Amu *et al*., 2006, Agematsu *et al*., 2000, Good-Jacobson *et al*., 2012). Also, MZ B cells are CD23- and have been shown to be CD27+ (Garraud *et al*., 2012) hence it is possible that the CD23-CD25+ subset also contain MZ B cells. Below is a table showing a summary of the functions of the sorted B cell subsets.

Table 5.3. Summary of functions of sorted B cell subsets

CD23+CD25-	CD23+CD25+	CD23-CD25+
Secretes IL-6	Secretes IL-6	Secretes low IL-6
Secretes IL-10	Secretes IL-10	Secretes IL-10
Secretes the most Ig	Secretes little Ig	Secretes little Ig
CD80-	$CD80^{low}$	$CD80^{high}$
Possibly contains FO B cells (Urashima *et al*., 1996)	Heterogeneous mixture containing T2-MZP and FO B cells (Garraud *et al*., 2012, Urashima *et al*., 1996)	Contains memory B cells or MZ B cells (Amu *et al*., 2006, Garraud *et al*., 2012, Good-Jacobson *et al*., 2012)

A table illustrating the functions of sorted subsets as observed

5.2.2. Phenotypic characterisation of B cell subsets in the spleen

5.2.2.1. Characterisation of B cells in the spleen

Our data indicates that after rShh treatment, there is an increased differentiation of B cells possibly into FO B cells at 18hours post-activation and regulatory B cells and memory B cells by 40hours post-activation. It was also observed that the different subsets appeared to have different functions associated with these different differentiation outcomes. We therefore wanted to carry out further characterisation of these subsets in terms of expression of B cell developmental markers. Using surface markers that are characteristic of the various B cell subsets in the spleen such as T1, T2,

T2-MZP and FO-I or FO-II and MZ B cells, the different developmental stages of B cells could be better identified in our culture system.

As before, B cells were purified by negative selection from the total splenic population. In order to identify the B cell population in the spleen that are emerging in our cultures, fresh uncultured purified B cells were first analysed and were stained with a combination of anti-IgM-^{PE}, anti-CD23-$^{Pe\text{-}Cy7}$, and anti-CD21-^{APC}. B cell populations were gated following the protocol and characterisation published by (Carsetti, 2004, Kleiman *et al*., 2015, Pillai and Cariappa, 2009, Rosado *et al*., 2014). B cells were first gated on whether they were CD23+ or CD23-. Expression of IgM and CD21 was then analysed on both the CD23+ and CD23- populations of B cells thus allowing identification of T1, T2, FO-I, FO-II and MZ B cells (Carsetti, 2004, Cariappa *et al*., 2007, Kleiman *et al*., 2015, Pillai and Cariappa, 2009).

Figure 5.9, shows dot-plots representing the peripheral B cell subsets in fresh purified B cells from the spleen. From our staining results, the B cells from the spleen were found to be majorly T2 and FO-II B cells comprising 39.5% of the population and a FO-I B cell population comprising 22.1%. Also present, were T1 population (17.8%), T2-MZP (8.6%). and MZ B cells of 10.7% (Figure 5.9).

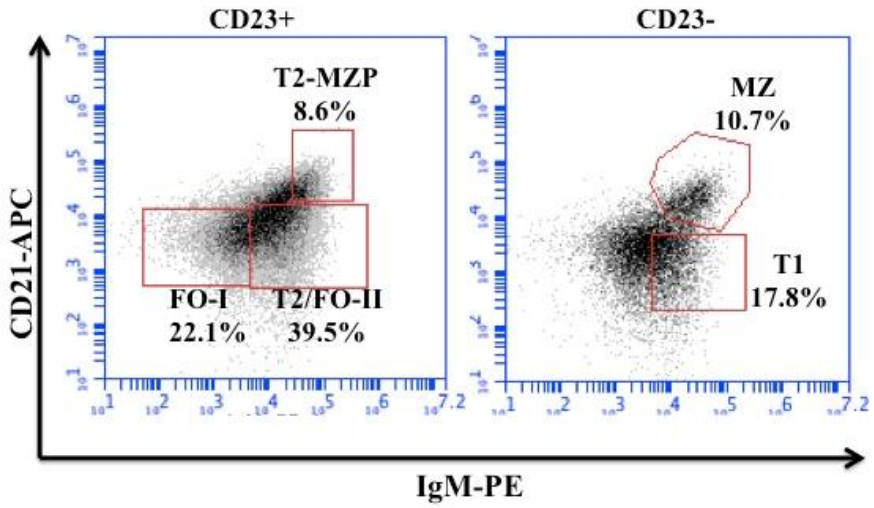


Figure 5.9. Peripheral B cell subsets

The dot-plots shown are from one experiment representative of three independent experiments in which fresh uncultured purified B cells were stained with anti-IgM, anti-CD21 and anti-CD23 in order to distinguish peripheral B cell subsets in the spleen.

5.2.2.2.Hh signalling increases B cell differentiation to FO and T2-MZP B cells

After identifying our populations of B cells in fresh splenic cell preparations to be majorly T2 and FO-II B cells, it became important to ascertain the developmental fate of the cultured B cell subsets at 18hours and 40hours post-activation stimulus. Purified B cells were activated with 5µg/ml anti-CD40/IgM with or without treatment with 0.05µg/ml rShh. At 18 and 40hours post-activation stimulus, the B cells were stained with combination of anti-IgM-PE, anti-IgD-FITC, and anti-CD23-PeCy7, anti–CD21-APC.

In our culture, as the B cells were activated with anti-CD40/IgM, thus B cells were activated via a T-dependent manner. Activation of B cells in a T-dependent manner drives B cell differentiation towards FO B cell differentiation and thus, MZ B cells would not be expected to emerge in increased numbers. T1 B cells would be expected to

die as they don't survive other than for a short period of time *in vitro* or in the spleen (Su *et al*., 2004).

Figure 5.10A, shows data on B cell differentiation at 18hours post-activation stimulus. In the unstimulated B cells, the major population of B cells, were T2 B cells and FO-II B cells which when the B cells are activated differentiate to become CD23+CD21^{mid}IgM^{low} FO-I B cells. However, treatment of B cells with 0.05µg/ml rShh induces increased B cell differentiation towards FO-I, from 41.6% in the control to 58.8% in treated B cells and this was significant at this time point (Figure 5.10B). This suggests that Hh signalling can increase B cell activation and differentiation towards FO-I B cells at 18hours post-activation stimulus.

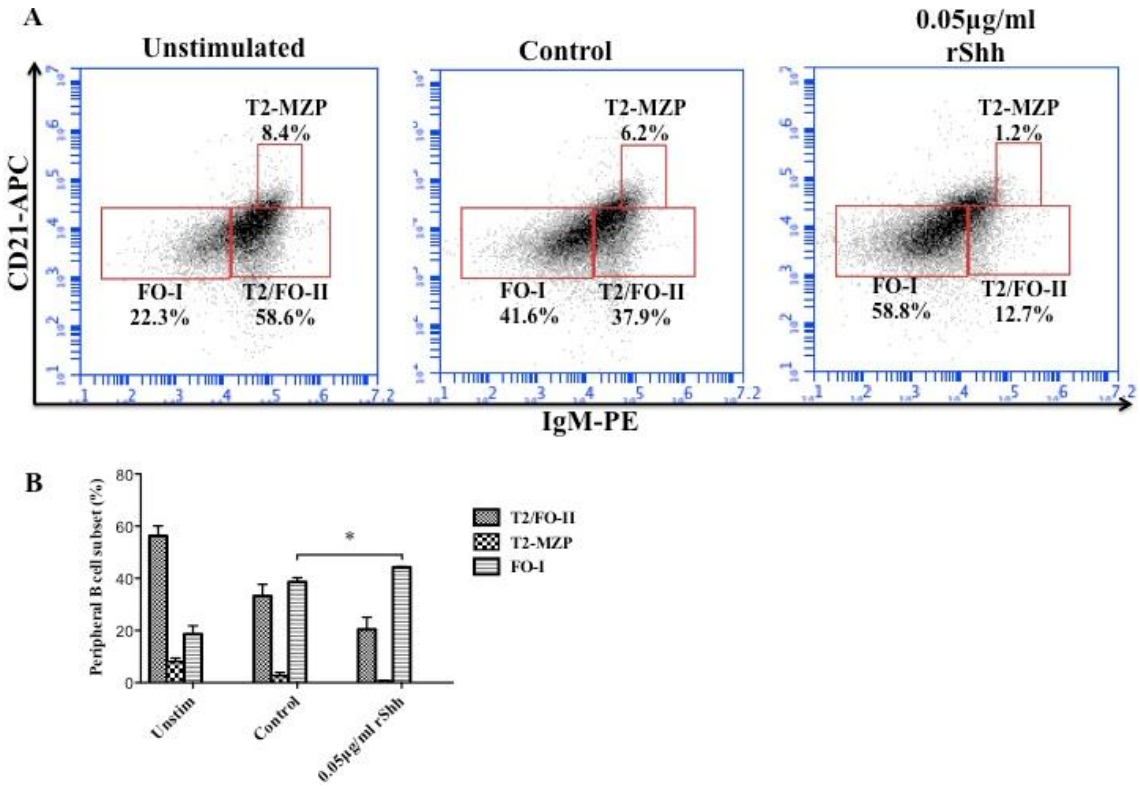


Figure 5.10. FO-I differentiation increases after treatment with rShh at 18hours post-activation

The dot-plots shown in (**A**) is representative of one experiment from three independent experiments and it shows CD21 and IgM expression on CD23+ peripheral B cells after treatment with rShh with a bar chart representing this data shown in (**B**). The results shown in the bar chart are derived from three independent experiments. The significance of the difference observed in FO-I B cells was assessed using Student *t* test *p≤0.05.

At 40hours post-activation (Figure 5.11A), differentiation towards the FO-I subset has decreased with treatment of rShh compared to the untreated control. However, the increased emergence of T2-MZP B cells as well as FO-II was identified following treatment with rShh. The presence of T2-MZP B cells increases in B cells treated with rShh compared to untreated control from 8.7% in the control to 13.3% in the rShh treated B cells, however, this difference was not significant (Figure 5.11B). T2-MZP have been identified to display regulatory properties, they have been shown to secrete the anti-inflammatory cytokine IL-10 (Blair *et al*., 2008). There was also a reduced FO-I population in B cells treated with 0.05μg/ml rShh, from 45.2% in the untreated control to 32.4% in treated B cells (Figure 5.11A).

In chapter 4, it was shown that treatment of B cells with rShh increased IL-10 secretion at 40hours post-activation stimulus. This increase in T2-MZP B cells observed in the presence of rShh could account for the increased IL-10 expression and secretion by B cells in the presence of Hh signalling.

These findings suggests that Hh signalling has the ability to accelerate B cells development to become FO-I, which is Btk signalling dependent, at 18hours post-activation stimulus (Pillai and Cariappa, 2009) and then either these cells are induced to apoptose or the signal may become weakened as cells remain in culture for 40hours post-activation stimulus and this leads to the differentiation towards T2-MZP and FO-II cells.

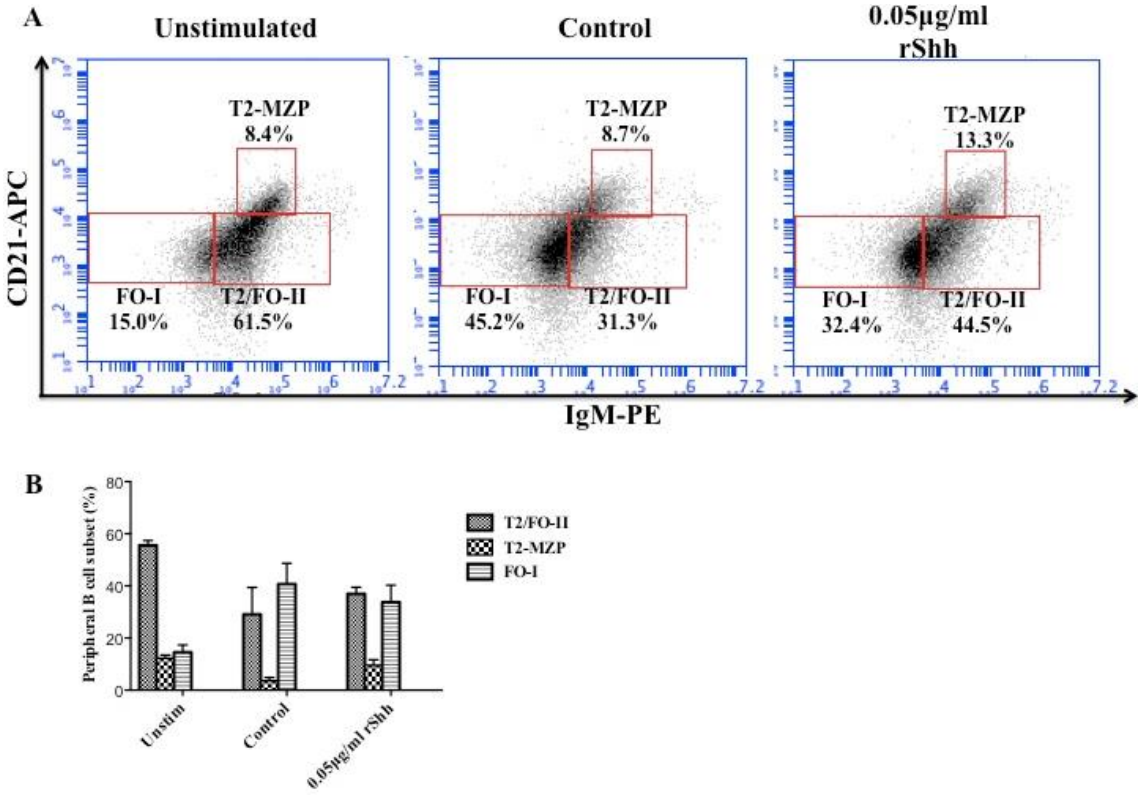


Figure 5.11. Treatment of B cells with rShh increases differentiation towards T2-MZP at 40hours post-activation stimulus

The dot-plots shown in (**A**) is representative of one experiment from three independent experiments and it shows CD21 and IgM expression on peripheral B cells after treatment with rShh with a bar chart representing this data shown in (**B**). The results shown in the bar chart are representative of three independent experiments.

5.2.2.3. Characterisation of sorted B cell subsets

After observing that treatment of B cells with rShh can affect the differentiation of B cells, it became important to better characterise the sorted B cell subset CD23+CD25-, CD23+CD25+ and CD23-CD25+, in order to ascertain if these subsets contain T2, FO-I, FO-II or, T2-MZP B cells according to our classification.

Purified B cells were cultured as previously described in Section 5.2.1.1. Sorted B cell population were then characterised using a combination of anti-IgM- PECy7 and anti-IgD-APC or CD21-APC.

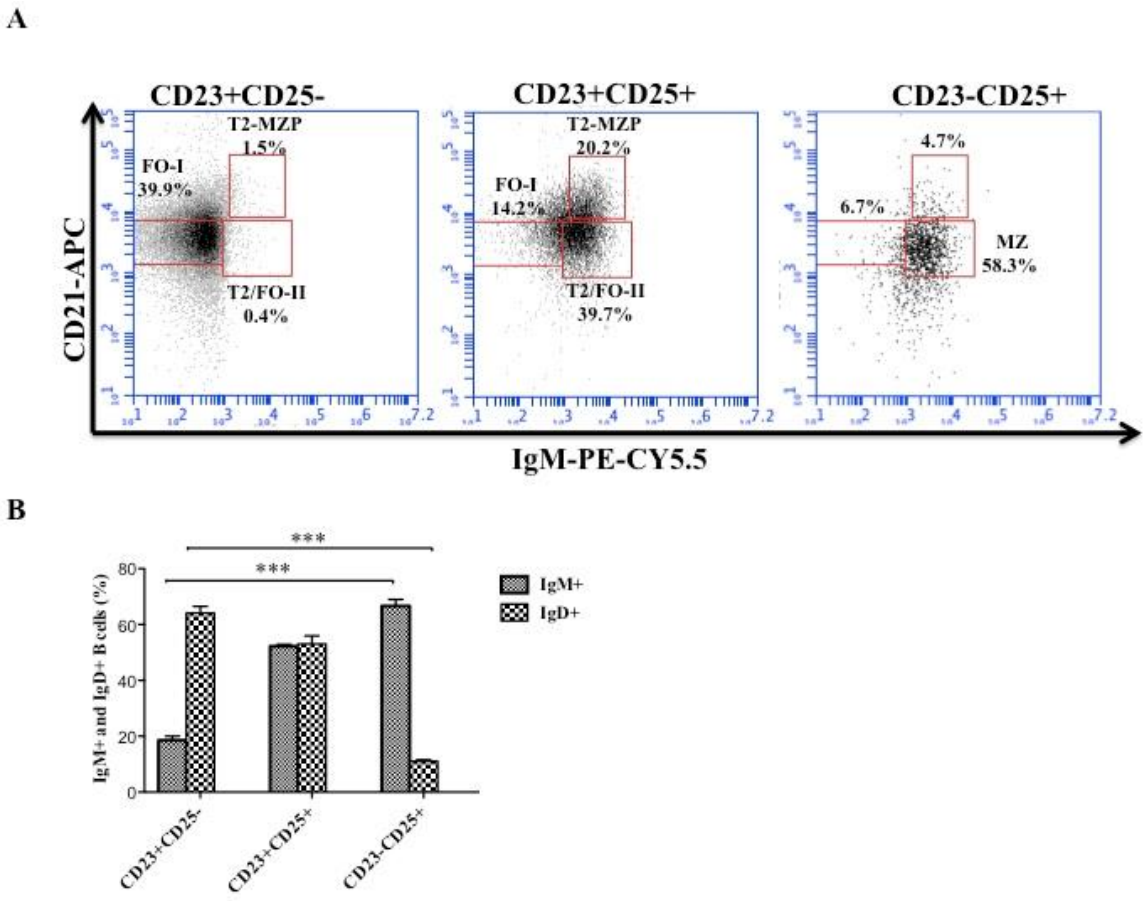


Figure 5.12. Characterisation of sorted B cell subsets

The dot-plots shown in (**A**) are representative of one experiment from three independent experiments and it shows CD21 and IgM expression on the sorted B cell subsets. The percentage of IgM+ and IgD+ B cells is shown in (**B**). The results shown in the bar chart are representative of three independent experiments. The significance of the difference observed in IgM+ and IgD+ B cells was assessed using Student *t* test ***$p \leq 0.001$.

Figure 5.12A, shows the expression of IgM and CD21 on sorted B cell subsets. CD23+CD25- was identified to be FO-I cells as they were IgM^{low} $CD21^{mid}$. There were no T2/ FO-II B cells neither any T2-MZP B cells present in this subset. CD23+CD25+ subset of B cells was found to contain a mix of FO-I, T2/FO-II and T2-MZP B cells. This suggests that FO-II are differentiating to become T2-MZP B cells as it has been reported that FO-II B cells form a reservoir and can differentiate to become T2-MZP (Cariappa *et al*., 2007). However, one unexpected finding was that CD23-CD25+ B cell population contained a mix of cells. This subset appears to contain 58.3% MZ B cells.

After analysing CD21 and IgM, we wanted to look at the levels of IgD on the different subsets, as memory B cells have been previously shown to be IgD negative (Tangye and Tarlinton, 2009, Tarlinton, 2006). Figure 5.12B, shows data for the expression of IgM and IgD on sorted B cell subsets. CD23+CD25- subset was identified to be $CD21^{mid}$ IgM^{low} (Figure 5.10A), however it was observed that this subset was also IgD^{high} (Figure 5.10B) thus, suggesting that this subset is FO-I B cells. The CD23+CD25+ was found to contain a mix of cells that were $CD21^{high}IgM^{high}IgD^{high}$ as well as $CD21^{mid}$,IgM^{high},IgD^{high} , this suggests that these subset contains T2-MZP as well as FO-I and FO-II B cells.

Interestingly, CD23-CD25+ was IgM^{high}, but had the lowest expression of IgD of all the sorted B cell subsets (Figure 5.10B). Memory B cells have been characterised as long-lived IgD^{-} B cells, thus indicating that they have undergone class switched recombination. Phenotypically, memory B cells are IgM+, CD80+, $CD23^{-}$ (Tangye and Tarlinton, 2009, Tarlinton, 2006) and also CD25+ (Amu *et al*., 2006). We have shown that after rShh treatment, an increase in memory-like B cells (subset III), which are CD25+, $CD23^{-}$, CD27+ (Chapter 4) and IgM+, $CD80^{high}$, and IgD^{low}. However, it is also possible that these CD23-CD25+ subset contains MZ B cells, as they have been described to have a high expression of CD80 (Cerutti *et al*., 2013), IgD^{low} CD23-, $CD21^{high}$(Allman and Pillai, 2008).

Taken together, it can be concluded that, CD23+CD25- (subset I) contains B cells that are predominantly FO-I, CD23+CD25+ (subset II) contains FOI, FO-II, T2 and T2-MZP cells and, finally, CD23-CD25- (subset III), possibly contains memory like B cells and MZ B cells. We have demonstrated, Hh signalling can increase B cell differentiation towards CD23-CD25+ (Chapter 4) indicating that there is an increase

differentiation of B cells towards memory like B cells and MZ B cells at 40hours post-activation stimulus following treatment with rShh.

5.3. Discussion

It has been reported in previous chapters that treatment of B cells with rShh can increase B cell activation and survival at 18hours post-activation and at 40hours the reverse was observed. At 40hours post-activation stimulus, three subsets were observed to arise and it was important to further characterise these subsets in order to identify the true function of each of the subset in isolation.

The findings in this chapter demonstrates that CD23+CD25-, CD23+CD25+ and CD23-CD25+ B cells, display different morphologies and functions that are modulated by Hh signalling. Additionally, it was observed that CD23+CD25- B cells were $CD23+CD21^{mid}IgD^{high}IgM^{low}$ thus characterising them predominantly as FO-I B cells (Kleiman *et al*., 2015), which were shown to have a reduced ability to be activated and were surviving less well after treatment with rShh (Figure 5.3). In the periphery, differentiation towards FO-I B cells is Btk dependent (Cariappa *et al*., 2007). We hypothesise therefore, that treatment of activated FO-I B cells with rShh can induce a stronger BCR signal to be transmitted into the cell and this might induce activation induced cell death (AICD) in the cells (Metzler *et al*, 2015) thus might explain the reduced presence of live cells in this subset (Figure 5.3). Furthermore, it has been shown that FDCs secrete Shh in the GCs, which might represent a physiological condition in which GC B cells evade apoptosis (Sacedon *et al*., 2005). It is also possible that CD23+CD25- B cells, which are mostly FO-I, either require the presence of another B cell subset to provide either an autocrine or paracrine Hh secretion in order to evade apoptosis or may have lost the ability to respond to Hh signalling. It would be

important to investigate further, whether FO-I B cells, express Hh signalling receptors Ptch and Smo, as it may be that they no longer express one of these receptors and have therefore lost the ability to respond to exogenous Hh.

After observing the effects of rShh treatment on the sorted B cells, it became important to investigate the effect of rShh treatment on peripheral B cell differentiation. Data obtained showed that treatment of B cells with exogenous rShh could accelerate the process of peripheral B cell differentiation. Hh treatment at 18hours post-activation stimulus increased B cell differentiation towards FO-I, which requires strong BCR signalling and can then switch differentiation towards T2-MZP by 40hours, which requires a weak BCR signal (Pillai and Cariappa, 2009). These findings suggest that Hh signalling can strengthen the immune response by B cells possibly by potentiating a stronger BCR signal thereby accelerating peripheral B cell differentiation. However, at a later time point, Hh signalling can down-regulate the response by increasing apoptosis of FO-I B cells. Hh signalling has previously been shown to regulate differentiation of peripheral T cells. It has been shown that, Hh signalling can influence the differentiation of $CD4^+$ T cells towards the Th2 subset. Furmanski *et al*. (2013) reported that Gli-2, which is part of the Hh signalling pathway, could modulate the transcription of genes in $CD4^+$ T cells, by up-regulating genes such as *Gata3* that would skew the differentiation of the T cells towards Th2 T cell phenotype and away from the Th1 phenotype (Furmanski *et al*., 2013).

In conclusion, the sorting assay used in this study revealed that the subsets have different characteristics, capable of secreting different levels of antibody and cytokines and that this is modulated by Hh signalling. Treatment of B cells with exogenous rShh accelerates B cell activation and differentiation to FO-I B cells at 18hours post activation stimulus and arrests B cell activation in the periphery whilst increasing B cell

differentiation towards T2-MZP B cells at 40hours post-activation stimulus. A situation where there is little or no Hh signalling present was investigated next in order to analyse whether the loss of a homologue of Hh would have an effect on B cell function and peripheral development *in vitro*.

5.4. Conclusion

It was demonstrated that CD23+CD25-, CD23+CD25+ and CD23-CD25+ B cells exhibit different B cell morphology, phenotype and function. Hh signalling was demonstrated to have an effect on the different subsets with Hh inducing death in CD23+CD25- B cells, which was characterised to be FO-I B cells. Additionally, this chapter demonstrates that treatment of B cells with rShh can have an effect on peripheral B cell differentiation. It is hypothesised that exogenous Hh signalling can affect peripheral B cell differentiation by strengthening the BCR signal. The next chapter extends findings of this chapter by investigating the effect of the loss of a Hh homologue on B cell development in the periphery.

Chapter 6: B cell development in the Dhh knockout mice

6.1. Introduction

6.1.1. Desert hedgehog protein (Dhh)

There are three homologs of mammalian Hh that share a common signalling pathway. However, each homolog has a distinct pattern of expression, thus, play different roles in development in different tissues (Varjosalo and Taipale, 2008).

Dhh is one such homolog of the vertebrate Hh family. Analysis of knockout mice in which the gene had been deleted revealed that it is essential for the maintenance of the male germ line and spermatogenesis (O'Hara *et al*., 2011). Mutations in Dhh in 46 human XY male patients resulted in symptoms ranging from mild to complete gonadal dysgenesis including bilateral streak gonads, normally developed mullerian ducts and female external genital (O'Hara *et al*., 2011). In the murine embryo, Dhh is expressed in an array of tissues including the testis (Bitgood *et al*., 1996), granulosa cells of the ovaries (Wijgerde *et al*., 2005), circulatory system, including the heart and blood vessels (Bitgood and McMahon, 1995), fetal liver (Cridland *et al*., 2009) and peripheral nerve tissue (Bajestan *et al*., 2006, Parmantier *et al*., 1999). In the peripheral nerve tissue, Dhh is expressed by schwann cells and it plays a role in nerve sheath formation (O'Hara *et al*., 2011). Dhh has also been found to be involved in follicle development (Russell *et al*., 2007). The extensive expression pattern of Dhh suggests it has a wide range of functions in development.

In the lymphoid tissues, Dhh is expressed in the thymus (Sacedon *et al*., 2003) and in the spleen (Perry *et al*., 2009, Lau *et al*., 2012). Dhh protein has also been shown to be expressed by non-hematopoetic cells of the spleen such as stroma (Hegde *et al*., 2008,

Perry *et al*., 2009). Thus, Dhh expression in the spleen may have an effect on the cells of the spleen including T cells, B cells and erythrocytes. Dhh has been shown previously to drive the normal process of erythropoiesis in mice as well as being involved in recovery from stress erythropoiesis (Lau *et al*., 2012). Using Dhh knockout mice (Dhh-/-), Lau *et al*., (2012), showed that there was abnormal erythropoiesis present in the Dhh-/- mice, as the spleens of these mice were bigger and there was increased presence of erythroblasts and reticulocytes in the Dhh-/- compared to the wild-type mice (Dhh+/+). These data suggest that Dhh is a negative regulator for erythropoiesis in mice (Lau *et al*., 2012).

Dhh has also been shown to play a role in T cells (Furmanski *et al*., 2013). $CD4^+$ T cells can differentiate to become either T-helper 1 (Th1) or T-helper 2 cells (Th2). Th1 can be characterised by their expression of T-bet and express cytokines such as IFN-γ. They have the capacity to control immune response against infection by activating $CD8^+$ T cells, B cells and macrophages. Th2 cells express *gata3* and are important in the immune response against extracellular pathogens (Paul, 2010). Using Dhh-/- mice, Furmanski *et al*., 2013, showed that CD4+ T cells isolated from the Dhh-/- mice had a reduced *gata3* expression and thus the T cells had an impaired ability to differentiate towards Th2 cells (Furmanski *et al*., 2013). The results obtained by Lau *et al*., 2012 and Furmanski *et al*., 2013, shows that in the absence of Dhh, there is reduced erythropoiesis and reduced differentiation to Th2 respectively, thus highlighting the importance of Dhh in cells of the immune system.

Dhh has also been implicated in the emergence of B cell chronic leukaemia (B-CLL); a lymphoma of B cells. B-CLL is one of the most prevalent leukaemias and it is characterised by a progressive accumulation of functionally incompetent B cells in the

peripheral blood, bone marrow (BM) and lymphoid organs (Decker *et al*., 2012). Decker *et al*., 2012, showed that Dhh could be simultaneously induced in an autocrine manner and this can support the survival of B-CLL cells via ERK (extracellular signal regulated kinase) phosphorylation thus involving Dhh signalling in the pathogenesis of B-CLL (Decker *et al*., 2012).

6.1.2. Objectives

In peripheral haematopoietic processes, loss of the *Dhh* has been shown to cause impaired differentiation and function (Lau *et al*., 2012, Furmanski *et al*., 2013). The aim of the work presented in this chapter is to investigate the effect the loss of Hh signalling would have on peripheral B cell development using the Dhh-/- mice as a model. The objectives of this chapter are:

- To investigate whether the loss of Dhh gene (*Dhh*) would have an effect on peripheral B cell subsets differentiation.
- To investigate the consequences of the loss of *Dhh* on B cell activation, survival, cytokine secretion and antibody production.
- To investigate whether there are any differences in the activities of rDhh and rShh treatment on B cell function.

6.2. Results

6.2.1. Peripheral B cell subsets in Dhh-/- mice.

After observing the effects of the addition of exogenous rShh to B cells in a mixed splenocyte culture as well as purified B cell cultures, we went on to look at a situation where one of the Hh is not expressed. To this end, we analysed B cell development in

mice deficient for the *Dhh* gene. In mice, where the *Shh* gene or the *Ihh* gene has been knocked out, the embryos suffer devastating effects resulting in embryonic death. The Shh-/- embryos exhibit axial mis-patterning as well as skeletal and lung defects while the Ihh-/- embryos die at mid gestation or before birth possibly as a result of yolk sac angiogenesis or defects in cartilage formation and patterning (Chiang *et al*., 1996, Cridland *et al*., 2009). However, when the *Dhh* gene is knocked out the mice are viable but males are infertile (Bitgood *et al*., 1996) hence we analysed adult Dhh-/- mice in our study. The Dhh-/- mouse was generated by removing exons 1, 2 and 3 which encode a highly conserved 19kDa peptide required for the signalling activities of Hh protein and replacing them with a construct encoding neomycin phosphotransferase. Hence, no functional protein would be produced in mice homozygous for this deletion (Bitgood *et al*., 1996). In Chapter 5, treatment of B cells with rShh was found to regulate peripheral B cell differentiation at different time points post-activation. Thus, we asked the question, whether there are any differences in representation of peripheral B cell subsets in the spleen of Dhh-/- mice compared to the Dhh+/+ control.

Purified splenic B cells isolated from Dhh-/- and Dhh+/+ mice were stained immediately with a combination of anti-IgM-PE, anti-CD23-PE-Cy7 and anti-CD21-APC before analysis by flow cytometry in order to identify the peripheral B cell subsets. Figure 6.1, shows dot-plots representing the peripheral B cell subsets in the Dhh+/+ and Dhh-/- mice. Peripheral B cell subsets were gated as described in Section 5.2.2.1. Little differences were observed between the subsets of B cells in the Dhh+/+ and Dhh-/-. However, there was an increased presence of the T1 population in the Dhh-/- compared to the Dhh+/+, from 28.0% in the Dhh+/+ to 32.8% in the Dhh-/-. There was also an increased T2/FO-II population, from 26.6% in the Dhh+/+ to 30.1% in the Dhh-/-. Fewer differentiated FO-I B cells and fewer MZ B cells were observed in the Dhh-/-

compared to the Dhh+/+. These results suggest that in Dhh-/-, there is probably an impaired differentiation of B cells in the spleen. There was an apparent build-up of the more immature B cells in the Dhh-/- compared to the Dhh+/+ suggesting that there may be an arrest in differentiation after T2 cell development.

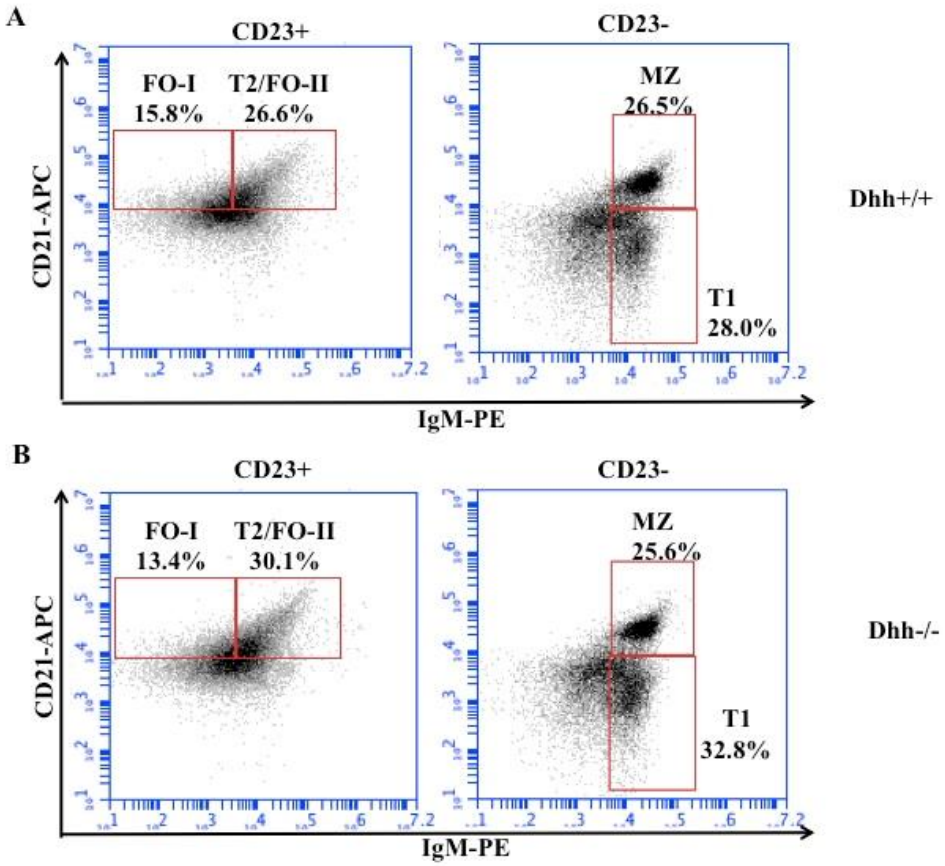


Figure 6.1. There is an increased immature B cells in Dhh-/-

The dot-plots shown are representative of one experiment where splenic purified B cells isolated from Dhh-/- and Dhh+/+ were stained using a combination of CD23, CD21 and IgM in order to characterise B cell subsets in Dhh+/+ (**A**) and Dhh-/- (**B**).

6.2.2. The activation and survival of B cells isolated from Dhh-/- mice compared to their littermate controls.

B cells cultured as a mixed splenocyte population as well as purified B cells were shown to display increased B cell survival as assessed by Annexin-V expression and increased B cell activation as judged by CD23 expression at 18hours post-activation

after treatment with rShh and the opposite was observed at 40hours post-activation in Chapter 3 and 4 respectively. Hence, it was of interest to investigate if there were any differences in survival and activation of B cells isolated from Dhh-/- compared to the Dhh+/+ littermates.

Purified B cells isolated from Dhh-/- and Dhh+/+ were activated with 5μg/ml anti-CD40/IgM before treatment with 0.05μg/ml rShh to both sets of cells. Exogenous rShh was added to both sets of cells in order to investigate whether addition of exogenous rShh to the cultures of B cells isolated from Dhh-/- mice could rescue any defects caused by lack of expression of Dhh. At 18hours and 40hours post-activation, cultured B cells were stained as described in Section 3.2.1.

Figure 6.2, shows data on the activation of B cells at 18hours (Figure 6.2A) and at 40hours (Figure 6.2B) post-activation stimulus. Surprisingly, at 18hours post-activation, there was an increased percentage of CD23+ B cells in B cells isolated from the Dhh-/- compared to its Dhh+/+ littermate. Treatment of B cell cultures obtained from Dhh+/+ mice with rShh also showed increased CD23+ B cells as was also observed previously in Chapter 3 and 4. However, interestingly, B cells isolated from Dhh-/- mice treated with rShh resulted in an apparent decrease in B cell activation as judged by a reduction in CD23 expression (Figure 6.2A).

Following 40hours post-activation (Figure 6.2B), CD23+ B cells in treated Dhh+/+ mice had decreased as previously observed in the case of the purified B cell (Chapter 4) and mixed cell experiments (Chapter 3). However, the number in percentage of CD23+ B cells isolated from the Dhh-/- mice had significantly increased by approximately 1.2 fold compared to B cells isolated from the Dhh+/+ mice and when treated with rShh,

this increase in CD23 staining was reduced back to baseline level. This data suggests that when rShh is reintroduced to the B cell cultures isolated from Dhh-/- the signal transduced by rShh acts to negatively regulate expression of CD23 both at 18 and 40hours post-activation. However, all of these observed trends were small and did not yield statistically significant differences between the different groups.

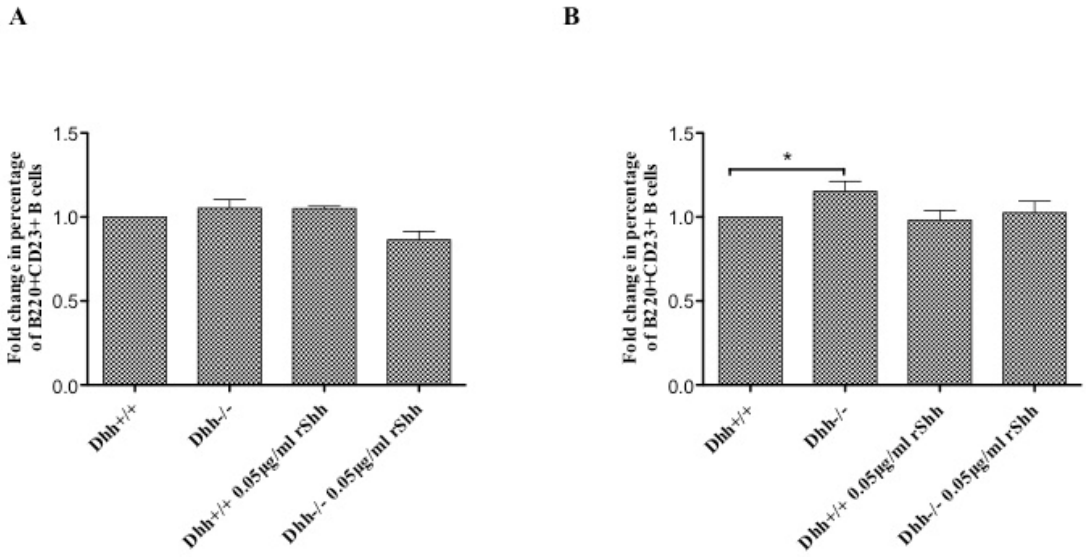


Figure 6.2. B cells isolated from Dhh-/- had increased activation

Purified B cells isolated from Dhh-/- and Dhh+/+ mice were activated with anti-5µg/ml CD40/IgM and were treated with 0.05µg/ml rShh. Cells were collected and analysed at 18hours (**A**) and 40hours (**B**) post-stimulation. B cell activation was determined using anti-CD23. The results shown in the bar chart are representative of six independent experiments. The significance of the differences observed in fold changes in CD23+ cells was assessed by Student *t*-test $*p \leq 0.05$.

Figure 6.3, shows data on survival of B cells isolated from Dhh-/- and Dhh+/+ at 18hours (A) and 40hours (B) post-activation. At 18hours post-activation (Figure 6.3A), there was a significant decrease in apoptosis of B cells treated with rShh in both strains of mice but at 40hours more death is seen in treated B cells isolated from Dhh+/+ (Figure 6.3B). B cells isolated from Dhh-/- mice also had a reduced apoptosis at 18hours post-activation, but unlike B cells isolated from Dhh+/+ mice, they also

survived better at 40hours. For example, at 40hours post-activation, there was a nearly significant decrease in apoptosis by approximately 40% in B cells isolated from Dhh-/- mice compared to the Dhh+/+ control. Interestingly, treatment of B cells isolated from Dhh-/- did not increase levels of B cell death (Figure 6.3B). These data are consistent with the idea that the death of B cells following 40hours post-activation stimulus, is in part, due to Hh signalling.

These results indicate that the negative regulatory effect observed at 40hours in our culture of mixed B cells and purified B cells with respect to activation and survival is lost when the *Dhh* gene is knocked out as the Dhh-/- B cells displayed increased B cell activation as judged by CD23 expression and increased B cell survival at both time points, thus indicating that the removal of *Dhh* gene can exert a positive effect for B cell function in respect of activation and survival.

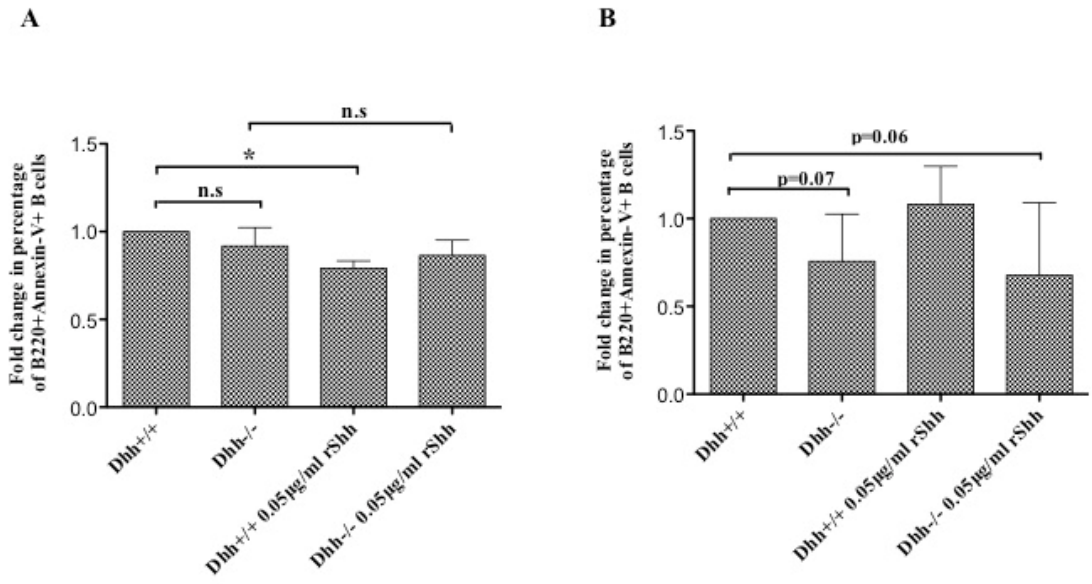


Figure 6.3. B cells isolated from Dhh-/- have increased survival

Purified B cells isolated from Dhh-/- and Dhh+/+ mice were activated with anti-CD40/IgM and were treated with 0.05µg/ml rShh. Cells were collected and analysed at 18hours (**A**) and 40hours (**B**) post-stimulation. B cell survival was determined using annexin-V. The results shown in the bar chart are representative of five independent experiments. The significance of the differences observed in fold changes in annexin-V+ cells was assessed by Students *t*test *$p \leq 0.05$.

6.2.3. B cell differentiation in Dhh-/- and Dhh+/+

In Chapter 4, it was observed that Hh signalling induces the differentiation of CD23-CD25+ B cells in purified B cell cultures at 40hours and the identification of the three different B cell subset, which include, CD23+CD25-, CD23+CD25+ and CD23-CD25. In Chapter 5, it was shown that CD23+CD25- contains mostly FO-I B cells, CD23+CD25+ contains a mix of FO-I, FO-II and T2-MZP B cells and CD23-CD25+ contains cells of the memory B cell phenotype and MZ. We therefore analysed whether there are any differences in the representation of these subsets at 40hours in the absence of *Dhh*. In order to do this, the differentiation of B cells was analysed using the expression of CD23 and CD25 following 40hours in culture.

Figure 6.4 shows the representation of the different B cell subsets in the Dhh-/- and Dhh+/+ cultures at 40hours. Compared to the B cells isolated from the Dhh+/+ mice, there was an increased presence of CD23+CD25- B cell subset (Figure 6.4A), and reduced number of CD23+CD25+ (Figure 6.4B) and CD23-CD25+ (Figure 6.4C) subsets in the B cells isolated from the Dhh-/-mice. For example, there was a significant increase of CD23+CD25- B cells by approximately, 1.2 fold in the case of the Dhh-/- mice compared to the Dhh+/+ littermate control (Figure 6.4A). Treatment of B cells isolated from the Dhh+/+ mice with rShh, caused a decrease in the number of CD23+CD25- subset (Figure 6.4A), and an accompanying increase in the number of CD23+CD25+ (Figure 6.4B) and CD23+CD25- (Figure 6.4C). Treatment of B cells isolated from Dhh-/- mice with rShh resulted in a reduced number of CD23+CD25-, however, not significant (Figure 6.4A) and a significantly increase number of CD23+CD25+ compared to the Dhh-/- control (Figure 6.4B).

These results suggest that in the case of the B cell isolated from Dhh-/- mice, there is an increased number of FO-I B cells, a reduced number of CD23+CD25+ containing FO-II, T2 and T2-MZP cells and a reduced presence of CD23-CD25+ containing memory B cells and MZ. Thus, suggesting that Hh signalling may play a role in diverting the differentiation of the T2 B cell towards either the FO-I B cell or towards the T2-MZP B cell.

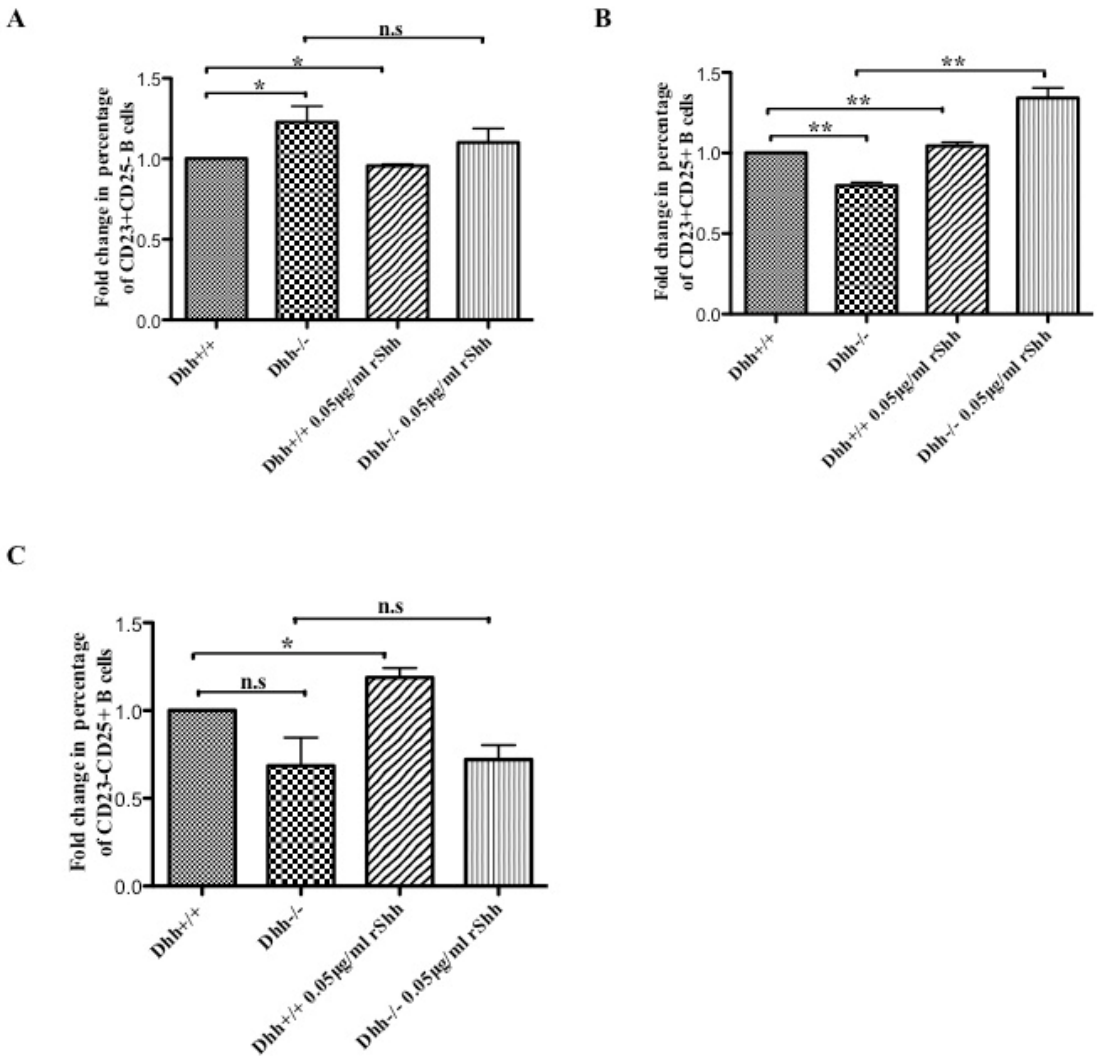


Figure 6.4. B cells isolated from Dhh-/- have increased differentiation to CD23+CD25- B cells

Purified B cells isolated from Dhh-/- and Dhh+/+ with or without treatment with 0.05µg/ml rShh were analysed for their representation CD23+CD25- (**A**), CD23+CD25+ (**B**) and CD23-CD25 (**C**) at 40hours post-activation. The results shown in the bar chart are representative of three independent experiments. The significance of the differences observed in fold changes in percentage of the B cell subsets was assessed by Students *t* test **p*≤0.05, ***p*≤0.01.

6.2.4. IL-10 intracellular staining and secretion in B cells isolated from Dhh-/- mice

We observed, that treatment of B cells with rShh at 40hours post-activation, caused an increase in the expression and secretion of IL-10 by purified B cells (Chapter 4, Section 4.2.4) as well as a reduced B cell post- T2 differentiation in B cells isolated from Dhh-/-

mice. It was of interest therefore to analyse IL-10 secretion by B cells isolated from Dhh-/- compared to Dhh+/+ mice.

Purified B cells isolated from Dhh-/- and Dhh+/+ mice were cultured as described in Section 4.2.8. In Chapter 4, we showed that IL-10 expression was restricted to B cells that are CD23+CD25+ and so we focussed on analysing this subset. Figure 6.5A shows data on IL-10+ B cells as assessed by flow cytometry. In CD23+CD25+ B cells, there is a significant reduction in IL-10+ B cells of approximately 40% in B cells isolated from Dhh-/- mice compared to Dhh+/+ mice. Addition of exogenous rShh to B cells isolated from Dhh-/- did not increase IL-10 expression by these B cells.

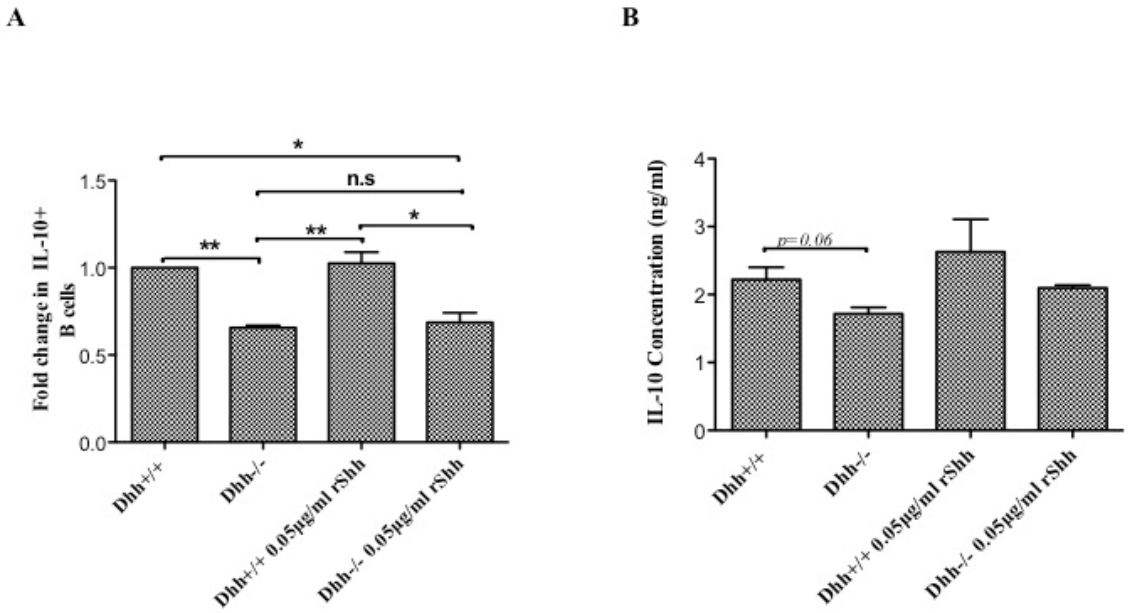


Figure 6.5. B cells isolated from Dhh-/- have reduced IL-10 secretion

Purified B cells isolated from Dhh-/- and Dhh+/+ mice were activated with anti-CD40/IgM and were treated with 0.05µg/ml rShh. Cells were collected and analysed for IL-10 expression (**A**) and supernatants were analysed for IL-10 secretion using ELISA (**B**) at 40hours post-stimulation. The results shown in the bar chart are representative of three independent experiments. The significance of the differences observed in fold changes in IL-10+ B cells and IL-10 secretion was assessed by Students *t* test *$p \leq 0.05$, **$p \leq 0.01$.

To validate the finding of the IL-10 intracellular staining study, IL-10 secretion was analysed in supernatants isolated from all cultures at 40hours. Figure 6.5B shows IL-10 secretion in B cells isolated from Dhh-/- and Dhh+/+ mice at 40hours post-activation. There was an increased secretion of IL-10 by treated B cells isolated from Dhh+/+ mice, but in the case of B cells isolated from Dhh-/- mice, there was less secretion of IL-10 compared to B cells isolated from Dhh+/+. For example, there is an increase in IL-10 secretion in B cells from 1.7ng/ml in the case of Dhh-/- to 2.3ng/ml in the case of the Dhh+/+ mice. This is consistent with the finding that at 40hours post-activation in B cells isolated from Dhh-/-, we are observing more B cells positive for CD23, which could be FO-I B cells and less differentiation towards T2-MZP, which is thought to be Bregs which secrete IL-10 (Blair *et al*., 2008). It therefore correlates that B cells isolated from Dhh-/- mice would secrete less IL-10 when compared to the Dhh+/+ littermate. These results suggest that B cells isolated from Dhh-/- are less able to differentiate towards T2-MZP B cells thus show reduced IL-10 secretion compared to Dhh+/+.

6.2.5. CD27 expression in B cells isolated from Dhh-/- mice.

Having observed that expression of CD27 is up-regulated in CD23-CD25+ in B cells treated with rShh (Chapter 4) and further characterising these cells to exhibit a memory B cell phenotype in Chapter 5 using CD80 and IgD. We therefore investigated if there are any differences in expression of CD27 in B cells isolated from Dhh-/- and Dhh+/+ mice

Figure 6.6, shows CD27 expression at 40hours post-activation on CD23-CD25+ B cells, there was a significant decrease of approximately 11% in CD27 expression on the B

cells isolated from Dhh-/- mice compared to Dhh+/+. Treatment of B cells isolated from Dhh+/+ with rShh increased CD27 expression as observed in the purified B cell culture (Chapter 4, Section 4.25). There was a slight decrease in CD27+ B cells in treated B cells isolated from Dhh-/- and compared to B cells isolated from Dhh-/-. These results suggest that B cells isolated from Dhh-/- have a reduced ability to differentiate towards B cells that display a memory B cell phenotype when compared to B cells isolated from Dhh+/+.

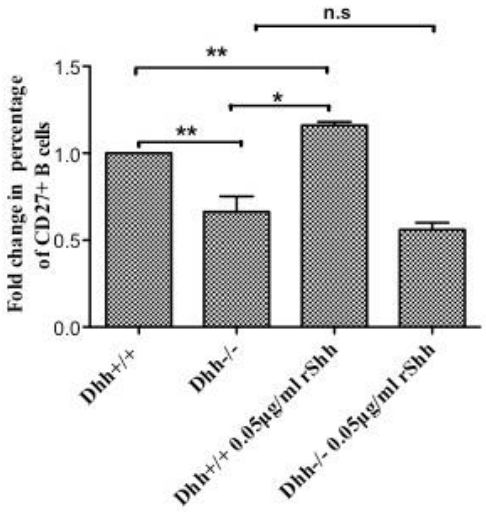


Figure 6.6. B cells isolated from Dhh-/- have reduced CD27+ B cells

Purified B cells isolated from Dhh-/- and Dhh+/+ mice were activated with anti-CD40/IgM and were treated with 0.05µg/ml rShh. CD23-CD25+ B cells were analysed for CD27 expression at 40hours post-activation. The results shown in the bar chart are representative of three independent experiments. The significance of the differences observed in fold changes in CD27+ B cells was assessed by Students *t*test *$p \leq 0.05$, **$p = 0.01$.

6.2.6. B cells from Dhh-/- produce increased amount of antibody

Having identified a possible role for Hh signalling in both up-regulating and down-regulating the production of antibodies by activated purified B cells in chapter 4, it was important to analyse what effect the absence of *Dhh* gene expression might have on antibody production. Hence, antibody production by B cells isolated from Dhh-/- was investigated.

Figure 6.7, shows antibody secretion by B cells isolated from the Dhh-/- and Dhh+/+ mice following 18 and 40hours post-activation. Interestingly, there is a massive increase in the production of IgG1 by B cells isolated from Dhh-/- compared to the Dhh+/+ at both time points. Consistent with previous data shown in chapter 4, treatment of B cells with rShh resulted in an up-regulation of IgG1 secretion at 18hours but these levels did not increase following 40hours in culture and indeed by this time point levels of secretion of IgG1 by untreated B cells had caught up. (Figure 6.9) This data is consistent with the idea that uncontrolled activation may occur in B cells leading to enhanced antibody production in the absence of the *Dhh* gene. Also, there was an increased number of the FO-I B cell observed in the cultures of B cells isolated from Dhh-/- mice following 40hours in culture when compared to the Dhh+/+ control, and thus these additional FO-I cells may go on to form germinal centres (Cariappa *et al*., 2007), leading to an increased antibody secretion.

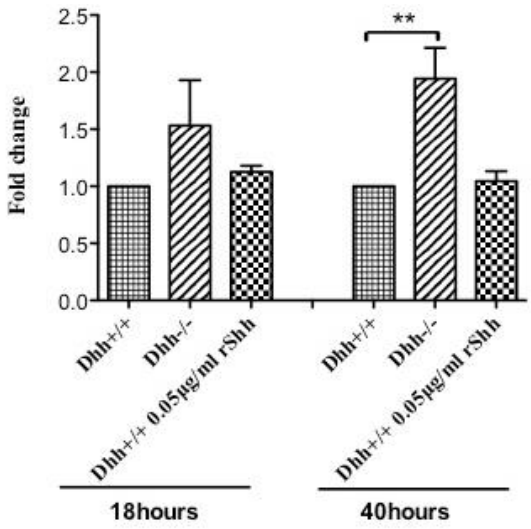


Figure 6.7. B cells isolated from Dhh-/- secrete increased IgG1

Purified B cells isolated from Dhh-/- and Dhh+/+ were activated with 5µg/ml anti-CD40/IgM and treated with 0.05µg/ml rShh. Supernatants were analyzed for IgG1 production after 18 and 40hours post-activation using ELISA. The results shown in the bar chart are representative of four independent experiments. The significance of the differences observed in fold change was assessed by Students *t* test **p≤0.01.

6.2.7. IL-6 secretion in Dhh-/- and Dhh+/+

Interleukin 6 (IL-6) was first identified as a B cell stimulating factor; it is involved in driving plasma cells to secrete more antibodies (Urashima *et al*., 1996). Following 40hours in culture, the B cells isolated from Dhh-/- mice showed an increased IgG1 secretion compared to the Dhh+/+ and the same phenomenon was observed in the presence of exogenous rShh. We would hypothesise therefore that this increased antibody secretion by B cells isolated from Dhh-/- mice might be accompanied by an increased secretion of IL-6 by these same B cells.

Figure 6.8, shows IL-6 secretion by B cells isolated from Dhh-/- and Dhh+/+ mice. As predicted, at 18 and 40hours post-activation stimulus, there was an observed trend with increasing secretion in IL-6 secretion by B cells isolated from Dhh-/- compared to Dhh+/+ mice. However, this increase in IL-6 secretion was not significantly different. For example, at 40hours post-activation, B cells isolated from Dhh+/+ mice secreted approximately 0.1ng/ml IL-6, which has increased to approximately 0.23ng/ml IL-6 in the case of the Dhh-/- mice. These data suggest that Hh signalling may act to decrease the secretion of IL-6 by B cells following stimulation via the BCR.

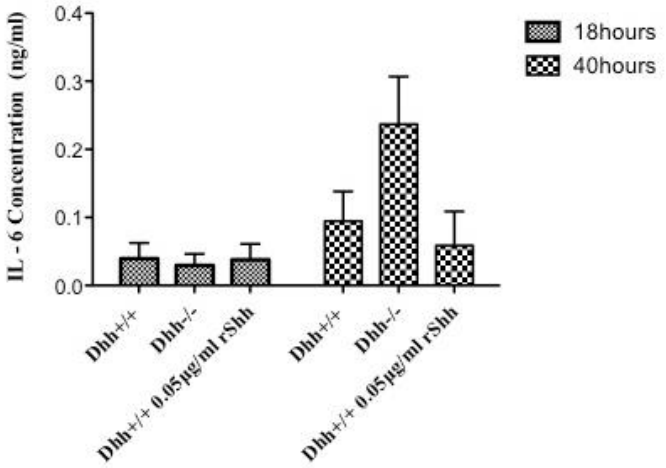


Figure 6.8. B cells isolated from Dhh-/- have an increased IL-6 secretion

Purified B cells isolated from Dhh-/- and Dhh+/+ mice were activated with anti-CD40/IgM and were treated with 0.05µg/ml rShh. Supernatants were analysed for IL-6 secretion using ELISA at 18 and 40hours post-stimulation. The results shown in the bar chart are representative of three independent experiments.

6.2.8. The effect of exogenous recombinant Dhh on B cell development

After observing the effect that the removal of *Dhh* gene had on B cell development, it was important to ascertain the effect that the addition of exogenous recombinant Dhh (rDhh) would have on B cell development and compare it with the effect of treating B cells with the closely related rShh protein.

Purified splenic B cells from BALB/c mice were activated with 5µg/ml anti-CD40/IgM before treatment with either 0.05µg/ml rShh or rDhh. Because a more pronounce effect on differentiation of B cells was observed at 40hours post-activation, the effect of rDhh would be investigated at this time point and compared with that induced by rShh. Following 40hours post-activation, B cells were stained with a combination of anti-CD23-^{PE}, anti-CD27-^{FITC}, anti-CD25-^{APC} and B220-$^{PE\text{-}CY5}$ or anti-CD23-^{PE}, Annexin-V-^{FITC}, anti-CD25-^{APC} and B220-$^{PE\text{-}CY5}$.

Figure 6.9, shows B cell activation (Figure 6.9A) and survival (Figure 6.9B) at 40hours as judged by CD23 and annexin-V respectively. As observed previously, addition of rShh decreases B cell activation as judged by CD23 expression following 40hours post-stimulation (Figure 6.9A). However, when the effect of rDhh is compared to that induced by rShh, there was less activation observed when rShh is added. This effect was also observed when B cell survival was analysed. Using Annexin-V, (Figure 6.9B), there was increased B cell death with treatment with both homologs of Hh as judged using annexin-V, however the effect is higher in the presence of rShh.

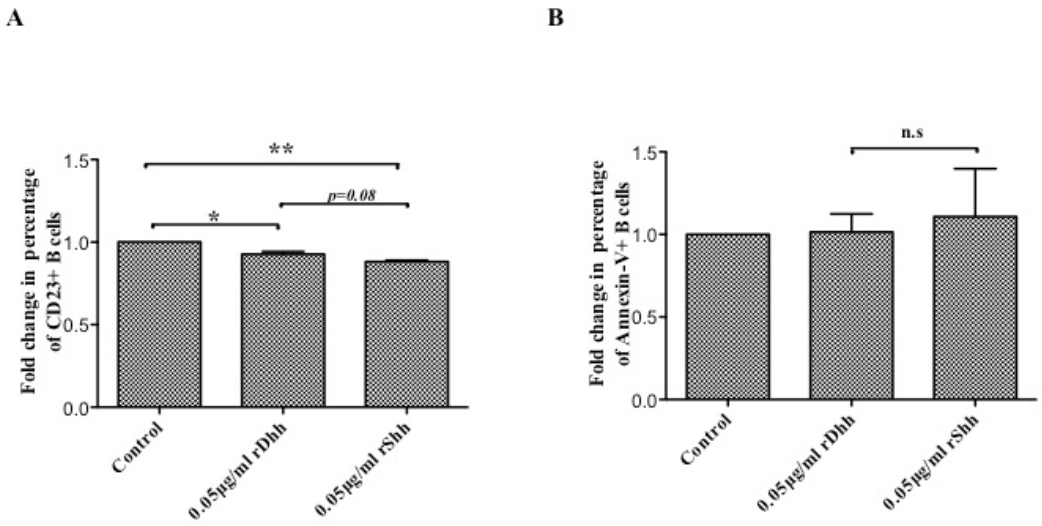


Figure 6.9. The effect of Hh signalling in B cells is more potent with treatment with rShh

Purified B cells were activated with 5µg/ml anti-CD40/IgM and were treated with 0.05µg/ml rDhh or rShh. Cells were collected and analysed for activation (**A**) and survival (**B**) at 40hours post-stimulation. B cell activation and survival was determined using anti-CD23 and annexin-V respectively. The result shown in the bar chart is representative of three independent experiments. The significance of the differences observed in fold changes in CD23+ cells was assessed by Student *t* test *p≤0.05, **p≤0.01.

After observing that the effect of Hh signalling on B cell activation and survival was more potent with treatment with rShh compared to rDhh, we investigated whether there would be any differences in the representation of the three subsets arising in our culture following 40hours in culture.

Figure 6.10, shows the different B cell subset representation at 40hours. There is a reduced CD23+CD25- B cell subset (Figure 6.10A), increased CD23+CD25+ (Figure 6.10B) and CD23-CD25+ (Figure 6.10C) in the presence of both rHh homologues compared to the control. This is consistent with results obtained that demonstrated that culture with rShh could reduce the representation of CD23+CD25-, which has been characterised to be FO-I at 40hours compared to the control. However, the effects are more potent in the presence of rShh compared to rDhh.

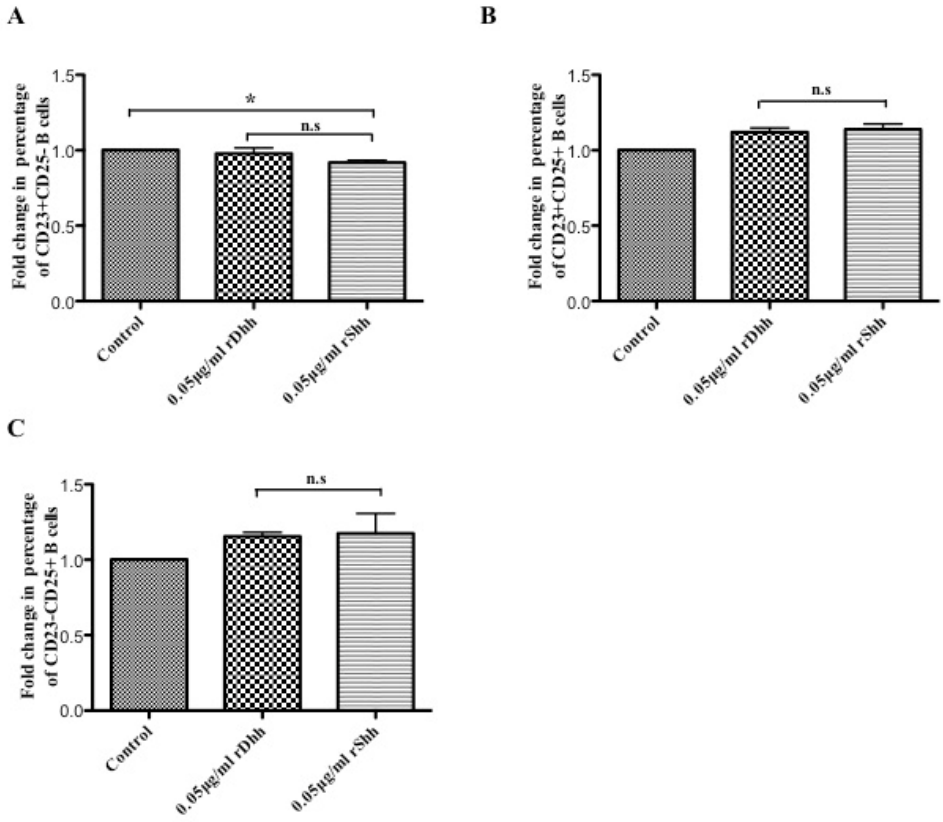


Figure 6.10. Representation of B cell subsets at 40hours

Purified B cells were activated with 5µg/ml anti-CD40/IgM and treated with either 0.05µg/ml rDhh or rShh and were analysed for their representation of CD23+CD25- (**A**), CD23+CD25+ (**B**) and CD23-CD25 (**C**) at 40hours post-activation. The results shown in the bar chart are representative of three independent experiments. The significance of the differences observed in fold changes in percentage of the B cell subsets was assessed by Students *t*-test *$p \leq 0.05$.

After observing that in the presence of rShh there is an increased B cell differentiation towards B cells that contain memory B cell phenotype and MZ in Chapter 4 and 5, we wanted to investigate whether there would be any differences observed when rDhh was added to the cultures in comparison to addition of rShh. CD27 expression was analysed on B cells that formed CD23-CD25+, following 40hours in culture as this population have been previously shown to be positive for CD27 (Chapter 4, Section 4.2.5). Figure 6.11, shows CD27 expression on purified B cells after treatment with either rShh or rDhh at 40hours post-activation. Interestingly, treatment of B cells with rDhh significantly reduced CD27+ B cells compared to the control and B cells treated with rShh. For example, control B cells contained 17.6 % CD27+ B cells, which decreased to 8.6% in B cells treated with 0.05μg/ml rDhh. Treatment of B cells with 0.05μg/ml rShh has increased CD27+ B cells to 33.0% (Figure 6.11A). There were increased CD27+ B cells when rShh is added to B cells compared to the stimulated control. These results were significant (Figure 6.11B).

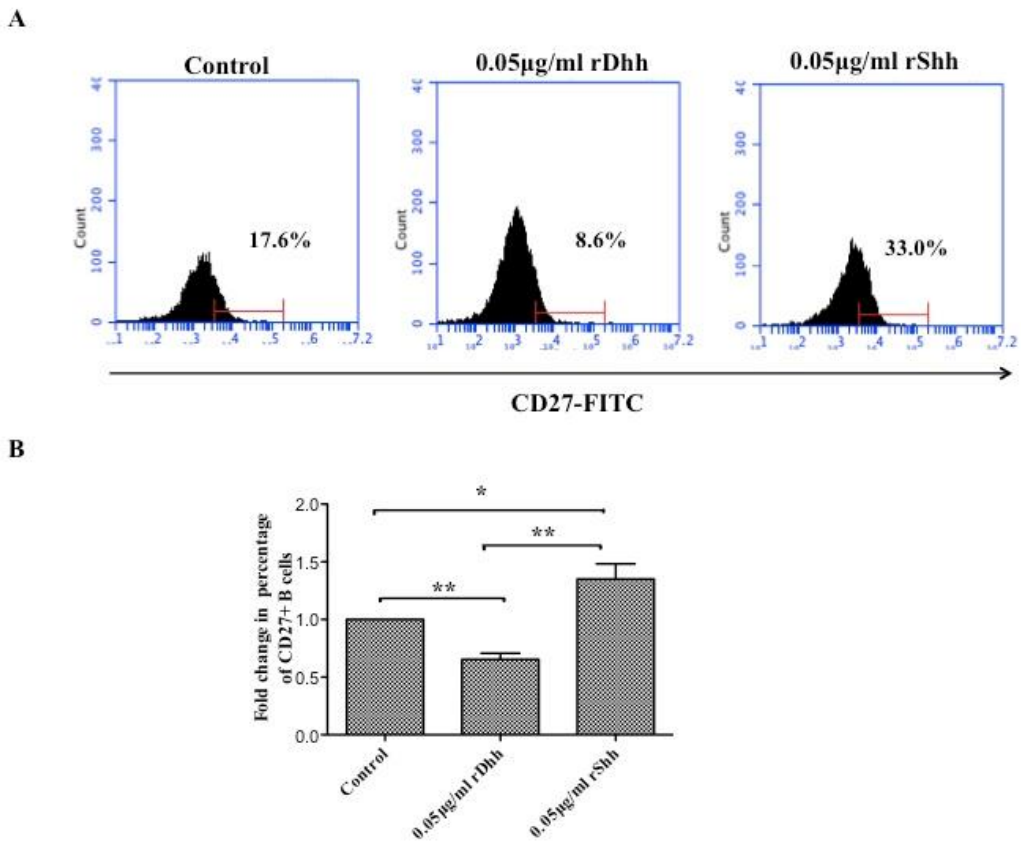


Figure 6.11. Treatment of B cells with rDhh reduces CD27+ B cells

The histogram (A) shown is from one experiment representing three independent experiments where purified B cells were activated with anti-CD40/IgM and were treated with either 0.05µg/ml rDhh or rShh. CD23-CD25+ B cells were analysed for CD27 expression and in (B) a bar chart of CD27+ B cells at 40hours post-activation. The results shown in the bar chart are representative of three independent experiments. The significance of the differences observed in fold changes in CD27+ B cells was assessed by Students *t*test *p≤0.05, **p≤0.01.

The results obtained suggest that B cells treated with rDhh, have a reduced ability to differentiate towards the memory B cell subset. This result was unexpected and requires further investigation.

After observing that in the presence of rDhh, there was reduced CD27 expression, we asked whether there would be any differences in IL-10 expression by B cells cultured together with rDhh as culture with rShh had been shown previously to increase IL-10 expression at 40hours (Chapter 4).

Figure 6.12, shows a preliminary data on IL-10+ B cells in the presence of rDhh. IL-10 expression was analysed on CD23+CD25+ B cells subset. There was no difference in IL-10+ B cells observed in B cells treated with rDhh and the control. Addition of 0.05µg/ml rShh showed increased intensity of IL-10 staining compared to the control as previously observed. For example, there were approximately, 11% of B cells expressing IL-10 in the control, which is similar to about 10% in the presence of 0.05µg/ml rDhh, This percentage increases in the presence of 0.05µg/ml rShh to approximately 17%.

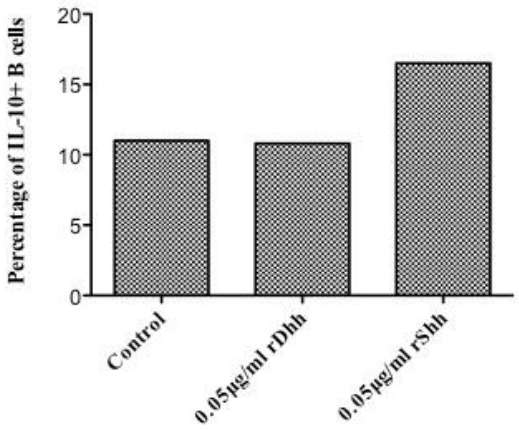


Figure 6.12. Treatment of B cells with rDhh did not increase IL-10+ B cell

Purified B cells were activated with anti-CD40/IgM and were treated with 0.05µg/ml rDhh or rShh. Cells were collected and analysed for the percentage of IL-10+ B cells 40hours post-stimulation. The result shown in the bar chart is representative of a single experiment.

The results observed in B cells treated with rDhh suggest that the different homologs of Hh protein have different effects on B cell function and that Shh is more potent than Dhh in our assays.

6.3. Discussion

In previous chapters, data presented demonstrated that treatment of B cells with rShh can increase the response of B cells by increasing differentiation towards FO-I, antibody production, cytokine secretion at earlier time point but also can subsequently dampen down to the response and drive B cell differentiation towards B cells that display a memory B cell phenotype at a latter time point thus suggesting that Hh signalling can regulate the immune response of B cells. It became of interest to investigate the effect of the loss of Hh gene would have on B cell development.

The percentages of peripheral B cell subsets were first investigated. The data presented in this chapter shows that there was more T1 and T2/FO-II B cells in number of percentage from the cells isolated from the spleen of Dhh-/- mice compared to the Dhh+/+. Thus, indicating that there was more immature B cells in the Dhh-/- compared to Dhh+/+. These results suggest that there is a partial arrest in B cell development in the spleen isolated from Dhh-/- mice. Removal of the *Dhh* gene has previously been shown to have a negative effect on development. Lau *et al*, (2012), showed that deletion of the *Dhh* gene led to impaired erythropoiesis (development of red blood cells) (Lau *et al*., 2012). Thus, it is possible that deletion of the *Dhh* gene arrests B cell development in the periphery, as there was an accumulation of immature B cells observed.

When B cells were activated, B cells isolated from Dhh-/- displayed increased activation as judged by CD23 and increased survival as judged as annexin-V at 18hours and 40hours post-activation stimulus. They also displayed an increased FO-I differentiation and less T2-MZP B cells. Differentiation of B cells to FO-I is characterised by strong BCR signalling and it is Btk-dependent (Pilla and Cariappa, 2009). It was also observed that B cells isolated from Dhh-/- displayed less ability to

differentiate into CD27+ B cells and also T2-MZP, hence less IL-10 secretion. Hence, deletion of *Dhh* gene results in impaired differentiation post-activation stimulus.

This apparent enhanced activation of B cells leading to a loss of regulation of antibody secretion may cause self-reactive antibodies to be secreted by B cells, which should otherwise have been deleted. Importantly, survival of B cells isolated from Dhh-/- mice was shown to be elevated following both 18hours and 40hours post-activation relative to the Dhh+/+ control suggesting that negative selection of autoreactive B cells in the spleen may have been impaired. This loss of regulation can lead to autoimmunity. The increased antibody production observed by B cells isolated from Dhh-/- mice might be due to the lack of the negative regulatory effect caused by Hh signalling. These results suggest that in the Dhh-/- mice, B cells are pushed earlier than would be expected to the FO-I phenotype and that there is less ability of B cells to differentiate to become more mature memory B cells suggesting an impaired B cell differentiation in the periphery. FO-I B cells have the ability to form GCs and secrete antibody (Cariappa *et al*., 2007) and hence, increased antibody production was observed by B cells isolated from Dhh-/- mice. This increased antibody production by B cells obtained from Dhh-/- might result in the development of autoimmune disease as it has been reported that increased Btk signalling which is required for FO-I differentiation can lead to auto-antibodies which causes lupus like symptoms (Kil *et al*., 2012).

Having observed the effects of loss of the *Dhh* gene on B cell development together, it is of interest to investigate the effects of addition of rDhh to purified B cells in culture and if there's any difference from the effects caused by rShh. The findings in this chapter demonstrate that treatment of B cells with rDhh had a weaker impact on B cell differentiation compared to rShh. It might be due to the fact that the B cells bind to both proteins with different affinities. Also, it has been previously shown that of all the Hh

homologs, Shh has the strongest activity, followed by Ihh and then Dhh as assesed using an alkaline phosphatase transcriptional reporter assay (Pathi *et al*., 2001).

The findings of this chapter suggests that although *Dhh* gene is required for proper development of B cell in the periphery, the other homologs of Hh might need to be present in the spleen or secreted by the B cells in order to get proper B cell development in the periphery. Next, differences in gene expression of B cells isolated from Dhh-/- and Dhh+/+ and after treatment with rShh shall be analysed using gene expression microarrays in order to access what differential transcriptome profiles underlay the phenotypic differences we observed in the Dhh-/- compared to Dhh+/+. We also will investigate the genes modulated by Hh signalling in B cells, with regards to survival, activation, and B cell differentiation.

6.4. Conclusion

In this chapter, it was observed that there is a partial arrest in B cell differentiation in the periphery of Dhh-/- mice resulting in a skewing in particular B cell subset representation. We showed that in the absence of the *Dhh* gene, there is an increased antibody production by B cells accompanied by an increased activation and survival of B cells. Hence, deletion of *Dhh* may form a mechanism by which autoimmune diseases may develop. It was also demonstrated that treatment of B cells with rDhh did not induce the differentiation of B cells displaying memory B cell like phenotype. The next chapter extends the findings observed in this chapter by carrying out a molecular gene analysis in order to assess whether there are any differentially genes expressed in B cells isolated from Dhh-/- and Dhh+/+ and after they are treated with rShh.

Chapter 7: Microarray analysis of the transcriptome of B cells isolated from Dhh-/-, Dhh+/+ and Dhh+/+ mice in the presence of exogenous Hh signalling

7.1. Introduction

7.1.1. BCR signalling pathway and components

The B cell receptor (BCR) complex is a transmembrane complex that is non-covalently associated with a disulphide linked Igα and Igβ heterodimer which each have a single immuno receptor tyrosine based activation motif (ITAM) located on their cytoplasmic tails, These are composed of two tyrosine residues (Hendriks *et al*., 2014, Seda and Mraz, 2015). Activation of the B cell via ligation of the BCR, leads to signalling via phosphorylation of the ITAMs on the Igα/β heterodimer. This chain of events then leads to subsequent activation of downstream kinases and transcription factors including Btk and nuclear factor of activated T cells (NFAT) (Hendriks *et al*., 2014).

Bruton's tyrosine kinase (Btk), a 77kD non-receptor tyrosine kinase that is expressed specifically in myeloid cells and B cells, is a key component of the BCR signalling pathway. The cystolic TEC kinase is activated when BCR signalling promotes its recruitment to the cell membrane via P13K mediated actions and subsequent phosphorylation by syk. This increases Btk kinase activity, which leads to an increase in calcium flux and activation of transcription factors NfκB and calcium dependent nuclear factor of activated T cells (NFAT) (Loder *et al*., 1999, Hendriks *et al*., 2014). In mice and humans, precise regulation of Btk expression levels is essential for normal B cell function and differentiation, as mutations in Btk, lead to an absence of mature B cells and agammaglobunemia (Hendriks *et al*., 2014, Seda and Mraz, 2015). Btk is also an

important regulator of B cell proliferation and cell survival and may therefore play a role in the development of various B cell malignancies (Hendriks *et al*., 2014).

BCR signalling is crucial for B cell development, however, the signal mediated by ligation of BCR and its signalling component need to be tightly regulated as overactive BCR signalling has been linked to the pathology of a number of diseases such as B cell chronic lymphoctic leukaemia (B-CLL) (Woyach *et al*., 2012).

7.1.2. Objectives

Data presented in Chapter 6, revealed that B cells isolated from Dhh-/- exhibited altered peripheral B cell differentiation compared to Dhh+/+. In Chapters 3, 4 and 5, Hh signalling has been shown to regulate B cell survival, activation, proliferation and differentiation. The aim of this chapter is to identify molecular targets that are regulated by Hh signalling using microarray analysis. The objectives of this chapter are:

- To identify differentially expressed genes in B cells isolated from Dhh-/- compared to Dhh+/+.
- To identify differentially expressed genes in B cells treated with exogenous rShh compared to untreated control using gene expression microarrays.
- To validate the differential expression of genes identified using gene expression microarrays by qRT-PCR.

7.2. Results

7.2.1. Gene expression in B cells isolated from Dhh-/-, Dhh+/+ and Dhh+/+ in the presence of Hh at 18hours and at 40hours post-activation stimulus

In order to investigate whether there are any differences in the transcriptional profile of B cells isolated from Dhh-/- compared to Dhh+/+ in the presence and absence of treatment with 0.05μg/ml rShh following 18 and 40hours post-activation stimulus, a whole exon expression microarray gene analysis was carried out on RNA isolated from these B cells. PCA was then applied to the data obtained from this analysis in order to identify areas of greatest variation in gene expression between the sample sets (Section 2.8). PCA uses a mathematical formula and procedures to transform the original data to a new co-ordinate data represented by principal components (PCs) (Ringner, 2008). PCs are the linear combinations of Eigen vectors and the variable. PCA approach ranks the largest Eigen value, where there is maximum variance, as the first principal component (PC1) and the smallest Eigen values, where there is the least variance, the last PC. Using PCA, samples can be plotted making it visually possible to access similarities and differences between samples and determine whether they can be grouped (Ringner, 2008).

Figure 7.1, shows PCA results generated from activated purified B cell samples isolated from Dhh-/-, Dhh+/+ B cells cultured with or without treatment with 0.05μg/ml rShh at 18 and 40hours post-activation stimulus. It was observed that the sample data separated most based on time. Principal Component (PC) 1 (Comp1, Row A, Column 1), the largest component for variation, shows that there are clear differences in gene expression profiles from 18 to 40hours in culture, for all samples taken at 18hours post-activation stimulus (Circles) all cluster together, while samples taken at 40hours (Triangles) also cluster together and separately from the 18hours samples (Figure 7.1).

This result suggests that the phenotypes in culture at 18hours post-activation stimulus are distinct from the phenotype observed at the latter time in culture. This result is to be expected, as all cells will have undergone extensive differentiation in response to the various stimuli during this time period.

More interestingly, in the case of PC3 (Comp 3(See Row C, Column 3) following 18hours post-activation stimulus (circles), it was observed that B cells isolated from Dhh-/- (red) cluster differently from B cells isolated from Dhh+/+ (black), thus indicating that there are genes within this component that are differentially expressed in B cell samples isolated from Dhh-/- compared to the Dhh+/+ littermate at this time point. However, there were no differences observed in the other components as no significantly separated clusters were identified between samples. This suggests that the genes grouped within Comp3 only contribute to the difference observed between B cells isolated from Dhh-/- and Dhh+/+ mice. Additionally, neither of the components could identify the samples according to treatment or time points and thus target genes expressed differentially in the 18hours samples and 40hours samples would need to be identified individually.

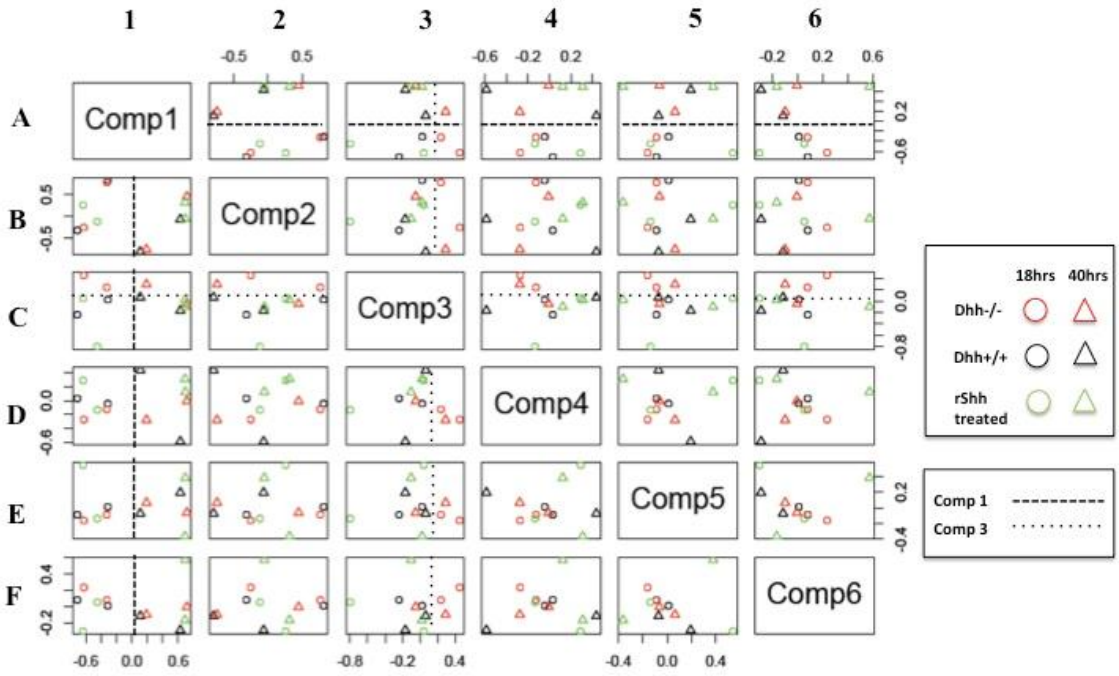


Figure 7.1. PCA analysis gene expressio of activated B cell samples 18 and 40 hours post-culture following rShh treatment

B cells were isolated from Dhh-/- and Dhh+/+ and were activated using 5µg/ml anti-CD40/IgM and treated with or without rShh (0.05µg/ml). After 18 and 40hours culturing, RNA samples were purified and analysed using microarray analysis. Comp1 showed that there are differences between the transcriptome profiles of B cell samples at 18hours and 40hours in culture. Differences were observed between the transcriptome profiles of B cells isolated from Dhh-/ and Dhh+/+ mice within Comp3.

In order to identify more easily whether there are any distinct differences in gene expression profiles at the different time points, a PCA was carried out on samples at the individual time points alone.

To simplify the analysis of the different sample Figure 7.2, shows PCA analysis of B cell samples following 18hours in culture. PC1, the largest component did not show any clear distinct differences in samples. However, when PC2 (Row B, Column 2) was analysed, it was observed that there were differences in the gene transcriptome profile of B cells isolated from Dhh-/- (red circles) and Dhh+/+ mice (black circles) as both samples separate out on this component. However, when the other components were

analysed, it was observed that there was no distinct differences between samples (Figure 7.2). There was also no clear differences observed between B cells isolated from Dhh+/+ treated with exogenous rShh and the untreated control at 18hours post-activation stimulus (Figure 7.2). This finding suggests that at 18hours post-activation, there are only differences in PC2 between the genes expressed in B cells isolated from Dhh-/- and Dhh+/+ mice.

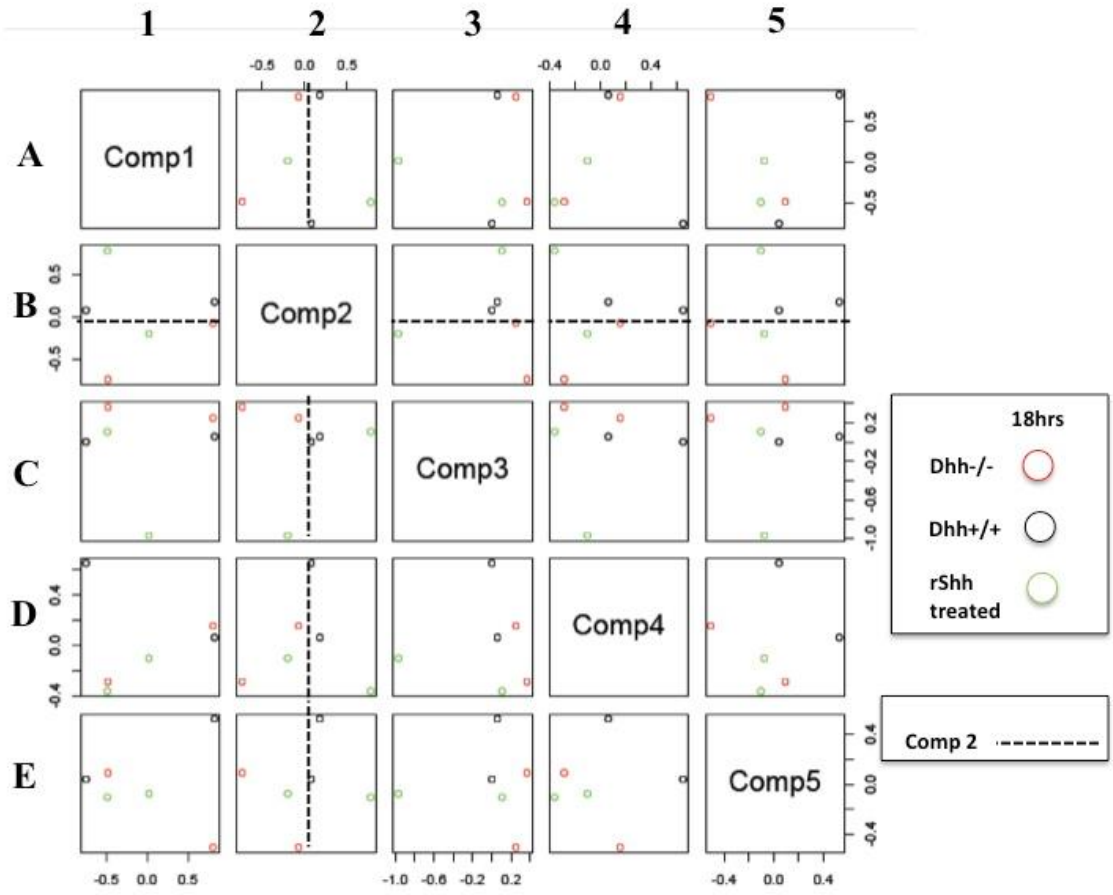


Figure 7.2. PCA analysis of B cell sample of 18hours post-activation stimulus

B cells were isolated from Dhh-/- and Dhh+/+ and were activated using 5µg/ml anti-CD40/IgM and treated with or without rShh (0.05µg/ml). After 18hours of culturing, RNA samples were purified and analysed using microarray analysis. Comp2 shows that B cell samples isolated from Dhh-/- have a different transcriptome profile from Dhh+/+ as represented by the broken line.

We also investigated more closely the differences in gene expression observed following 40hours in culture using PCA. Figure 7.3, shows PCA analysis of the different sample sets following 40hours in culture. It was observed, that there was no clear differences in gene expression between samples in PC1, PC2, and PC3. However, in the case of PC4 (Row D, Column 4), there was a significant difference in gene expression profile in purified B cells isolated from Dhh-/- and Dhh+/+. Interestingly, in PC4 (Row D, Column 4), the samples derived from Dhh+/+ samples and rShh-treated Dhh+/+ samples are all grouped together, suggesting the genes contained within PC4 can reflect the difference in transcriptome profiles between the B cells isolated from wild-type and knockout mice. This data may include the genes that are responsible for the impaired B cell development observed in the Dhh-/- at 18hours and 40hours post-activation stimulus as shown in Chapter 6. Additionally, when considering PC5 (Row E, Column 5) (Figure 7.3), it was observed that B cells isolated from Dhh+/+ that were treated with exogenous rShh significantly separate from the untreated control at this time point and thus may represent the different phenotypes observed between the untreated control and treated B cells as shown in Chapter 4.

Taken together these data suggest that the transcriptional profile of B cells vary between the different sample sets following 18 and 40hours in culture, also that there are differentially expressed genes in B cells isolated from Dhh-/- and Dhh+/+ mice. Thus, it became important to identify more closely genes that are differentially expressed between these two conditions. To do this, PCA analysis was carried out on B cells isolated only from Dhh-/- and Dhh+/+ following 18 and 40hours post-activation in culture.

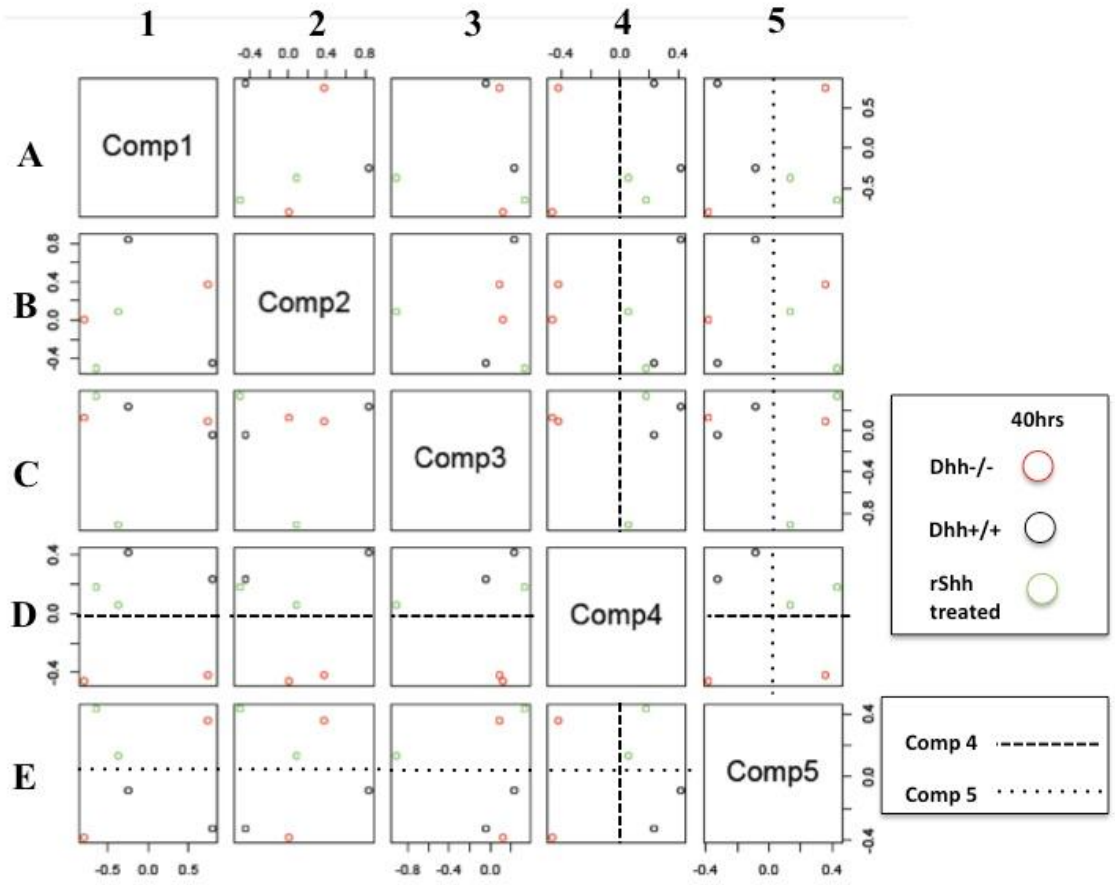


Figure 7.3. PCA analysis of B cell sample at 40hours post-activation stimulus

B cells were isolated from Dhh-/- and Dhh+/+ and were activated using 5µg/ml anti-CD40/IgM and treated with or without rShh (0.05µg/ml). After 40hours culturing, RNA samples were purified and analysed using microarray analysis. Differences were observed between the transcriptome profiles of B cells isolated from Dhh-/- and Dhh+/+ mice within Comp4. Comp5 showed that there were differences in transcriptome profile of B cells treated with rShh and the untreated control.

7.2.2. Differentially expressed genes in B cells isolated from Dhh-/- and Dhh+/+ at 18 and 40hours

PCA was first carried out between B cells samples isolated from Dhh-/- and Dhh+/+ following 18hours in culture to identify whether there are any differentially expressed genes. Figure 7.4A, shows a 2-dimensional PCA analysis at 18hours post-activation. At 18hours post-activation stimulus, PC1 the largest component and PC3 the smallest

component for variation did not show any differences between the B cell samples. However, PC2 (Comp 2), the second largest component for variation showed that samples of B cells isolated from Dhh-/- were distinguished from samples from the Dhh+/+. It was observed that there were 32% of genes that were differentially expressed between B cell samples isolated from Dhh-/- and Dhh+/+ mice. Thus, suggesting that the transcriptome profile of B cells isolated from Dhh-/- differs from Dhh+/+ littermate and the gene candidates generated from PC2 is causing the variation between both profiles.

The genes involved in the Hh signalling pathway including *Smo, Ptch, Gli1* and *Gli3* were investigated specifically (Figure 7.4B). These genes all had low PC2 scores suggesting that they are expressed differently in the transcriptome profile of B cells isolated from Dhh-/- and Dhh+/+ mice and expression of these genes may be more likely associated with B cells isolated from Dhh+/+ mice (Figure 7.4B). This suggests that there is a reduced expression of *Smo, Gli1* and *Gli3* in B cells isolated from Dhh-/- compared to Dhh+/+ littermate. It has been reported that upon activation of B cells, Smo expression is up-regulated (Sacedon *et al*., 2005), thus indicating that the B cells become Hh responsive. The reduced expression of Smo observed in B cells isolated from Dhh-/- suggests that they are less Hh responsive. Additionally, Gli-1 is itself a target gene of the Hh signalling pathway and Gli3 is activated by Smo signalling and antagonises the Hh signalling pathway (Briscoe and Therond, 2013). This suggests that the reduced Smo signalling in B cells isolated from Dhh-/- leads to a reduced activation of the Gli proteins. Thus suggesting that they are less Hh responsive compared to the wild type.

Interestingly, expression of *Btk* (Bishop, 2004), *Nfatc1* (Bhattacharyya *et al*., 2011) and the B cell activation marker, *CD69* (Patterson *et al*., 2006) all had high PC2 scores

(Figure 7.4B) thus indicating that there is an increased gene expression in B cell samples isolated from Dhh-/- compared to Dhh+/+ mice.

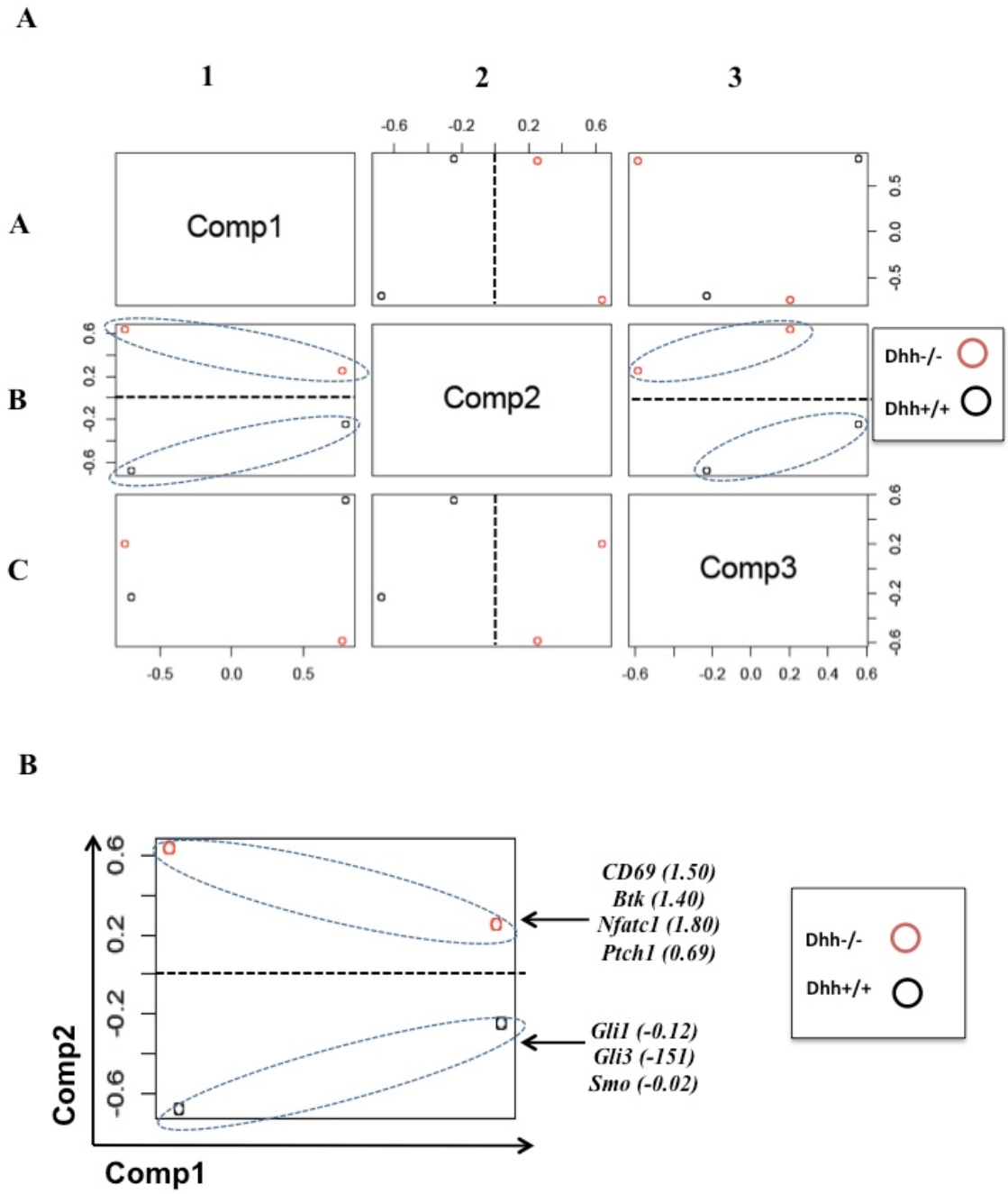


Figure 7.4. PCA analysis of B cell samples isolated from Dhh-/- and Dhh+/+ at 18hours post-activation stimulus

B cells were isolated from Dhh-/- and Dhh+/+ and were activated using 5µg/ml anti-CD40/IgM. After 18hours of culturing, RNA samples were purified and analysed using microarray analysis. PCA analysis (**A**) and candidate genes identified from Comp2 (**B**).

We performed the same closer inspection of PCA analysis of samples collected following 40hours post-activation stimulus (Figure 7.5A). Analysis of PC1 and PC2 did not show any difference in B cell samples isolated from Dhh-/- compared to Dhh+/+. However, when PC3 was analysed it was observed that 24% of the genes were causing variation in B cells isolated from Dhh-/- and Dhh+/+. This suggests that the genes contained within PC3 contribute to the different gene expression profile of B cells isolated from Dhh-/- compared to Dhh+/+.

In Chapter 6, it was demonstrated that there were differences observed in B cell activation following 40hours in culture in B cells isolated from Dhh-/- compared to Dhh+/+ mice. It was also observed that there was an increased IL-6 secretion and reduced IL-10 secretion at this time point in B cells isolated from Dhh-/- compared to the Dhh+/+. There was also an increased B cell differentiation towards FO-I B cell and the transition from T2 to FO-I B cell is known to be, Btk dependent. This made Btk a good candidate gene for further investigation.

Figure 7.5B shows candidate gene contained within PC3 that are differentially expressed following 40hours in culture. It was observed that IL-6 had a low PC3 score thus indicating that changes in its gene expression may contribute to the phenotype observed in B cells isolated from Dhh-/-. Also, IL-10 had a high PC3 score thus suggesting it is associated with B cells isolated from Dhh+/+ compared to Dhh-/- (Figure 7.5B). These results confirm the findings observed in Chapter 6. Other genes, found to be differentially expressed between B cells isolated by PCA at 40hours, include *CD37,* a marker, whose ablation can inhibit humoral production (Knobeloch *et al*., 2000), pro-proliferation protein; *Ccnd2*, anti-apoptotic protein; *Bcl2*, which all had a

low PC3 score, thus are more associated with B cell samples isolated from Dhh-/- (Figure 7.5B).

The results discussed above indicate that there are clear differences in gene expression in B cells isolated from Dhh-/- and Dhh+/+ mice at different time points, and expression of these genes thus might account for the differences observed in their respective B cell maturation in the spleen. However, expression of these candidate genes needs to be validated using qRT-PCR.

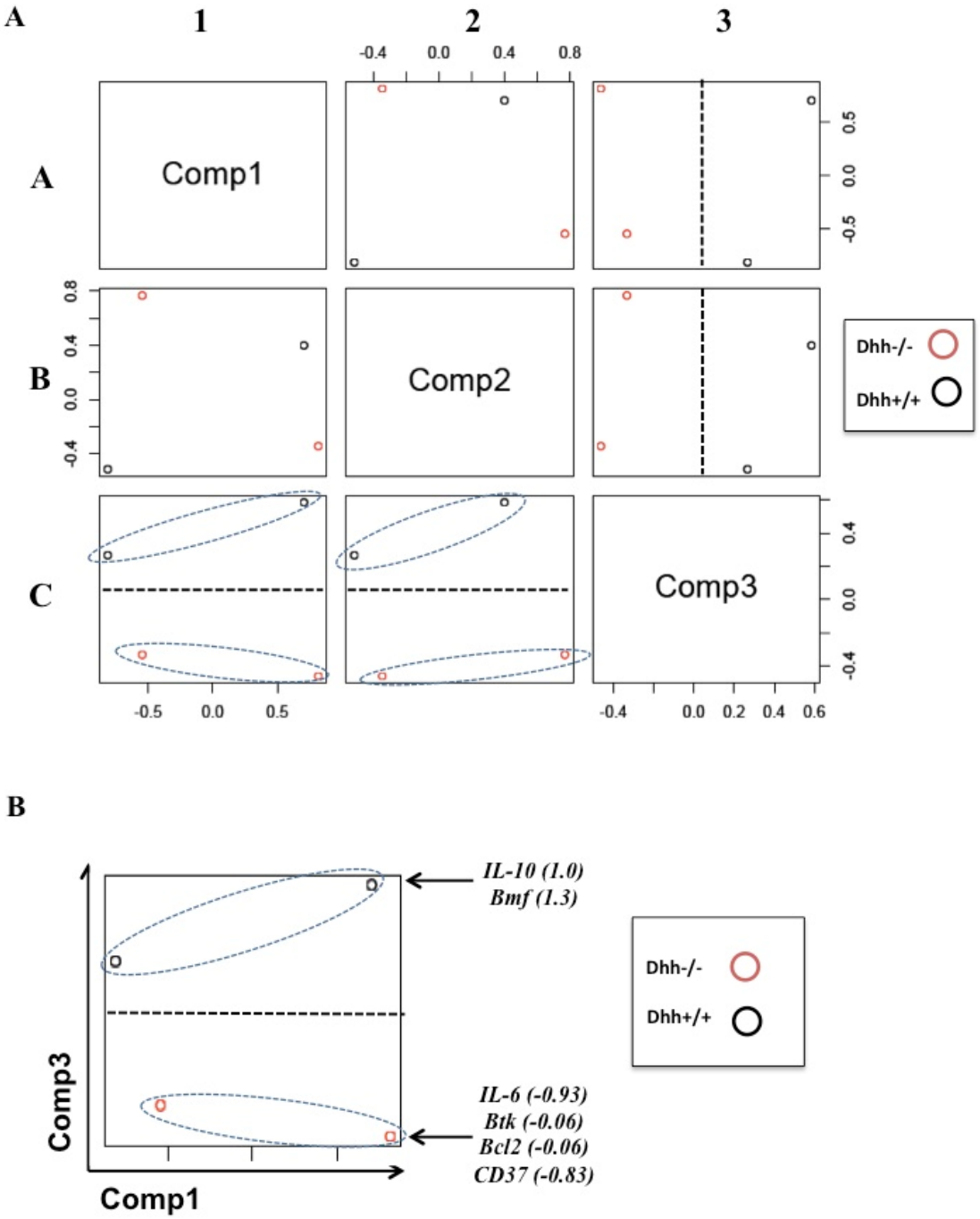


Figure 7.5. PCA analysis of B cell isolated from Dhh-/- and Dhh+/+ at 40hours post-activation stimulus

B cells were isolated from Dhh-/- and Dhh+/+ and were activated using 5µg/ml anti-CD40/IgM. After 18hours of culturing, RNA samples were purified and analysed using microarray analysis. PCA analysis (**A**) and candidate genes identified from Comp2 (**B**).

7.2.3. Genes differentially expressed in B cells isolated from Dhh+/+ after treatment with exogenous rShh

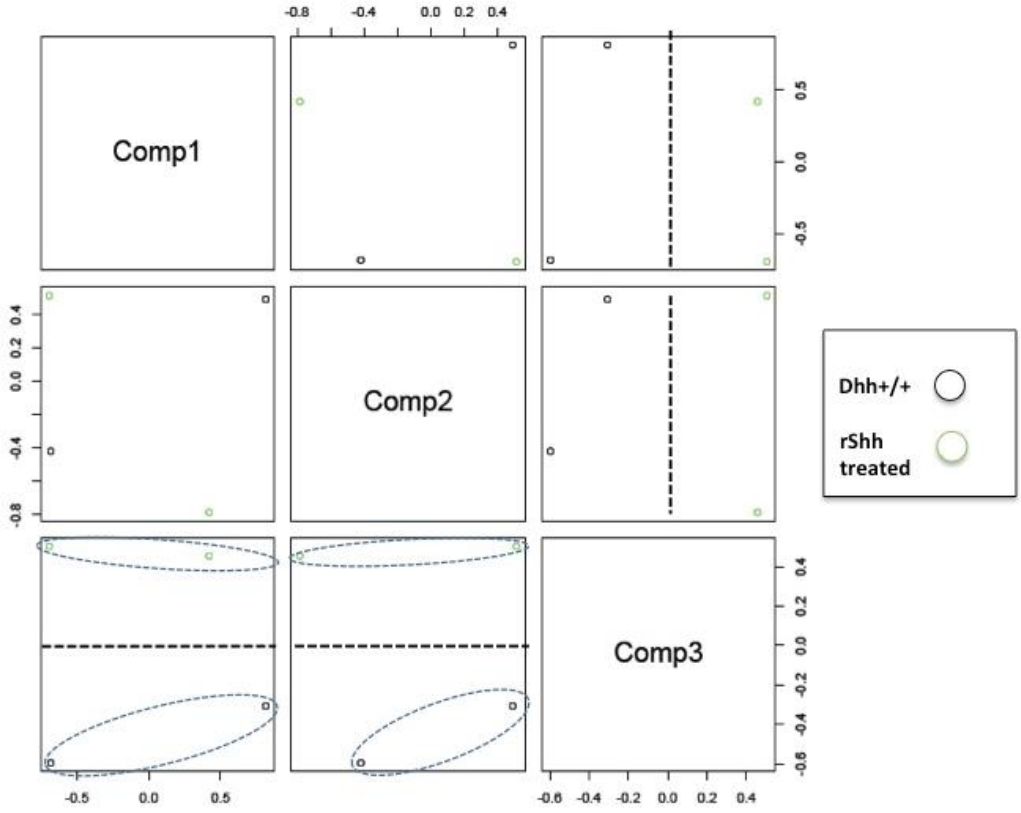
In previous chapters (Chapter 3, 4 and 5), the treatment of B cells with exogenous rShh has been shown to regulate B cell activation, survival and differentiation at different time points. Thus, it was equally important to identify the target genes regulated by Hh signalling in B cells when treated with exogenous rShh at the different time points in order to understand the molecular mechanisms that underpin these observations. Data presented in Figure 7.3, showed using PCA analysis that there are a number of differentially expressed gene profiles between B cells isolated from Dhh-/-, Dhh+/+ treated with exogenous rShh and Dhh+/+ control following 40hours in culture. Hence, a further PCA analysis was carried out in order to identify the genes causing variation between rShh treated and untreated B cells isolated from Dhh+/+ mice at both time points.

Figure 7.6A, shows the PCA analysis of the data obtained at 18hours post-activation showing data from B cell samples isolated from Dhh+/+ and with treatment with rShh. The largest component, PC1 did not show any significant differences, neither did PC2. However, analysis of PC3 showed that 30% of genes varied between the rShh treated and untreated samples. Figure 7.6B, shows PCA analysis of data obtained at 40hours post-activation stimulus showing data from B cell samples isolated from Dhh+/+ and with treatment with rShh. It was observed that neither PC1, PC2, PC3 showed any variability between the rShh treated B cell samples and the untreated control.

The results obtained suggest that treatment of B cells with exogenous Hh can activate different signalling pathways involved in the regulation of B cell function at 18hours post-activation and these genes may contribute to the phenotypic differences observed in B cells treated with exogenous rShh at 40hours post-activation stimulus. However, by

40hours post activation stimulus the patterns of gene transcription between treated and untreated samples have stabilised and are closely related. Thus, candidate genes generated within PC3 at 18hours (Figure 7.6A), were selected to be analysed further.

A



B

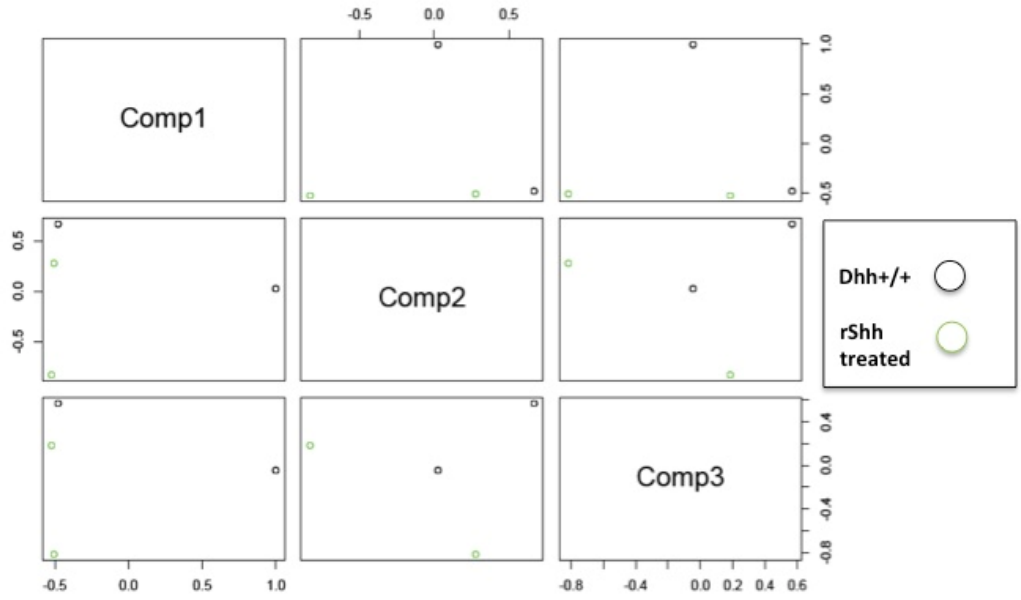


Figure 7.6. PCA analysis of Hh treated B cells and the control at 18hours and 40hours post-activation stimulus

B cells were isolated from Dhh+/+were activated using 5µg/ml anti-CD40/IgM and treated with or without rShh (0.05µg/ml). After 18hours (**A**) and 40hours (**B**) of culturing, RNA samples were analysed by micro-array analysis.

The gene list generated in respect of PC3 was investigated closely. Genes of interest that were identified from this analysis included genes involved in the BCR signalling pathway, as well as the induction of B cell differentiation and apoptosis, Interestingly, expression of other morphogens such as BMP also varied indicating that there is an interplay between these two morphogens families in regulating peripheral B cell development (Figure 7.7A).

We found that genes involved in preventing the apoptotic pathway such as the pro-survival factors *Bcl2* and *Traf2* as well as those genes with pro-apoptotic activity such as *Bnip3* (Liu and Frazier, 2015), were identified as genes contributing to the variation in gene expression between samples. It was observed that *Traf2*, an anti-apoptotic protein (Bishop, 2004) had a high PC3 score (Figure 7.7A), thus indicating that major changes in its gene expression is contributing to the phenotype associated with B cells isolated from Dhh+/+ in the presence of exogenous rShh.

Further verification of *Traf2* expression using qRT-PCR showed that by 18hours post-activation stimulus, there was a significant increase in expression of approximately 6fold in the rShh treated cells compared to the untreated control (Figure 7.7B). Interestingly, the levels of *Traf2* decreased significantly between 18 and 40hours post-stimulus in B cells treated with rShh. These results suggest that Hh signalling can modulate B cell survival via the up-regulation of anti-apoptotic protein such as *Traf2* at 18hours post-activation stimulus. This is consistent with the increased survival of B cells observed using annexin-V staining after 18hours in our culture as shown in Chapters 3 and 4. The down-regulation of *Traf2* expression at 40hours post-stimulus is also consistent with the annexin-V data, suggesting that Hh signalling can induce B cell apoptosis via down-regulation of *Traf2*. Overexpression of Traf2 activates the NfκB and

MAPK pathways, which can then increase B cell survival (Bishop, 2004). Also, Traf2 expression has been shown to favour FO B cell differentiation and survival (Perez-Chacon *et al*., 2012), and it has been reported that Traf2 expression leads to the synergistic activation of B cells, and production of immunoglobulin and IL-6 (Bishop, 2004). Thus, the increased *Traf2* expression observed in B cells treated with rShh, might play a role in the increased FO-I differentiation driven by exogenous rShh (Chapter 5) and thus lead to the increased IL-6 secretion and antibody production (Chapter 4) observed in our culture in treated B cell samples.

The expression of *Bnip3* was also verified using qRT-PCR. BCL2/ adenovirus E1B 19kDa interacting protein (Bnip3) is a pro-apoptotic protein that contributes to cell death through the activation of the mitochondrial pathway of apoptosis. Bnip3 has a c-terminal transmembrane domain, which is required for mitochondrial targeting and for its pro-death function. Upon activation of Bnip3, it localises to the mitochondria, where it collapses mitochondria membrane potential, increases reactive oxygen species, and increases mitochondrial swelling thus leading to activation of cell death. Interestingly, over expression of Bnip3 has been shown to occur in a number of tumours including follicular lymphoma indicating that *Bnip3* may act as both a tumour suppressor as well as a tumour promoter (Sington *et al*., 2007).

Figure 7.7C, shows qRT-PCR data on *Bnip3* expression in purified B cells treated with rShh and untreated B cells after 18 and 40hours post-activation stimulus. At 18hours post-activation, there were low levels of *Bnip3* expressed in both the treated and untreated samples. Interestingly, by 40hours post-activation stimulus, expression of *Bnip3* had increased in both samples but it had increased significantly by approximately 5-fold in B cells treated with exogenous rShh compared to the untreated control only.

This correlates with the induction of a significant increase in cell death in the presence of rShh following 40hours in culture as shown in Chapter 3 and 4.

These results suggest that Hh signalling can regulate B cell survival following 18 and 40hours post-activation stimulus via the up-regulation of the anti-apoptotic protein *Traf2* and the pro-apoptotic protein *Bnip3* respectively thus indicating that Hh signalling can induce both survival and death in B cells. The finding that a morphogen can induce apparently opposing physiological processes is not a new finding and has previously been published as occurring during thymocyte development (Outram *et al*., 2009) and may reflect a complex negative feedback process maintained by Hh signalling.

Other genes, identified using microarray gene analysis as being differentially expressed include genes whose expression is involved in the BCR signalling pathway, such as *Btk* and *Nfatc1* (Figure 7.7A**).** Further verification using qRT-PCR, showed that both *Btk* and *Nfatc1* are differentially expressed in B cells treated with rShh compared to untreated B cells.

Figure 7.7D shows qRT-PCR data of *Btk* expression in purified B cells treated with rShh and the control untreated at 18 and 40hours post-activation stimulus. At 18hours post-activation, there was a nearly significant 3-fold increase in *Btk* expression in B cells treated with rShh compared to the untreated control. At 40hours post-activation stimulus, *Btk* expression had become very similar between the treated and untreated samples. In the control untreated B cells, *Btk* expression had increased, however, there has been a decrease in *Btk* expression in B cells treated with rShh. These results support the findings observed in chapter 5, which showed that the treatment of B cells with rShh

drives an increased B cell differentiation towards Btk dependent FO-I B cells at 18hours post-activation stimulus compared to the untreated control (Pillai and Cariappa, 2009) and by 40hours a decrease in Btk dependent FO-I

Figure 7.7E, shows qRT-PCR data for *Nfatc1* expression in untreated B cells as well as B cells treated with rShh. At 18hours post-activation, *Nfatc1* expression is increased in B cells treated with rShh by approximately 1.6 fold compared to the control, however, this was not significant. At 40hours post-activation stimulus, the levels of *Nfatc1* have decreased in B cells treated with 0.05μg/ml rShh but the control has expressed more *Nfatc1* between 18 and 40hours post-activation stimulus. This pattern of expression mirrors the expression observed with *Btk* expression and suggests that their expression may be co-regulated. It has been reported that mice with complete deletion of *Nfatc1* showed an increase in IL-10 secreting B cells and they exhibited a mild clinical course of experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis (Bhattacharyya *et al*., 2011). The data presented in this chapter, showed that at 40hours post-activation stimulus, treatment of purified B cells with exogenous rShh decreased *Nfatc1* expression and thus may correlate with the increased IL-10 secretion observed in our purified B cell culture in the presence of rShh (Chapter 4).

These results indicate that treatment of B cells with exogenous rShh can potentiate the BCR signal possibly by inducing an up-regulation of expression of kinases involved in the BCR signalling pathway and, as a result, enhancing survival. However, this signal might be too strong and thus induce activation induced cell death (AICD) that can lead to the up-regulation of pro-apoptotic protein such as Bnip3, which in turn may increase cell death at 40hours post-activation stimulus.

Data presented in Chapter 3, demonstrated that the effect of Hh signalling in B cells is, in part, due to the effects of BMP signalling occurring downstream of the Hh signal. Additionally, PCA analysis of microarray data obtained from samples derived from treated and untreated samples identified a pattern of differential expression for *Bmp*2/4 causing variation between control and treated samples (Figure 7.7A). Further verification using qRT-PCR, revealed that at 18hours post-activation stimulus, there is an increase, albeit not significant, in gene expression of *Bmp2* (Figure 7.7F) and *Bmp4* (Figure 7.7G) in B cells treated with exogenous rShh compared to untreated control. At 40hours post-activation stimulus, gene expression of *Bmp*2/4 had decreased in B cells treated with rShh compared to control.

These data suggests that treatment of B cells with rShh, can up-regulate BMP expression and therefore the BMP signalling pathway at 18hours post-activation stimulus. The downstream effects of the activated BMP signalling pathway may then only be observed at the later time-point of 40hours in culture. These data indicate that the effects of rShh on peripheral B cell development may occur, in part due to an involvement of an interplay with another morphogen, BMP.

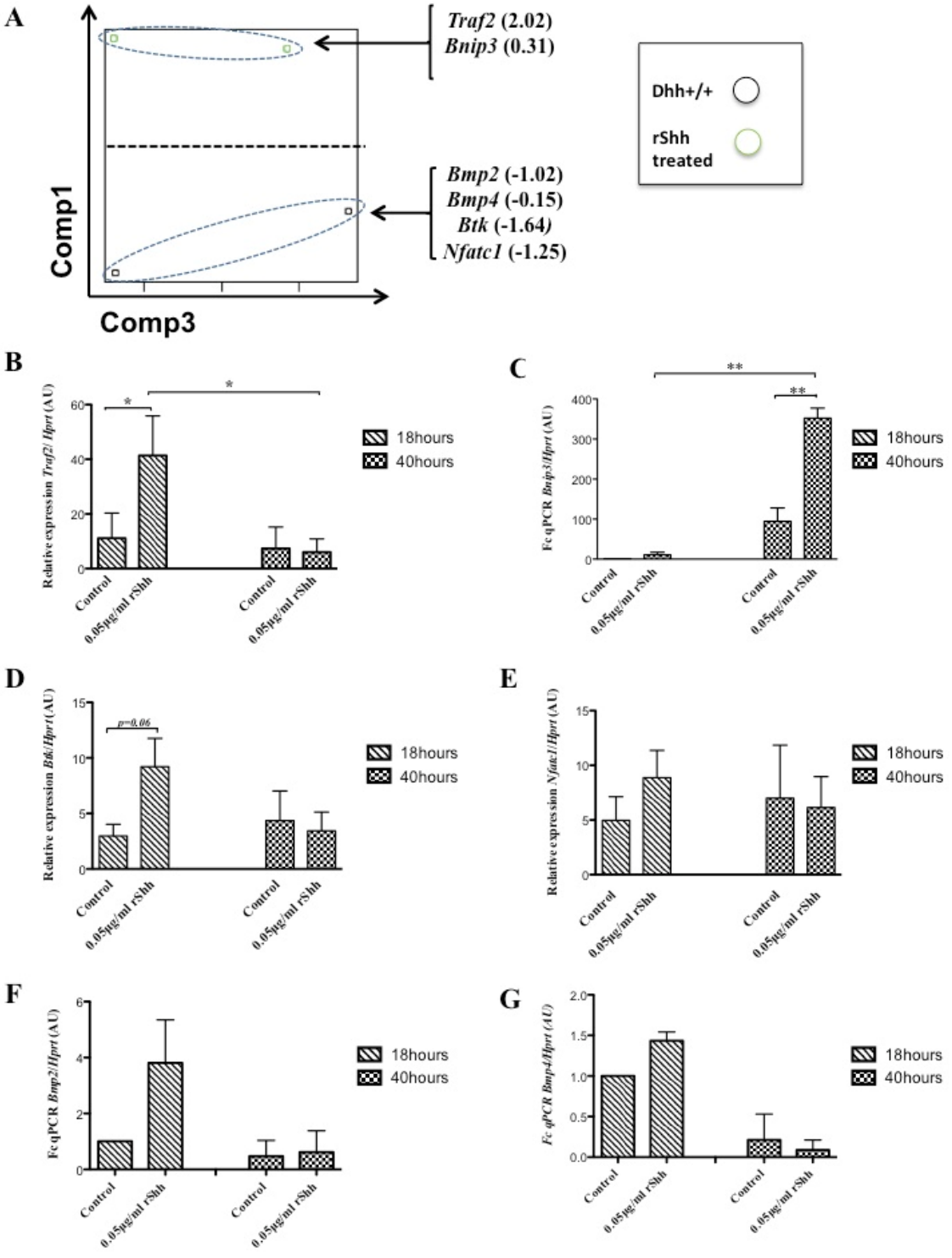


Figure 7.7. qRT-PCR verification of candidate genes

The PCA shows the different candidate genes identified from PC3 at 18hours post-activation stimulus in B cells treated with rShh and the control (**A**). Purified B cells were activated and treated with rShh. At 18 and 40hours, RNA was analysed for expression of *Traf2* (**B**), *Bnip3* (**C**), *Btk* (**D**), *Nfatc1* (**E**), *Bmp2* (**F**) and *Bmp4* (**G**) using qRT-PCR. The bar chart is representative of two or three biological experiments. The significance of the gene expression was analysed by Student *t* test (**$p \leq 0.01$ *$p \leq 0.05$).

7.3. Discussion

It has been reported in previous chapters that treatment of B cells with exogenous rShh can regulate peripheral B cell differentiation and survival. Additionally, in Chapter 6, it was shown that there was an impaired B cell development in B cells isolated from Dhh-/- compared to Dhh+/+. The aim of this chapter was to investigate the molecular targets of Hh signalling that is used in regulating peripheral B cell development. In this study, we used microarray gene expression analysis to investigate whether there are any differences in the transcriptome profile of B cells isolated from Dhh-/- and Dhh+/+mice. Also, to investigate the differences in gene expression profile of B cells treated with rShh and the untreated control.

The data presented in this chapter reveals that, using PCA, we could identify set of differentially expressed genes in B cells isolated from Dhh-/- compared to Dhh+/+ at 18 and 40hours post-activation stimulus. These genes might be responsible for the impaired B cell development observed in the Dhh-/- as well as the altered levels of Ig and cytokine secretion observed in cultures (Chapter 6). Candidate genes identified would be required to be verified using qRT-PCR in order to identify whether the findings in relation to altered gene expression were significant. The data presented here also demonstrates that treatment of B cells with exogenous rShh resulted in the differential expression of genes such as *Traf2*, *Btk*, *Nfatc1 Bnip3* and *Bmp2/4* in treated samples compared to the untreated control at 18hours post-activation stimulus. However, by 40hours in culture, our PCA analysis revealed that there was no significant difference between treated and untreated B cell samples, thus indicating that while Hh target genes are induced at 18hours in our culture system by 40hours in culture, they have exerted their biological effects and have been subsequently down regulated.

These findings indicate that treatment of B cells with rShh at 18hours post-activation stimulus could strengthen BCR signalling thus accelerating B cell activation, differentiation and survival with an associated up-regulation of gene expression of *Btk*, *Nfatc1* and *Traf2*. Increased Btk expression may drive the expansion of B cells towards the *Btk*-dependent, FO-I B cell rather than to the *Btk* independent MZ B cell. Consistent with this idea, phenotypic analysis of the differentiation of B cells in culture showed that in the presence of exogenous rShh, there was an increase in the presence of FO-I B cells at 18hours post-activation stimulus (Chapter 5). However, by 40hours post-activation stimulus, possibly due to the increased strength of the BCR signal induced at 18hours by rShh treatment; there is an increased induction of apoptosis in B cell possibly due to the induction of activated induced cell death or via the up-regulation of pro-apoptotic protein *Bnip3,* resulting in the reduced presence of FO-I B cells. This is accompanied by decreased *Btk and Nfatc1* expression at this later time point.

It has been reported that auto-reactive and anergic B cells rely on Btk signalling in order to mature and survive during onset of the autoimmune disease, Type 1 diabetes (Bonami *et al*., 2014). Also, over-expression of Btk can induce spontaneous formation of GC and plasma cell formation, eventually giving rise to autoantibodies, which leads to development of lupus like symptoms in mice (Kil *et al*., 2012). It would be of interest, therefore, to identify if the increased antibody production observed in the presence of rShh protein reported in Chapter 4 contains within it a higher proportion of autoantibody compared to the untreated control.

Additionally, it was demonstrated in this chapter that treatment of B cells with rShh could lead to an increased expression of *Bmp2* and *Bmp4* at 18hours post-activation stimulus. In B cells, BMP signalling has been shown to inhibit antibody production and

increase B cell apoptosis (Huse *et al*., 2011). Thus, the induction of expression of these proteins, might contribute to arrested production of antibody as well as the increased apoptosis observed in treated B cells at 40hours post-activation stimulus. This suggests an interplay between the two morphogens families, Hh and BMP, in regulating peripheral B cell development.

7.4. Conclusion

In conclusion, the findings in this chapter indicate that Hh signalling may induce an accelerated B cell mediated immune response in response to antigens by up-regulating target genes involved in transmitting the signal through the BCR as well as in the control of survival and death of B cells. It is possible that this programme of accelerated development can also induce B cell death thus resulting in down-regulation of these effects in order to avoid the onset of pathology. However, in autoimmune diseases such as SLE and multiple sclerosis and B cell lymphomas, Hh signalling might have lost the ability to negatively regulate these effects, or the negative effects are silenced in these pathologies.

Chapter 8: General discussion and future directions

8.1. Discussion

Hh signalling has been reported to regulate a plethora of functions in a wide variety of tissues. In T cells, Hh signalling has been shown to play a role in both thymic (Outram et al., 2000) and peripheral T cell development (Chan *et al*., 2006, Rowbotham *et al*., 2007). However, little is known about the role of Hh signalling in peripheral B cell development. With this gap in the literature in mind, the research presented in this thesis shows an analysis of the effect of Hh signalling in peripheral B cell development using mouse splenic B cells as a model.

Firstly, the effect of rShh treatment on B cell activation and survival in a mixed splenocyte population was investigated (Chapter 3). It was demonstrated that treatment of B cells in a mixed splenocyte population with exogenous rShh could increase B cell activation as judged using CD23 and survival as judged using annexin-V at 18hours post-activation stimulus. This finding is consistent with the findings of Sacedon *et al* 2005. However, by 40hours post-activation stimulus, it was observed that treatment of B cells with rShh increased B cell apoptosis and arrested, or decreased activation (Chapter 3, Figure 3.4). This suggested that Hh signalling could regulate both activation and survival of B cells. The increased apoptosis observed with treatment with rShh at 40hours in our culture was hypothesised to involve the actions of another morphogen, BMP. Using noggin, a BMP inhibitor, it was demonstrated that the effect of Hh treatment on B cells at 40hours post-activation stimulus was inhibited upon addition of Noggin to the cultures (Chapter 3, Figure 3.6). These data suggested that Hh signalling

can have an effect on B cell development and this may be mediated, in part, by BMP signalling.

In the following chapter (Chapter 4), the effect of rShh treatment on purified B cells was explored in order to investigate whether or not the observed effects in the mixed splenocyte cultures was intrinsic to B cells or required the presence of another cell type in the spleen. The findings in Chapter 4, demonstrated that treatment of purified B cells with rShh can also increase B cell activation (as judged by CD23 expression) and survival (as judged using Annexin-V) at 18hours post-activation stimulus and at 40hours post-activation stimulus, rShh treatment arrested, or decreased, activation and survival as observed in the mixed cell culture (Chapter 4, Figure 4.1 and 4.2). These data indicated that the effects of Hh treatment observed in B cells, were intrinsic to B cells and did not require the presence of any other cellular subset in the spleen. Analysis of the late B cell activation marker, CD25, revealed that treatment of purified B cells with exogenous rShh could increase expression of this marker at 40hours post-activation stimulus. Further analysis of the co-expression of early B cell activation marker, CD23, and late B cell activation marker, CD25, lead to the identification of three subsets named subset I, II and III, which are, CD23+CD25-. CD23+CD25+ and CD23-CD25+ respectively. It was observed that rShh treatment could increase the representation of CD23-CD25+ B cells at 40hours post-activation stimulus and decrease the representation of subset I (CD23+CD25-). Further characterisation of these subsets revealed that subset I CD23+CD25- was negative for the Breg marker, IL-10 and human memory B cell marker, CD27. However, subset II CD23+CD25+ was positive for IL-10, and rShh treatment increases the percentage of IL-10+ B cells at 40hours post-activation stimulus. Further verification using ELISA, showed that rShh treatment of purified B cells could increase IL-10 secretion compared to the untreated control.

Subset III CD23-CD25+ B cells were negative for IL-10 expression and positive for expression of the human memory B cell marker, CD27, with rShh treatment increasing the percentage of CD27+ B cells at 40hours post-activation stimulus. These results suggest that Hh signalling can regulate B cell differentiation towards B cells that express IL-10 and CD27. The production of antibody and IL-6 was also investigated. It was demonstrated that rShh treatment of B cells could increase antibody secretion and IL-6 secretion at 18hours post-activation stimulus and at 40hours post-activation stimulus, an arrest in secretion was observed. However, it must be considered, that the data observed in respect to antibody and IL-6 secretion at 40hours post-activation stimulus could be due to the observed increase in apoptosis.

Having observed in Chapter 4, that three subsets were arising in our culture and that following treatment with rShh, the representation of these subsets was altered, further characterisation of these subsets was carried out in order to identify the true identity and functions of these subsets. In Chapter 5, CD23+CD25-, CD23+CD25+ and CD23-CD25+ B cells were sorted and analysed in isolation. It was demonstrated that CD23+CD25- B cells were FO-I B cells (Pillai and Cariappa, 2009), with treatment of Hh inducing B cell death in these cells by 40hours in culture (Chapter 5, Figure 5.3 & 5.12). CD23+CD25+ B cells were shown to be a mix of cells including FO-I, T2/FO-II and T2-MZP, while CD23-CD25+ B cells were shown to contain MZ B cells and memory B cells as these cells were $CD80^{high}$ (Chapter 5, Figure 5.7). Additionally, at 18hours post-activation stimulus, treatment of B cells with rShh increased B cell differentiation towards FO-I B cells and at 40hours post-activation stimulus; it was observed that there is a decrease in FO-I representation and an emergence of T2-MZP B cells. These results suggested that treatment of B cells with exogenous Hh could affect the differentiation of peripheral B cells. It is possible that exogenous Hh treatment can

strengthen the BCR signal, which could, in turn, increase B cell activation, survival and differentiation towards the Btk dependent FO-I cell at 18hours in our culture, however, at 40hours in culture, due to the strong signal induced in FO-I B cells, they are induced to die, possibly as a result of activation induced cell death (AICD), hence increased apoptosis at 40hours post-activation stimulus was observed.

The investigation was furthered by investigating the effect of the loss of Hh signalling on B cell development using Dhh knockout mice as a model. In Chapter 6, it was demonstrated that B cells isolated from Dhh-/- exhibited a skewed peripheral B cell development. At 40hours post-activation stimulus, it was observed that they had an increased representation of Btk dependent FO-I, increased IL-6 and IgG1 secretion, and decreased IL-10 secretion when compared to the wild-type littermate. These results indicated that the loss of *Dhh*, can affect peripheral B cell development.

In order to elucidate the molecular mechanism by which Hh signalling regulates B cell development and to investigate whether there are any differences in the transcriptome profile of B cells isolated from Dhh-/- and Dhh+/+, a microarray analysis was performed (Chapter 7). It was demonstrated using PCA, there were differences in the transcriptome profile of B cells isolated from Dhh-/- compared to the Dhh+/+ control. It was observed that genes including *CD37*, *Btk*, *IL-10*, and *IL-6* were differentially expressed between B cells isolated from Dhh-/- and the Dhh+/+. It was also demonstrated using qRT-PCR that treatment of B cells with exogenous rShh can lead to increased expression of the BCR signal kinase, *Btk*, transcription factor, *Nfatc1*, anti-apoptotic protein, *Traf2* at 18hours post-activation stimulus. At 40hours post-activation stimulus, it was observed that there was a decrease in expression of these genes, however, it was demonstrated that there was also an increase in pro-apoptotic protein

Bnip3. Exogenous Hh treatment was also shown to increase the expression of *Bmp2/4*. These results suggest that Hh signalling could regulate peripheral B cell development via strengthening the BCR signalling pathway and by inducing expression of BMP.

In summary, it was demonstrated in this thesis, that treatment of B cells at 18hours post-activation with exogenous Hh can potentiate the BCR signal by increasing components of the BCR signalling pathway including *Btk*, *Nfatc1* and *Traf2*. This increased expression could lead to an increased B cell activation and survival and ultimately, an increased differentiation towards FO-I, which can secrete antibody and IL-6. However, at 40hours post-activation stimulus, the increased BCR signal (induced at 18hours post-stimulus with exogenous Hh treatment) could possibly induce activation induced cell death in FO-I B cells or increase the expression of pro-apoptotic proteins such as *Bnip3*, which, then leads to an increased apoptosis and an arrest in antibody production and IL-6 secretion and a decrease in *Btk*, *Nfatc1* and *Traf2* expression. The reduced *Btk* expression at 40hours post-activation stimulus, might lead to the B cells differentiating into Btk independent T2-MZP, and MZ B cells which can secrete IL-10 as well as memory B cells at this latter time point. Figure 8.1, shows a model of events observed occurring with Hh treatment.

However, in the case of B cells isolated from Dhh-/-, there is an increased differentiation of B cells towards the Btk dependent FO-I at 40hours post-activation stimulus, which leads to an increased IL-6 and IgG1 secretion at this time point. It is possible that peripheral B cell development require the presence of Dhh to mediate the apparent arrest in B cell development observed at the later time point in our cultures and for the ability to divert B cell differentiation away from FO-I and towards other B cell lineages. Hence, it can be assumed that lack of exogenous Hh treatment or the absence of one of the homologue of Hh protein, can lead to the BCR signal not being potentiated

in these cells and therefore activation induced cell death is not induced. This could result in increased survival of the FO-I B cells. It was observed using PCA, that there was an increased expression of Btk in B cells isolated from Dhh-/- compared to the wild-type. This increased Btk expression may drive increased Btk dependent differentiation of FO-I B cells with the associated decrease in differentiation towards T2-MZP and MZ B cells observed in B cell cultures isolated from Dhh-/- mice. This hypothesis is also consistent with the observed reduced IL-10 secretion by B cells isolated from Dhh-/- mice. This apparently unregulated immune response by B cells isolated from Dhh-/- could lead to the development of autoimmunity as over-expression of Btk, has been shown to lead to the survival of auto-reactive B cells which cause symptoms of auto-immune diseases (Kil *et al*., 2012). This possibility needs to be investigated and the findings used to develop potential new therapeutics for use in the treatment of B cell mediated autoimmune disease. A proposed model of events of the role of Shh in peripheral B cell development is shown in Figure 8.1.

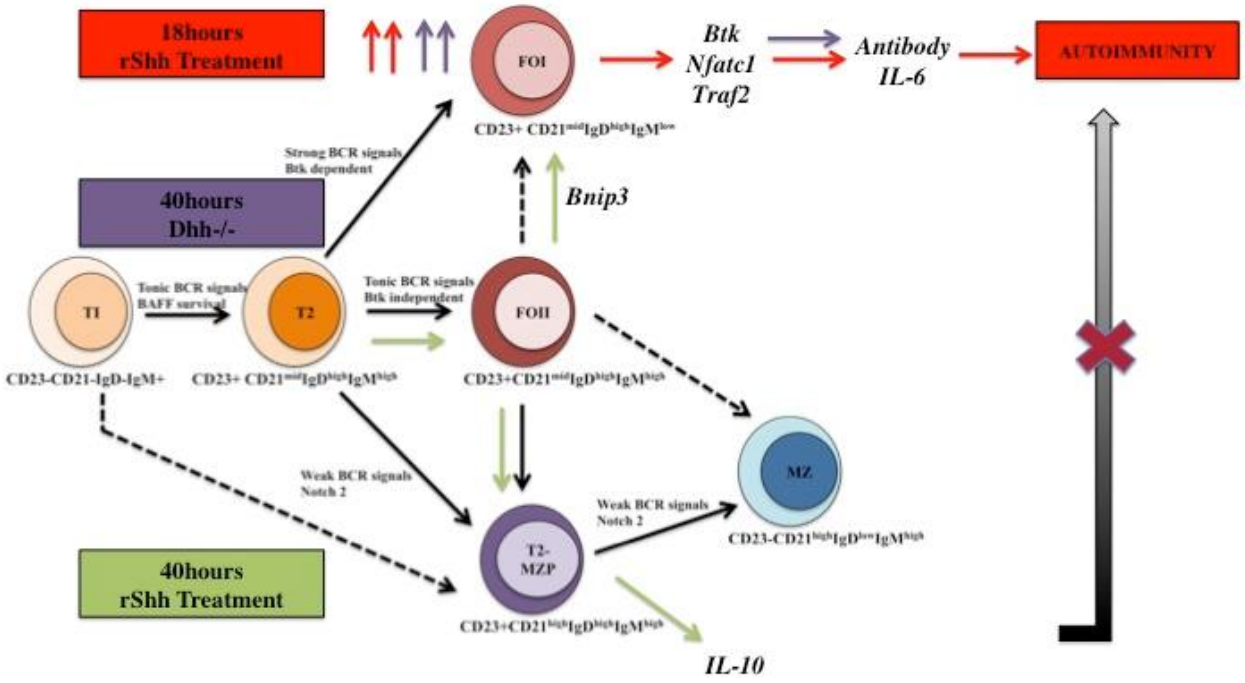


Figure 8.1 A proposed model of events illustrating the effect of exogenous Hh on peripheral B cell development.

A schematic model illustrating the effect of rShh observed in peripheral B cell development, 18hours (Red), 40hours (Green) and Dhh-/- 40hours (Purple). At 18hours, Hh treatment can drive FO-I differentiation characterised by an increase in *Btk, Nfatc1, Traf2* as well as antibody and IL-6 secretion. At 40hours, Hh treatment is leading to an increased B cell death via the induction of pro-apoptotic protein *Bnip3*. There is also an increased differentiation towards T2-MZP B cells that can secrete IL-10. However, in Dhh-/-, the effect observed at 40hours, was similar to the effect of Hh treatment at 18hours and if unregulated can lead to autoimmunity. Perhaps, the effect observed at 40hours with rShh, can possibly be used as an interventional therapy in autoimmunity.

8.2. Future directions

This thesis provides new evidence that Hh signalling may be able to strengthen the BCR signal thus accelerating the immune response and subsequently lead to a down-regulation of the immune response via different mechanisms including the up-regulation of apoptosis.

In 2005, Sacedon *et al*., (2005), showed that all GC B cells express Ptch and 30% co-expressed Smo, which increases following B cell activation. Shh signalling was shown to be a physiological requirement for GC B cell survival (Sacedon *et al*., 2005).

Additionally, it was reported in this thesis that treatment of isolated CD23+CD25- B cells, which were characterised to be FO-I B cells, with rShh, lead to an increased death. Hence, it is possible, that FO-I B cells may have lost the ability to respond to a Hh signal or no longer express the receptors for Hh. It is also possible that they require the presence of another B cell subset to secrete Hh protein. Thus, it would be important to characterise the different peripheral B cell subset including, T1, T2, FO-I, FO-II, T2-MZP and MZ in terms of their expression for the Hh signalling component including Ptch, Smo and Gli proteins. Using, flow cytometry, the expression of Ptch and Smo could be investigated in the peripheral B cell subsets. Using RT-PCR, the expression of the Gli proteins in the different peripheral subsets could be investigated. Also, it was observed that treatment of B cells with exogenous rShh, could increase the expression of genes including *Bnip3*, *Traf2*, *Btk*, *Nfatc1* and *Bmp2/4*. It would be important to investigate whether these genes are induced differently in the different peripheral subsets observed in our culture including CD23+CD25-, (subset I) CD23+CD25+ (subset II) and CD23-CD25+ (subset III). Thus, it would be important to sort the subsets, and examine them in isolation for their expression of these genes. A pilot experiment has been carried out, although inconclusive, it remains very promising.

Additionally, it would be important to investigate whether the increased differentiation to T2-MZP B cells at 40hours post-activation stimulus that can secrete IL-10 could have any therapeutic effect. It has been reported by Evans *et al*., (2007), that T2-MZP B cells were able to prevent and ameliorate disease in a mouse model of experimental arthritis (Evans *et al*., 2007). It is possible that the increased IL-10 observed following rShh treatment of B cells could have the ability to suppress autoimmune disease. An in-vivo study using a mouse model in which a pro-inflammatory disease has been induced could be used to investigate whether the disease would be milder following treatment with

exogenous Hh. Using a different mouse model for autoimmunity such as the EAE mouse model (a mouse model for multiple sclerosis) Exogenous Hh could be given intravenously, in order to investigate whether; it would increase differentiation towards T2-MZP B cells that can secrete IL-10 and reduce disease pathology. This would open the possibility of using Hh signalling for therapy in autoimmunity.

The microarray analysis done in this study to identify whether there are any differences in the gene expression profile in B cells isolated from Dhh-/- compared to Dhh+/+ was carried out with a limited amount of samples and thus needs to be supported with further experiments. However, it was observed that there were differentially expressed genes in B cells isolated from Dhh-/- compared to Dhh+/+ including *CD37*. CD37 is a tetraspanin that is expressed in mature B cells and has been reported to be highly expressed in B cell lymphomas including, B-CLL and leukaemia. Inactivation of CD37 has been shown to lead to a reduced secretion of antibody (Flinn, 2011). In this thesis, it was demonstrated that there was increased IgG1 secretion in B cells isolated from Dhh-/- compared to the wildtype. It is possible that the increased IgG1 secretion is due to an increased expression of CD37 in B cells isolated from Dhh-/-. Hence, the expression of this gene should be validated using RT-PCR. Additionally, there was increased FO-I differentiation in B cells isolated from Dhh-/- compared to the wild-type. It would be important to investigate the expression of the components of the BCR signalling pathway, including *Btk*, *Nfatc1* and *Traf2* in B cells isolated from Dhh-/- mice.

It was also demonstrated in this thesis, that exogenous rShh treatment could increase the expression of BMP proteins (*Bmp2/4*). Thus indicating the effect of Hh signalling may, in part, due to downstream BMP signalling. An investigation into the exact mechanism by which Hh and BMP signalling interact to regulate B cell development would be of

interest as BMP signalling is regulated by a number of secreted proteins including twisted gastrulation which is produced by activated B cells (Tsalavos *et al*., 2011). It would be important to investigate whether the effect observed in this thesis with rShh treatment on B cells at 40hours post-activation stimulus would be any different in the absence of BMP proteins. Using a BMPR1a knockout mouse, these effects could be investigated, as there is a possibility that Hh and BMP signalling pathway interact at checkpoints during peripheral B cell development. This study would influence future studies and open the possibility for use in therapeutics in B cell mediated immune diseases.

8.3. Significance of findings

The results described in this study provide a new understanding of the role Hh signalling plays in the regulation of peripheral B cell development. It demonstrates for the first time that Hh treatment of B cells with rShh can potentiate the B cell immune response but can also lead to increased death of B cells. Thus causing a subsequent down-regulation of the immune response, which can prevent the onset of disease. This study also reveals some of the molecular targets of the Hh signalling pathway that is used in regulating peripheral B cell development. Moreover, treatment of B cells with exogenous Hh induced the increased expression of BMP proteins thus indicating an interplay for these morphogens in regulating peripheral B cell development. Taken together, these data suggest that, it is possible that targeting the Hh signalling pathway could be used as an interventional therapy for the treatment of B cell autoimmune diseases and B cell lymphoma.

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Appendix

Appendix 2.1

A melting curve analysis was carried out to ensure the product of primer were the expected size and not the result of primer dimer. The image below shows an example of a melting curve.

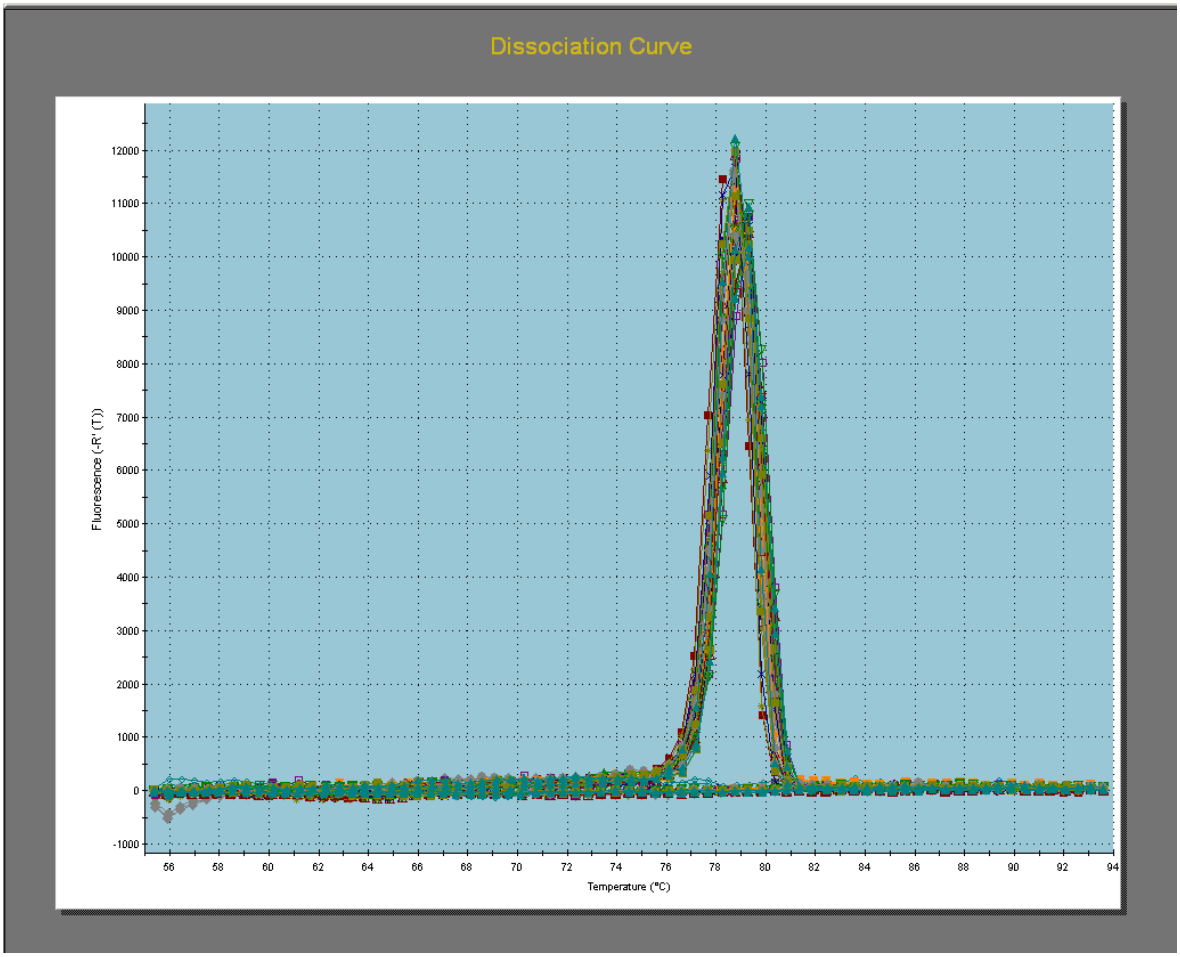
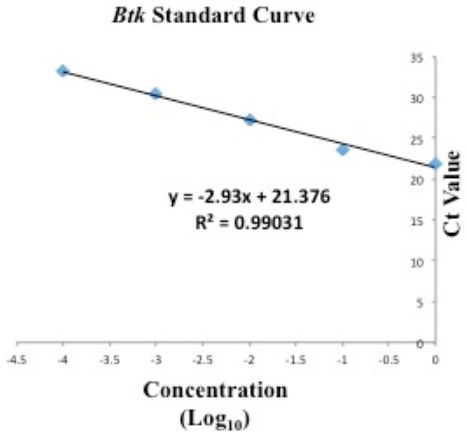


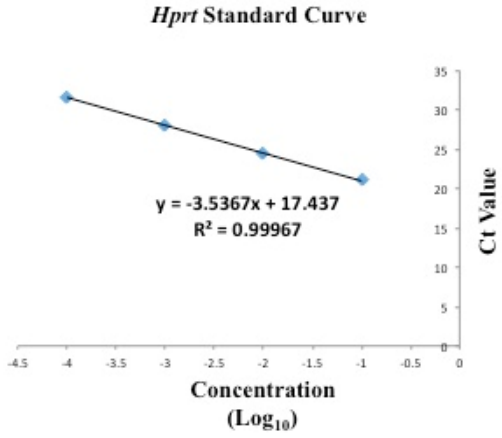
Figure 2: Image of a melting curve

Change in fluorescence (y axis) with increasing temperature (x axis) is measured. As the temperature increases, the two strands of the amplicon breaks to form single stranded DNA triggering the fluorescent intercalating dye to dissociate from the DNA and stop fluorescing.

Appendix 2.2

Below shows a worked example of the calculation of relative expression using the relative standard method. Standard curves are generated for both the gene of interest (in this example, Btk) and the house keeping gene, *Hprt*. The standard curve is shown below. A 1:10 serial dilution of the genes was carried out. The standard curves for both genes were generated by plotting the Ct values against the Log_{10} concentrations of the gene.





The table below shows the average Ct value of the different samples.

Sample	**Average Ct** ***Btk***	**Average Ct** ***Hprt***
Control 18hours	23.21	22.34
0.05µg/ml rShh 18hours	22.49	22.25
Control 40hours	22.63	22.81
0.05µg/ml rShh 40hours	22.52	22.05

The equation obtained from the standard curve for *Btk* and *Hprt* was used to calculate the gene concentration in the different samples, shown below in the table.

Sample	Concentration *Btk*	Concentration *Hprt*
Control 18hours	0.2360	0.0411
0.05µg/ml rShh 18hours	0.4156	0.0436
Control 40hours	0.3723	0.0303
0.05µg/ml rShh 40hours	0.4070	0.0496

Relative gene expression in each sample was obtained by dividing the concentration of the gene of interest (*Btk*) by the housekeeping gene (*Hprt*). The relative expression is shown below.

Sample	Relative expression *Btk*
Control 18hours	5.744
0.05µg/ml rShh 18hours	9.539
Control 40hours	12.305
0.05µg/ml rShh 40hours	8.201