The Interplay between Hedgehog Protein and Bone Morphogenetic Protein in B cell Development

Martina Neville

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I. Abstract

Morphogens play a significant role in the modulation of cell fate and development and are expressed in all tissue at some point during development. Recent studies suggest that expression of two morphogens, Hedgehog (Hh) and Bone Morphogenetic Protein (BMP), may influence immune cell development in the periphery which is consistent with the finding that Hh is expressed by splenic stromal cells. In addition, preliminary data obtained at UEL suggest that Hh signalling may provide a positive signal in early peripheral B cell development in terms of activation and survival but may then deliver a later negative immunoregulatory signal capable of dampening down the immune response. This may occur as a consequence of the ability of Hh signalling to upregulate expression of BMP. Although Hh and BMP signalling have both been shown to play a role in immune cell development independently, little is known regarding the interplay between the two morphogens and the role this may play in directing an immune response to antigen. In this study murine splenic B cells were cultured in the presence of a signal for B cell activation (anti-IgM and anti-CD40) together with recombinant Sonic Hedgehog protein (rShh) and a BMP inhibitor, Noggin. These experiments were designed in order to elucidate whether it is necessary for Hh signalling to upregulate the expression of BMP for an effective B cell response to take place. We observed that expression of both Hh and BMP is required for efficient activation and survival of B cells at 18 hours post stimulus whilst expression of BMP plays a more dominant role in the later stages of B cell development. Abrogation of BMP signalling led to a significant skewing in the development of certain B cell subsets. Further analysis revealed a potential role for BMP-2 in particular during splenic B cell development, expression of which was significantly upregulated in the presence of exogenous Hh. Overall, our findings suggest that there is indeed an interplay between Hh and BMP signalling in driving appropriate B cell development.

II. Acknowledgements

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V. Abbreviations

Mg	Micrograms
Ml	Microlitres
-/-	Knockout
+/+	Wildtype
APC	Allophycocyanin
BCR	B Cell Receptor
BMP	Bone Morphogenetic Protein
Btk	Bruton's Tyrosine Kinase
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
dNTP's	Deoxynucleotide Triphosphate
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
KO	Knockout
Mg	Milligrams
Ml	Millilitres
M	Moles
mRNA	Messenger Ribonucleic Acid
MZ	Marginal Zone
Ng	Noggin
PBS	Phosphate Buffered Solution
PCR	Polymerase Chain Reaction
PE	PhycoErythrin
PRR	Pattern Recognition Receptor
rSHh	Recombinant Sonic Hedgehog
RT-PCR	Real-time Polymerase Chain Reaction
SD	Standard Deviation
TD	T cell-dependent
TGF-B	Transforming Growth Factor Beta
WT	Wildtype
2-BME	2-Beta Mercaptoethanol

1. Introduction

1.1. The Immune Response

1.1.1. B cell Development

B cells undergo early development within the unique microenvironment of the bone marrow, originating from progenitor cells known as haematopoietic stem cells (HSC) (Murphy, 2012). In a structured sequence of events regulated by the surrounding scaffold of stromal cells and the factors secreted by stromal cells, a series of Ig receptor editing phases take place culminating in the expression of a mature antigen receptor on the B cell surface (BCR). These processes involve the induction of V(D)J rearrangement and somatic hypermutation, according to the nature and strength of the signal. Thus, B cells develop, guided by the expression of, amongst other factors, RAG-1 and 2, from stem cells through to pro-B cells, pre-B cells and, finally, mature B cells in the bone marrow (Murphy, 2012). Once mature, the B cell may exit the bone marrow and move to the peripheral lymphoid tissue as transitional T1 B cells where they undergo further differentiation in response to their specific antigen.

Peripheral B cells may fall in to three major groups: B1 cells, B2 cells and regulatory B cells (Bregs). The latter cell has been only recently identified and is known to have the ability to dampen down immune responses. B1 cells are mostly found within the peritoneal spaces and within the gastro-intestinal tract, B2 cells are predominantly found within the spleen and lymph node whilst a specific location for Bregs has yet to be fully elucidated. As previously mentioned, B cells within the periphery originate from a transitional subset (T1) and the strength and type of signal received will determine cell fate. T1 cells are an immature subset of B cells which may mature to acquire T2 status or, upon Toll-like receptor (TLR) stimulation, develop into marginal zone B cells (MZB) acting as innate-type cells (Guerrier *et al*., 2012) (Figure 1.1). Numerous studies have elucidated that T1 cells readily undergo apoptosis in

response to a tonic signal through the BCR whilst a strong signal will lead to polarisation of a T1 cell to become a T2 cell (Pillai and Cariappa., 2009). T2 cells are a highly diverse group of cells which have the capacity to further polarise into marginal zone precursors (MZP), follicular type I or follicular type II cells. Importantly, T2 cells cannot be activated by TLR activation alone; at this stage in development, BCR stimulation and co-stimulation must also take place in order to elicit an efficient immune response.

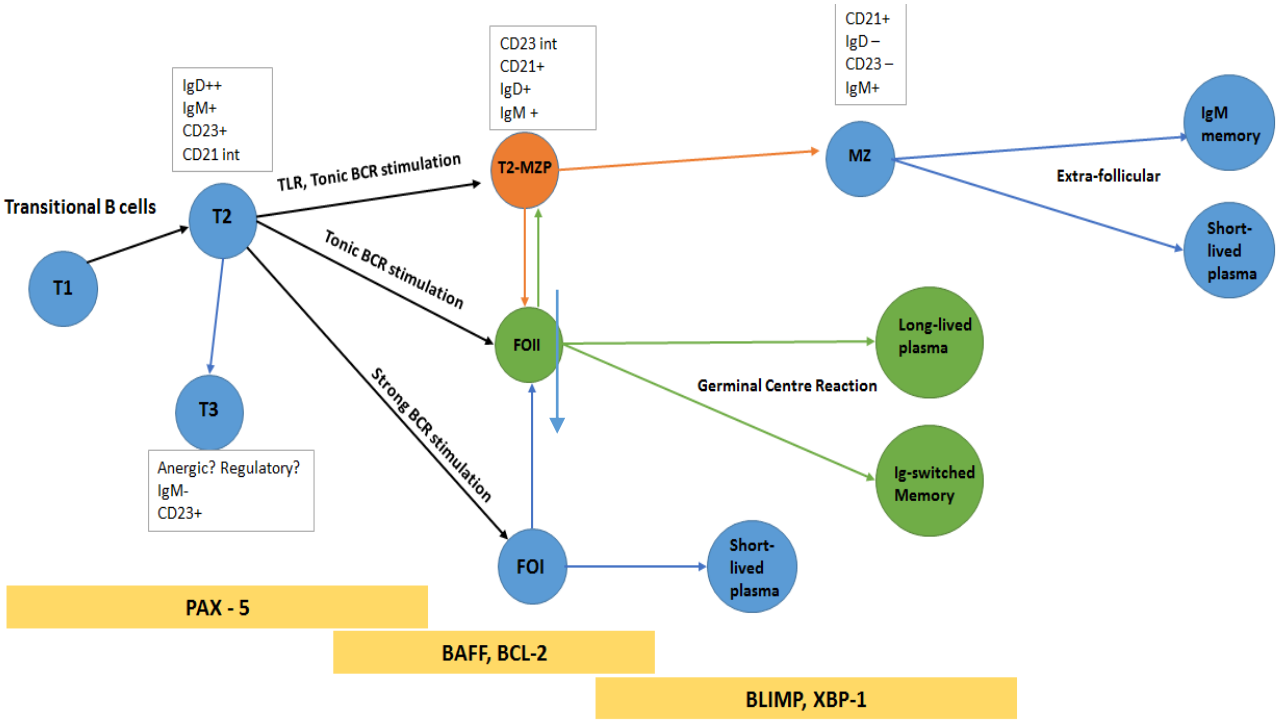


Figure 1.1. Splenic B Cell Development
Development of B-cells in response to antigen stimulation. Stage-specific transcription factors shown in yellow boxes.

Crucially Loder *et al* (1999), using mice lacking the Ig-α cytoplasmic tail required for BCR signalling, noticed that the T2 and mature B cell repertoire had failed to develop whilst the T1 population remained present at normal levels of around 20 per cent of splenic B cells indicating that signalling through the BCR is required for the

transition from T1 to T2 B cell. In addition, mice lacking the gene for Lyn, a src family kinase also required for appropriate signalling through the BCR, had normal numbers of pro-, pre- and immature B cells in the bone marrow in contrast to mature B cells which had reduced 10-20 fold (Allman *et al*., 2001). Furthermore, an inverse relationship between the strength of BCR signalling and marginal zone cell development was noted by Cariappa *et al* in 2001. Marginal zone B cells respond rapidly, to stimulation via innate immune receptors such as TLR indicating that their purpose is fit for the location in which they reside. Marginal zone B cells are the first point of contact for many blood-borne antigens following entry in to the body therefore it is essential they have the ability to develop and proliferate in direct response to stimulation through the pattern recognition receptor (PRR). Thus far, MZB cells have been assumed to be limited in their abilities to migrate into the primary lymphoid follicles, however, a recent study (Ferguson and Corley., 2005) discovered that marginal zone B cells, in fact, traffic antigens across the mantle zone, into the follicles to stimulate follicular B cell and T cell help.

Naïve mature B cells enter the peripheral circulation, recirculate through the lymphoid organs and migrate to the spleen, residing in primary follicles until an antigen is encountered. Upon BCR cross-linkage and T-cell co-stimulation via CD40, highly specialised germinal centres are formed providing the B-cells with a site where the cytokines and growth factors required for clonal expansion and differentiation are expressed. Within the germinal centres B-cells become activated undergoing class switching mediated by activation-induced cytidine deaminase (AID) and affinity maturation - a process whereby the production of low affinity antibodies is replaced by the production of specific high affinity antibodies that will clear an invading infection more efficiently. Activated B-cells have the propensity to form short-lived plasma cells, long-lived plasma cells (LLPC), memory cells or regulatory B-cells; the latter

being a critical factor in abrogating the immune response. Current research has shown that B cells expressing a high affinity BCR prior to GC formation will go on to act as short term plasma cells whilst those with a lower affinity are required to undergo class-switching in order to increase their affinity for an antigen (Cariappa and Pillai., 2007).

In addition to phenotypic changes during B cell development, a number of transcriptional modifications occur characterised by the expression of a number of transcription factors such as Pax-5, BAFF and BLIMP-1. Expression of PAX-5 is associated with immature B cell development and ensures a state of self-renewal. Additionally, Debnath et al (2008) noted that loss of PAX-5 correlates with a decrease in expression of CD21 and CD23 indicating that PAX-5 is a redundant transcription factor in memory and plasma cell differentiation. BAFF is a survival factor, expression of which is important in the transitional period between the naïve T1 B cells developing into activated follicular B cells. BAFF expression itself reduces expression of the pro-apoptotic factor, Bim via an NF-kB dependent pathway leading to increased cell survival. BLIMP-1 is a transcription factor with repressor activity, typically repressing PAX-5 expression. Repression of PAX-5 leads to the expression of, an endonuclease IRE-1, which subsequently splices a 26-nucleotide intron from an inactive isoform of XBP-1, an mRNA present in all cells, subsequently leaving an active form. Expression of XBP-1 is associated with the unfolded protein response and regulates levels of ER stress during increased levels of protein folding such as that during antibody production, thus is associated with plasma cell development. Interestingly, XBP-1 has also been shown to play a role in the regulation of IL-6 expression; a cytokine also involved in plasma cell differentiation (Iwakoshi *et al*., 2003). Presently, there is an absence of knowledge regarding the role of specific transcription factors associated with memory cell development and function although a study by Tomayko et al in 2009 suggested

that memory cells have characteristics in common with naive B cells and this is likely to be a result of the need for longevity and self-renewal.

1.1.2. Immune Resolution

Resolution of the adaptive immune response following immune insult is a critical stage in restoration of immune homeostasis. The numbers of effector cells and secretion of their associated cytokines are downregulated, in order to prevent aberrant or collateral damage to the host, as inefficient termination is a well-documented cause of auto-immune and auto-inflammatory disorders. Termed the resolution phase, numerous processes exist to ensure that basal levels of immunity are restored in a timely manner: secretion of suppressive cytokines; expression of inhibitory ligands leading to anergy and induction of apoptosis; and polarisation of regulatory T and B cell subsets. To date, regulatory subsets of T cells, dendritic cells and macrophages have been well characterised. However, the characterisation of B cells possessing regulatory characteristics, such as the expression of IL-10, remain largely elusive despite a significant amount of research within the area. Phenotypically, B10 cells or Bregs, as they are more commonly known due to the fact that they all secret IL-10, appear to lack distinct characteristics. However, a small number of studies using murine models of lupus suggest that regulatory B cells have features which mimic naive cells and are $CD24^{+}$ $CD1d^{+}CD19^{+}$ (Blair *et al*., 2010). A significant proportion of research suggests that IL-10 is the factor responsible for driving immune resolution.

IL-10 is a 18.5kDa protein which acts as a pleotrophic cytokine involved in the regulation of immune reponses. Historically, IL-10 has been associated with the suppression and inhibition of the immune response and a number of inhibitory mechanisms have been described including: inhibition of pro-inflammatory cytokine production, downregulation of co-stimulatory ligands, such as the B7 family, and the development of antibody-forming B cells (Saraiva and O'Garra., 2010). Nonetheless, in

recent times a paradoxical role as a pro-inflammatory and survival factor has been revealed particularly in autoimmune diseases such as Systemic Lupus Erythromatosus (Park *et al*., 1998). The exact molecular mechanisms of IL-10 remain controversial however many studies agree IL-10 signalling activates STAT3 and immunomodulates predominantly through an NF-κB-dependent manner (Niemand et al., 2003)

1.2. Man versus Mice

Currently, a noteworthy amount of research has focused on B cell development within disease models in mice and in humans, however, specific B cell-mediated immune responses in normal healthy mice remain elusive. There are notable differences between markers associated with human and mouse B cells such as the variance in memory cell markers CD27 in humans, and CD80 in mice (Tomayko *et al*., 2008; Wang *et al*., 2006). In addition, variance between gender and strain of mouse are likely to influence results in studies utilising murine disease models. Exposing precise differences in specific models will ensure that research characterising immune diseases, such as Lupus, in mice is consistent and reliable. With this in mind, we will examine the role of morphogens on T cell dependent (TD) B cell development in BALB/c female and C57BL/6 male mice and elucidate any notable differences.

1.3. Morphogens

1.3.1. Morphogen Dynamics in Developing Cells

Morphogens are evolutionarily conserved extracellular cell surface or secreted proteins displaying high pleotropic tendencies and governing a multitude of developmental processes including cell fate, patterning and organogenesis in many vertebrates. Unsurprisingly, expression of many morphogens has been shown to be constitutive during embryogenesis whilst tightly regulated thereafter by the presence of numerous regulatory proteins dependant on the specific type. Morphogens transduce signals by varying mechanisms which include: secretion from a local source such as

cells of the extracellular matrix (ECM), or by diffusion according to Fick's law and ligand-internalisation via cognate receptors (Tanaka *et al*., 1997). Consequently, a dynamic long-range concentration gradient capable of influencing cell fate is formed; the stability of a gradient is essentially reliant on a steady-state arrangement of synthesis and decay at its source (Eldar *et al*., 2003) and receptor kinetics (Porter *et al*., 1996). The formation of a 'threshold' in which cells will or will not respond forms the basis of morphogen signalling. Subsequent target cell responses are dependent on their intricate spatial and temporal arrangement within a concentration gradient and recent studies have shown that stochastic noise within gradients may cause disease (Karim *et al*., 2012). Essentially, morphogens are enigmatic entities which appear to exert effects in a context-dependent manner and disruptions within the complex communication network of morphogens is widely documented to cause severe developmental defects. Studies investigating the role that morphogens play in the development and maintenance of B-cell homeostasis has, thus far, been largely empirical and more research is required to elucidate the part that such disruptions may play in immune disorders. Systemic Lupus Erythematosus, an autoimmune disease characterised by massive infiltrates of inflammatory mediators, has been shown to be governed by plasma cells which recognise and respond to auto-antigens such as nucleic acids (Lui *et al*., 2011; Minowa *et al*., 2011). Multiple Myeloma, a plasma cell neoplasm whereby 'false' antibodies are synthesised leads to severe immunodeficiency and immune complex deposits within the kidney which may cause acute renal failure. Interestingly Hedgehog (Hh) signalling is often found to be dysregulated in patients suffering from multiple myelomas. Therefore, specific mechanisms involved in the development of B cells within the periphery need exposing in order to understand immune diseases such as those mentioned. Within the last decade, morphogens such as Hh and Bone Morphogenetic Protein (BMP) have been found to play a role in many different aspects

of cellular development and a novel role for morphogens in self-renewing tissues has been revealed. Furthermore, research has revealed the possibility of synergism between the two morphogens as a consequence of signalling cross-talk (Bitgood and McMahon, 1995; Guo and Wang., 2009 and Reichert *et al*., 2013); thus the role of morphogens in peripheral immune cell development must be explicated. The various components of the Hh signalling pathway have been detected within both the Thymic (Outram *et al* 2000, Sacedon *et al*., 2003) and Splenic (Sacedon *et al*., 2005) environment. A significant proportion of research has already shown that Hedgehog proteins influence central and peripheral T-cell (Sacedon *et al*., 2003, Outram *et al*., 2009) and that aberrant hedgehog signalling may lead to various haematopoietic neoplastic disorders such as Lymphoma and Multiple Myeloma (Hegde *et* al., 2008; Irvine and Copland., 2012). Similarly, BMPs are thought to play a role in the development of many haematopoietic cells and Sacedon *et al* (2005) revealed that within the stromal niche, follicular dendritic cells within the spleen may express such morphogens. BMP, historically, has been associated with regulatory/inhibitory functions such as decreased proliferation and increasing cell death and, in recent years, been shown to negatively regulate the hedgehog signalling pathway as previously documented (Kersten *et al*., 2005; Seki and Hata., 2004). Furthermore, BMP 6 has been shown by Kersten et al, in 2005, to induce the expression of the Inhibitor of Differentiation family of proteins in B cells leading to the abrogation of peripheral B cell development. Nevertheless, a recent paper revealed a more complex role for BMP in the development of immune cells in regards to activation and survival (Martinez *et al*., 2015).

1.3.2 The Origins of Hedgehog Proteins

Hedgehog protein is a widely studied morphogen initially discovered in the fruit fly *Drosophilia melanogaster* whereby genetic mutational analysis of the hedgehog gene led to formation of a spiky ‘sonic hedgehog’ appearance in the progeny (Nusslein-

Vilhard and Wieschaus, 1980). Within mammals, there are three well established homologues of Hedgehog proteins – Desert, Indian and Sonic which are structurally conserved, however, a single variation in amino acid sequence was noted in 1995 by Kumar *et al*, indicating that their affinity for specific Hh signalling receptors could vary to some extent and may also be responsible for variability of Hh expression in specific tissue types. Hedgehog plays a critical role in embryonic development with the differential expression of the *Hh* gene governing cell fate, patterning and organogenesis. Hedgehog proteins are synthesised as precursor proteins, approximately 45 kDa, which are subject to internal cleavage by the C-terminus leaving the 20 kDa N-terminus which has previously been documented to contain all signalling capabilities (Porter *et al*., 1996). Previous work has suggested that nucleophilic attack by the C-terminus (Cysteine 258 – Asparagine 471) leads to the generation of a thiol group between Glycine 257 and Cysteine 258 which is subsequently modified by the addition of a cholesterol moiety (Tanaka *et al*., 1997). The modification leads to association of the Hh-N-terminus with the cell membrane. Furthermore, Porter *et al*., 1996 proposed that autolytic processing acts as a regulatory mechanism for controlling the spatial arrangement of Hh protein thus influencing gradient formation.

All Hh proteins are thought to signal through an identical canonical pathway whereby Patched, a twelve-pass transmembrane receptor, is the ligand receptor and Smoothened is the seven transmembrane signal transducer of Hh signalling. In the absence of a Hh ligand, the pathway is typically inhibited; Patched inhibits Smoothened signalling in to the cell, whilst in the presence of the Hh ligand, Smoothened translocates to the membrane leading to constitutive signal transduction. This signalling pathway culminates in the nucleus in the activation of the Gli family of transcription factors (Dorsam and Gutkind., 2007). The cumulative level of Gli within the nucleus and the relative ratios of Gli proteins to one another determines the expression of a

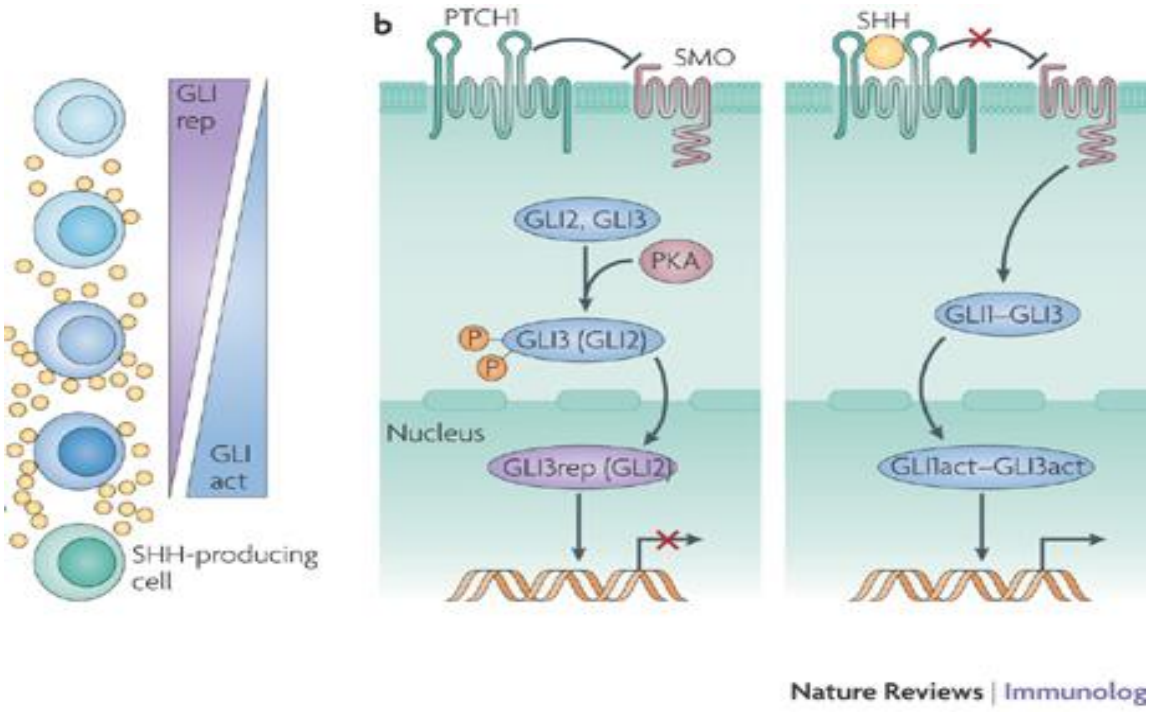


Figure 1.2. Hedgehog Signalling Pathway.

Schematic representing mechanisms of Hh signalling. In the absence of a Hh ligand, Patched binds to Smoothened inhibiting signal transduction. When a Hh ligand is present, Patched release Smoothened allowing a signal to transduce into the cell. Hh signalling leads to the activation of the Gli family of transcription factors. (Crompton *et al*., 2007)

1.3.3. BMP Signalling

Bone Morphogenetic Proteins are evolutionarily conserved proteins originating from the TGF-β family. BMP's play a significant role in organogenesis, osteogenesis and are secreted by local cells within the bone marrow microenvironment, in particular, stromal cells. BMPs, like many morphogens, are highly complex pleotropic molecules, expressed differentially in almost all tissue types. Genetic abnormalities within BMP genes and the associated regulatory elements cause widespread life-limiting diseases such as pulmonary hypertension (Newman *et al*., 2001) and fibrodysplasia ossificans progressiva (Kaplan *et al*., 2001).

A significant role for BMP in the development of haematopoietic cells has recently been described. To date there are twenty known homologues of mammalian BMPs of which BMP 2, 4, 6 and 7 have been the most extensively studied in immune cells (Huse *et al*., 2011; Kersten *et al*., 2005). All are synthesised as an inactive precursor protein subsequently cleaved by furin, an endopeptidase, leaving the active C-terminal signalling protein. Active BMP's are secreted into the extracellular matrix where they go on to form homo/hetero-tetramers with other BMP's (Cui *et al*., 2001). BMP 2 has been shown to hetero-dimerise with both BMP 4 and 6 leading to differential regulation of downstream target gene expression. BMP's transduce signals through two receptor types: type I and type II; both serine/threonine receptors in which both components are required for effective signalling. Upon binding, the type II receptor forms a hetero-tetramer with type I receptors and phosphorylates the Receptor-activated Smads (R-Smads), 1, 5 and 8. Phosphorylation of the type I receptor leads to recruitment of a common-mediator Smad (Co-Smad/Smad4) which leads to formation of a signalling complex (Figure 1.3). This complex traverses the nuclear membrane leading to the transcription of a number of BMP target genes. BMP signalling is unique from TGFβ in that different combinations of Smads undergo phosphorylation.

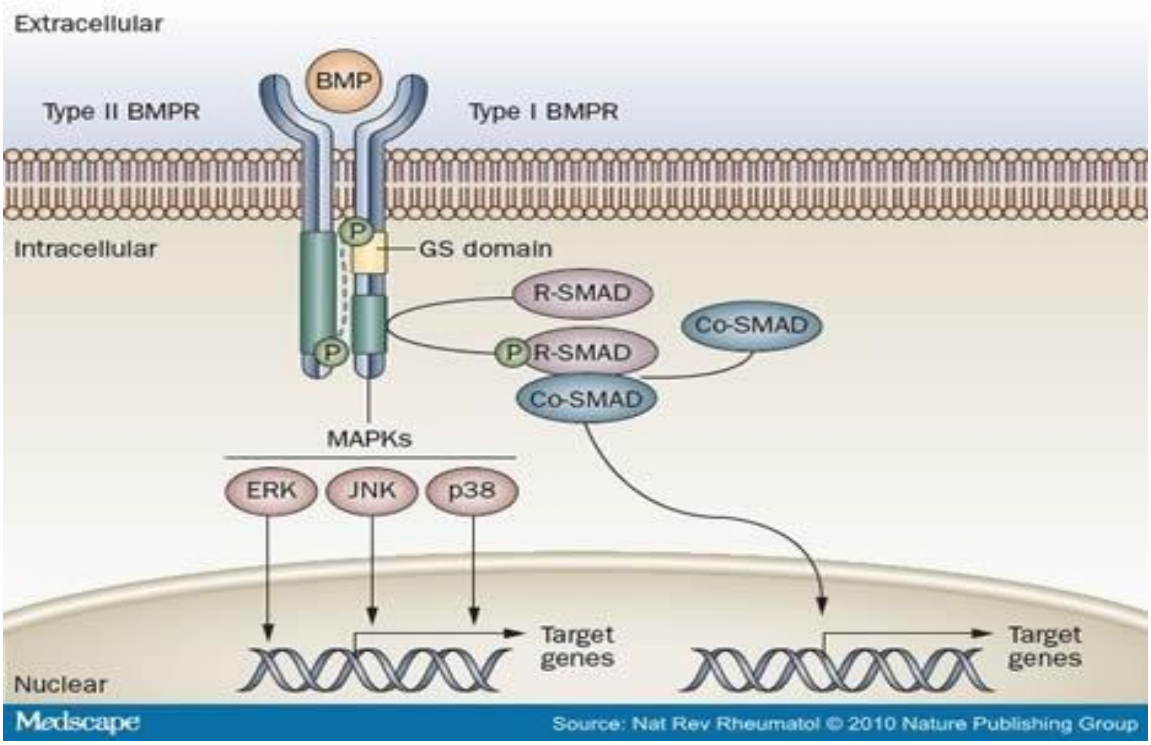


Figure 1.3. BMP Signalling Pathway.

Schematic representing BMP signal pathway. BMPs signal through mainly through BMPRI and BMPRII leading to recruitment of the Smad family of proteins. Smad proteins form a complex which tranverses the nuclear membrane and leads to transcription of target genes (Shore and Kaplan., 2010)

1.3.4 Morphogen signalling in peripheral B cell development

Recently, a small number of studies have suggested that Hh and BMP may be key mediators of peripheral B cell development. Sacedon et al (2005) labelled spleen tissue sections using both B220 and Shh to determine whether Shh proteins are expressed by B cells. Interestingly, after overlapping stained sections they found that germinal centre B cells themselves are not capable of expressing Hh proteins, however, follicular dendritic cells displayed a capacity for Hh expression. Furthermore, Hh protein expression was minimal within primary follicles suggesting that Hh may be upregulated upon BCR stimulation. Consequently, this implies that Hh may be essential in directing a B cell mediated immune response and, additionally, that Hh responsive cells do not necessarily express Hh. Moreover Huse et al, in 2011, noted that BMP abrogated IgA, IgG and IgM secretion in human peripheral B cells, however, the effects of BMP's were highly differential; BMP 6 abrogated plasma cell differentiation whilst BMP 7 inhibited Ig production significantly. In addition to this, Noggin, a BMP specific inhibitor, inhibited BMP 2, 4 and 7 reversing the effects previously observed. Interestingly, BMP 6 was found not to be inhibited by the addition of Noggin suggesting that the inhibitor may have widely varying affinities for the BMP family of proteins. Finally, BMP – 6 has been shown to inhibit proliferation in human naïve and memory B cells, and induce apoptosis in memory B cells alone by induction of Smad 1, 5 and 8 phosphorylation and upregulation of the inhibitor of differentiation 1 (ID1) protein (Kersten *et al*., 2005).

1.4. Aims

The paucity of research focussed on the role of morphogens in peripheral B-cell development and the simultaneous effects on B cell plasticity means that this area of research remains largely uncharacterised. Preliminary data from UEL (Umukoro et al,

unpublished) suggests that, following activation of B cells in culture using a stimulus that mimics encounter with antigen, at 18 hours post-stimulus, hedgehog provides a positive signal for differentiation and survival in B-cells with an increase in expression of surface markers associated with activation together with an increase in immunoglobulin secretion, however, this signal appears to be abrogated at 40 hours with a reduction in immunoglobulin secretion and an increase in cell death. It is hypothesised that this negative signal may be due to the upregulation of expression of the Hh target gene, BMP, thus, in addition to BMP being a target of hedgehog, BMP may well be a negative regulator of the B-cell immune response.

Specific aims

- To elucidate whether preliminary data (Ukumoro, 2012) suggesting that, an early survival signal at 18 hours post-stimulus delivered by Hh also provides a negative inhibitory signal at 40 hours post-stimulus, is affected by the addition of a BMP-specific inhibitor, Noggin. To characterise the B cell repertoire phenotypically in response to both morphogens thus revealing the role of Hh and BMP in skewing the direction of the B cell immune response.
- To determine whether the response of B cells to Hh signalling, observed within a mixed splenocyte population, are maintained in a purified B cell population thus indicating that these effects are intrinsic to B cells.

2. Methods and Materials

2.1. Animal Experimentation

Wild type BALB/c female and C57Bl/6 male mice were purchased from Harlan Laboratories UK, maintained at the animal facility in University House building located at the Stratford campus and allowed access to food and water *ad libitum* in line with the *animals (scientific procedures) act 1986.* Splenectomies were performed between 4 – 8 weeks old.

BMPR1α* KO** and ***BMPR1α* WT** mice were bred and maintained within the animal facility at the Institute of Child Health, University College London. BMPR1α floxed alleles from a transgenic mouse line and Cre recombinase, under the Vav promoter, from an additional transgenic line were crossed in order to ensure that the BMPR1α gene would be knocked out exclusively in haemopoietic cells. ***BMPR1α KO and ***BMPR1α*** mice were used in collaboration with Professor Tessa Crompton at ICH/UCL.

2.2. Cell culture

Following dissection, spleens were homogenised in 10ml AIM-V (Life Technologies), using frosted glass slides, to obtain a single cell suspension. Cells were seeded at a density of 2.5 – 3.5 x 10^6 per 200μls and cultured in AIM-V medium in 96 – well flat – bottomed treated plates at 37°C with 5% CO_2. 2-β-mercaptoethanol was added to AIM-V prior to cell culture to maximise lymphocyte growth and reduce reactive oxygen species. Activation stimuli anti-F(ab')2 IgM and anti-CD40 (ebioscience) were added at a final concentration of 5μg/μl to mimic an *in vivo* immune response. Recombinant Sonic Hedgehog Protein (ebioscience) was added to all wells excluding unstimulated, stimulated control and stimulated Noggin (Ng). Increasing

concentrations of rSHh were used: 0.005µg/ml and 0.05µg/ml to observe the effects of a concentration gradient on surrounding cells. Recombinant Noggin (ebioscience), a BMP inhibitor, was added at a final concentration of 10µg/ml.

The purpose of the activation stimuli is to mimic an *in vivo* immune response to antigen; stimulation of IgM mimics the signal delivered to the BCR by antigen and anti-CD40 mimics the signal provided by a helper T cell.

2.3 Isolation of B-cells using Negative Selection

B-cells were be isolated from the splenocyte population using the EasySep negative magnetic bead selection kit (Stemcell Technologies) and and subject to previous cell culture conditions in order to elucidate whether morphogen responsiveness is intrinsic to B-cells as opposed to alternate cells contained within the splenocyte population. Briefly, cells were centrifuged, supernatant removed and pellet resuspended in 2ml 2% Foetal Calf Serum (FCS)/Phosphate Buffered Solution (PBS). Cells were counted using a haemocytometer and, dependent on cell count, between 50-100µl of rat serum added. Following this, 50µl of a B cell isolation cocktail was added and incubated at 4C for 15 minutes. Biotin was added at 50µl/ml and incubated again for 15 minutes at 4C. 100µl of magnetic beads were added after homogenisation and incubated for 5 minutes at room temperature. Finally, tubes containing samples were inserted into the Easysep magnet and incubated at room temperature for 5 minutes, after which, cells were poured off into a new sterile tube. Cells were counted and resuspended to yield a final cell count of $3.5x10^6$/ml. B cell purity ranged between 93% and 99% when assessed by flow cytometry.

2.4 Analysis of cell surface markers using Flow Cytometry

At 18 and 40 hours post-stimulus cells were stained using fluorescence-conjugated antibodies to specific cell markers and analysed using flow cytometry in

order to determine cells types and the status of cell survival. Briefly, FC receptors were blocked using anti CD16/32, prior to staining, to prevent non-specific binding. 10ul of CD16/32 was added to 190µl PBS supplemented with FCS at a final concentration of 2%. Cells were incubated on ice for 10 minutes. Staining using fluorescence-conjugated antibodies to B220, CD23, CD25, CD80, CD138, IgD, IgM and Annexin-V followed this. Antibodies were diluted 1:100 using 2% FCS/PBS, and incubated on ice for 60 minutes, as this had previously been optimised and considered the most efficient staining protocol. Mixed splenocytes were gated on B220 to ensure the population majority consisted of B-cells. All antibodies were purchased from eBioscience excluding CD138 which was purchased from BD Pharmingen.

Figure 1.1 Table of CD Markers.

CD Marker	**Function**	**Antibody**	**Supplier**
B220	Murine CD45 receptor	Tri-colour	Ebioscience
CD23	Low affinity IgE receptor. Expressed on naïve and activated B cells	FITC	Ebioscience
CD25	IL-2α receptor component. Expressed on mature, differentiating B cells	PE	Ebioscience
CD80	Co-stimulatory ligand expressed on germinal centre and memory B cells.	APC	Ebioscience
CD138/Synedan 1	Adhesion molecule expressed on plasmablasts.	APC	BD Pharmingen
IgD	Immunoglobulin expressed on immature and naïve B cells	FITC	Ebioscience
IgM	Immunoglobulin differentially expressed on immature and mature B cells	PE	Ebioscience
CD23	As before	Tri – colour	Ebioscience
Annexin V	Binds to phosphotidylserine on cells undergoing apoptosis	FITC	Ebioscience

2.5 Enzyme Linked Immunosorbent Assay (ELISA)

Using supernatants from the cells at both 18 and 40 hours, an IL-10 ready-SET-go ELISA (eBioscience) was carried out in order to detect the secretion of IL-10, thus indicating which subset of B-cells had developed under the influence of Hh and BMP signalling. Briefly, 40µl of capture antibody was added to 10mls 1X coating buffer and 100µls pipetted into each well of a Corning Costar ELISA plate. Plates were sealed and incubated overnight at 4°C. After 24 hours, wells were aspirated and washed four times using 250µls ELISA wash buffer (1XPBS + 250µl 20% Tween) in each well. Plates were left for 1 minute between each wash. Following this, wells were blocked using 200µls of a 1:5 dilution of ELISA diluent, sealed and incubated for 1 hour at room temperature. Whilst plates were incubating, 20µls of IL-10 standard was added to 5mls diluent to make a stock solution of 4ng/ml. A series of standards were prepared by adding 400µls diluent each to 4 eppendorfs then 400µls of stock solution added to the first Eppendorf. Subsequent serial dilutions ensued in order to create a calibration curve. ***BMPR1α KO*** and ***BMPR1α WT*** samples were diluted 1:10 to allow for accurate extrapolation of data. 100µls of each standard or sample were added to wells in triplicate, sealed and incubated overnight at 4°C. Finally, wells were washed 5 times using ELISA wash, 100µls of detection antibody added to wells and incubated at room temperature for 1 hour. After washing five times, 100µls HRP was added and incubated at room temperature for 30 minutes. Washing was performed seven times with 1-2 minutes between each wash. 100µls of 1X TMB solution added to wells and incubated for 15 minutes at room temperature. 50µls of 1M H_2SO_4 was utilised as stop solution and absorbance measured at both 450nM and 540nM.

2.6 Gene Expression Analysis

2.6.1 RNA Isolation and cDNA Synthesis

RNA was isolated from B cells using an RNA isolation kit (Ambion) with the addition of 2-βME to lysis buffer, as stated in the protocol provided. Briefly, cells were centrifuged for 10 minutes at 12,000g at supernatant removed and stored at 4°C for later ELISA analysis. Pellets were re-suspended in 300µls of lysis buffer and vortexed until pellets were homogenised. RNA was isolated from cells using an RNA isolation kit with an additional DNase1 stage to remove genomic DNA. RNA was stored at 80°C. cDNA was generated using the iScript cDNA Synthesis kit (Biorad) consisting of 400ng RNA, 4µls iScript reaction mix, 1µl reverse transcriptase and the remaining volume, up to a total of 20µls, nuclease-free water. Thermocycler was programmed at 42°C for 65 minutes and 85°C for 5 minutes. cDNA was used instantly or stored at 4°C overnight.

2.6.2 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RT-PCR was performed using cDNA generated from ***BMPR1α*** KO and ***BMPR1α*** WT mice. Embryo head was used as a positive control as it is known to express high levels of morphogens and the housekeeping gene, HPRT. A standard curve using HPRT and embryo head cDNA was created in order to quantify expression levels. Briefly, a series of 1 in 10 dilutions of embryo head cDNA were performed in order to create a standard curve using HPRT and BMP 2 primers. Following this, cDNA from ***BMPR1α KO*** and ***WT*** mice was diluted 1 in 50 to allow for efficient extrapolation of data from the standard curve generated. iTaq Universal SYBR Green Supermix (Biorad) containing DNA polymerase, dNTPs, $MgCl_2$ and SYBR green dye was used at a 2X dilution. A total reaction volume of 20µls with 10µls of SYBR Green, 0.4µls exon-exon spanning primers to eliminate genomic DNA, 2µls cDNA and 7.6µls nuclease-free H_2O. Primer concentrations had previously been optimised and a 1:50 dilution was deemed the optimal to prevent primer-dimer formation. Dissociation

curves were created and analysed at each step to ensure primers anneal at specific temperature and time. All primers were Qiagen quantitect and primer sequences are protected.

2.7 Statistical Data Analysis

Prior to inferential statistical tests, all data was tested for normality using the Shapiro Wilks and Kolmogornov-Smirnov tests. The unpaired Student's T-Test was utilised to deduce whether differences between groups were statistically significant. Data was considered statistically significant at the following values: $p=<0.05$, $p=<0.01$ and $p=<0.001$.

3. Results

3.1 The role of Hh and BMP signalling within a heterogeneous splenic population

In order to elucidate whether Hh signalling influences B cell development, cells isolated from BALB/c female mice were first stimulated with anti-IgM and anti-CD40 to recapitulate antigen presence by BCR cross-linkage and co-stimulation mimicking T-cell help, and additionally, in the presence and absence of rShh. Initially, B cells were analysed from within a heterogeneous splenic cell population w and the gating strategy used is shown in figure 3.1.

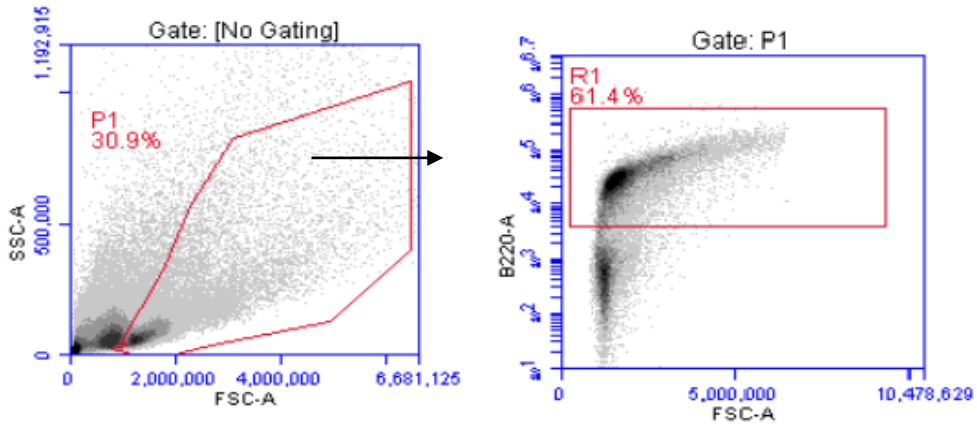
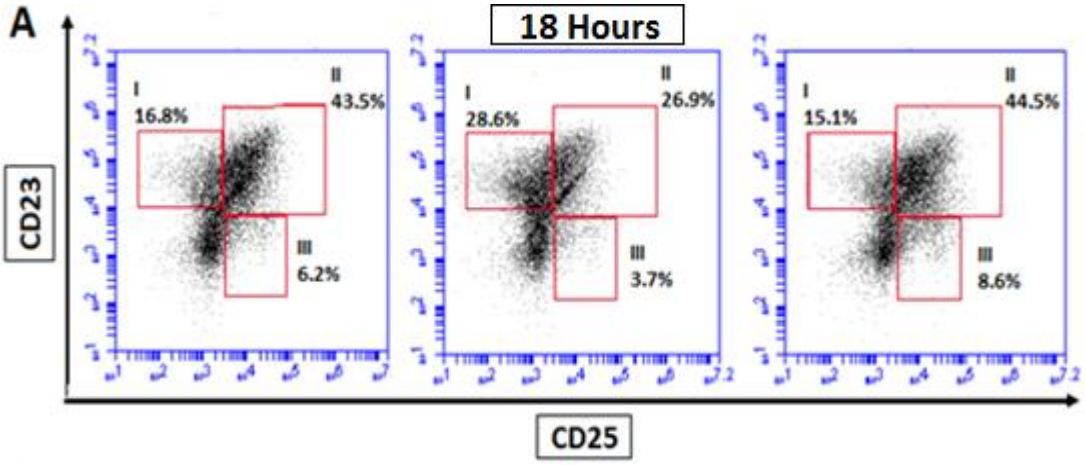


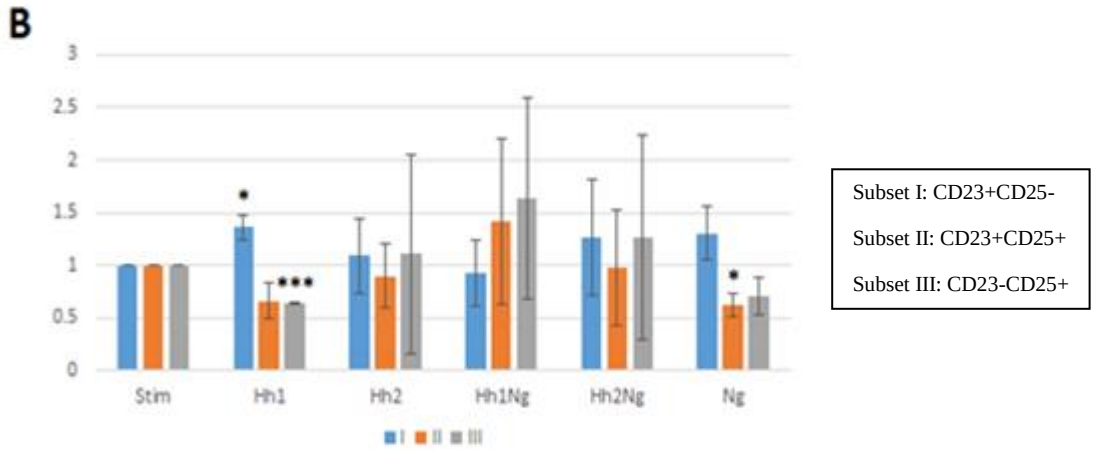
Figure 3.1. Gating strategy for B220+ B cells.

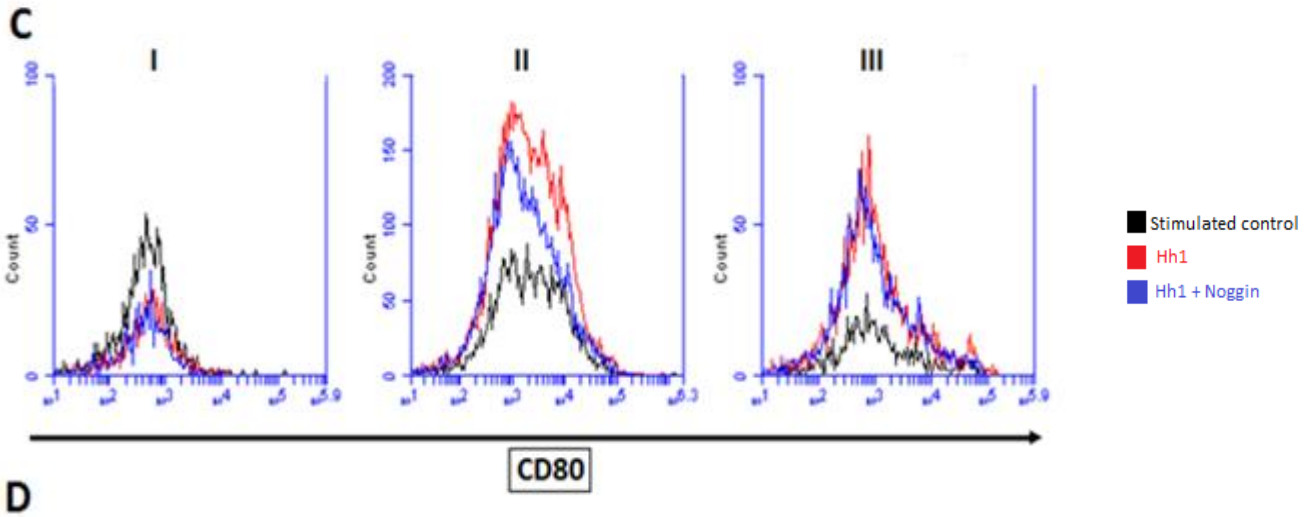
Splenic cells from BALB/c female mice were first gated using forward and side scatter to isolate live cells then gated on B220+ cells to isolate B

The Developmental status of the gated B cells was then assessed by analysing expression of the activation marker CD23, differentiation marker CD25 and Annexin V for survival. A significant increase in activation, shown in figure 3.2A, was observed at 18 hours as judged by an increase in the presence of the CD23+CD25 subset in the presence of 0.005µg/ml rShh ($p=0.04$) as previously described by Umukoro et al typically by 16.8% to 28.6%.

3.2


A
18 Hours
CD23
CD25
I
II
III
16.8%
43.5%
6.2%
28.6%
26.9%
3.7%
15.1%
44.5%
8.6%


B
Stim
Hh1
Hh2
Hh1Ng
Hh2Ng
Ng
I II III
Subset I: CD23+CD25-
Subset II: CD23+CD25+
Subset III: CD23-CD25+


C
I
II
III
Count
CD80
Stimulated control
Hh1
Hh1 + Noggin

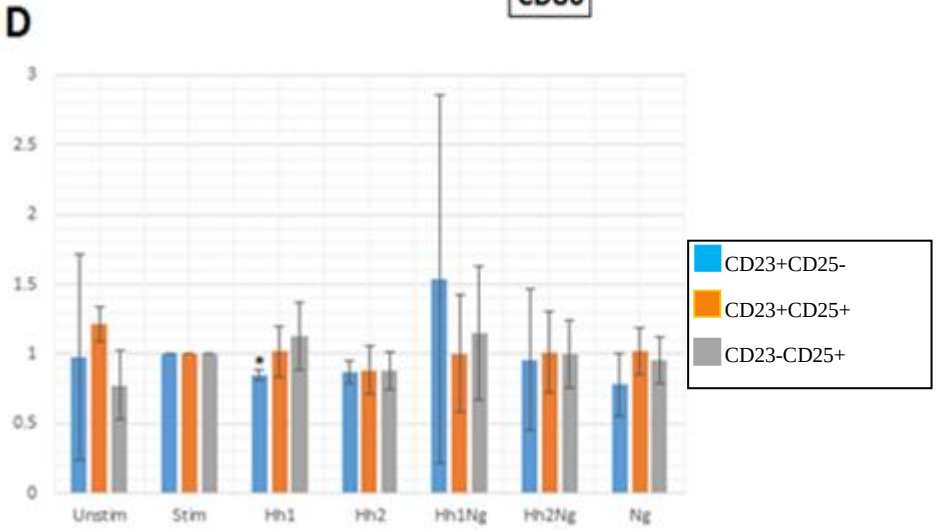

D
Unstim
Stim
Hh1
Hh2
Hh1Ng
Hh2Ng
Ng
CD23+CD25-
CD23+CD25+
CD23-CD25+

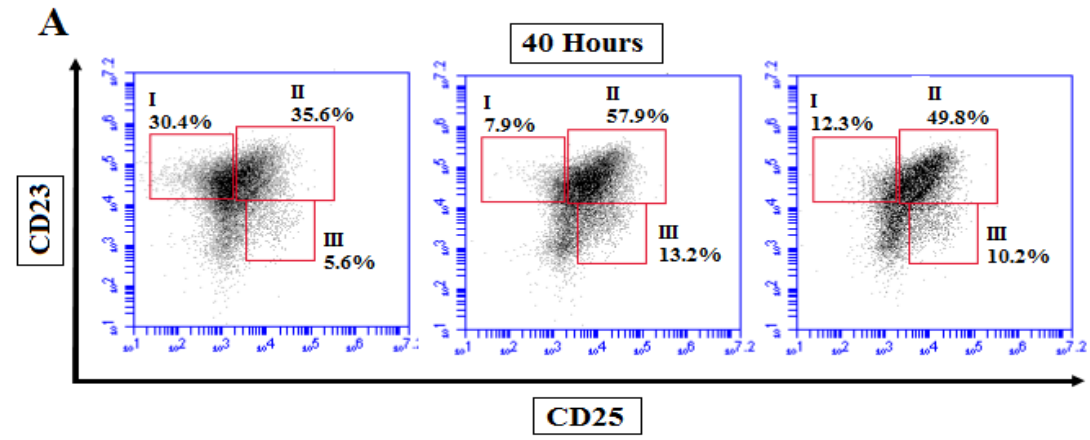
Figure 3.2. CD23 and CD25 at 18 hours within a mixed cell population.

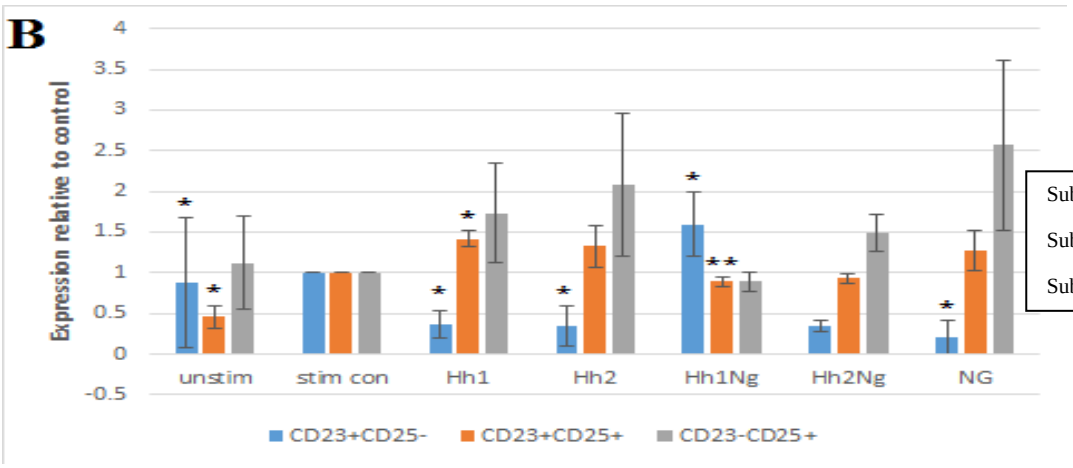
Splenocytes from BALB/c female mice were stimulated with anti-IgM and anti-CD40, and co-cultured for 18 hours in the presence or absence of rShh and Noggin. Hh1=0.005μg/ml and Hh2=0.05μg/ml. Scale representative of expression normalised to stimulated control. Error bars represent +/- SD. N=3. **A)** Dot plots showing cells stained for CD23 and CD25. Left = stimulated control, mid = Hh1 and right = Hh1 + Noggin. **B)** Bar chart depicting CD23 and CD25 expression relative to control. **C)** Histograms depicting CD80 expression within subsets gated as I) CD23+CD25- II) CD23+CD25+ III) CD23-CD25+. **D)** Bar chart showing levels of CD80 within previously defined subsets.

This enhanced activation could be abrogated by the addition of a BMP inhibitor, Noggin, suggesting that both Hh and BMP signalling are required to take place for these early events in B cell activation and that the synergy requires BMP signalling to take place downstream in order to mediate its effects. In addition, expression of CD25 was reduced significantly at 18 hours in the presence of 0.005μg/ml rShh ($p = < 0.001$) (Figure 3.2B) and upregulated in the presence of Noggin suggesting that Hh signalling may act to enhance activation but prevent further differentiation and this would appear to require the presence of BMP signalling to take place. However, expression levels of CD25 are low at this time point (Amu *et al*., 2010), thus, it may be too early to observe draw any real conclusions about differentiation. Co-culture of B cells with the higher dose of rShh, 0.05 μg/ml decreased expression of the co-stimulatory marker, CD80 in all subsets, all results were approaching significance (figure 3.2C) The addition of Noggin to the same samples did not significantly recover development although expression of CD80 was increased in all. Interestingly, in cells cultured with the lower dose of rShh, 0.005μg/ml there was a significant decrease in CD80 expression in the CD23+CD25- population ($p=<0.05$) whilst a simultaneous increase in the expression of CD80 was observed in the CD23-CD25+ subset (Figure 3.2D). The addition of Noggin to the cultures containing 0.005μg/ml rShh did not affect the numbers of a CD23-CD25+CD80+ population whilst the presence of the CD23+CD25- CD80+ population greatly increased although variability within samples for this particular subset was high.

CD80 would not normally be expected to be expressed at this early stage of the immune response and we suggest this is caused by aberrant signalling due to the inhibition of BMP signalling. This aberrant signalling may then skew the development of B cells by upregulating the expression of CD80 prematurely in the immune response thereby inducing the emergence of a memory B cell population. This data suggest that both Hh and BMP signalling play a role in the early activation of B cells in response to a TD antigenic stimulus.

3.3

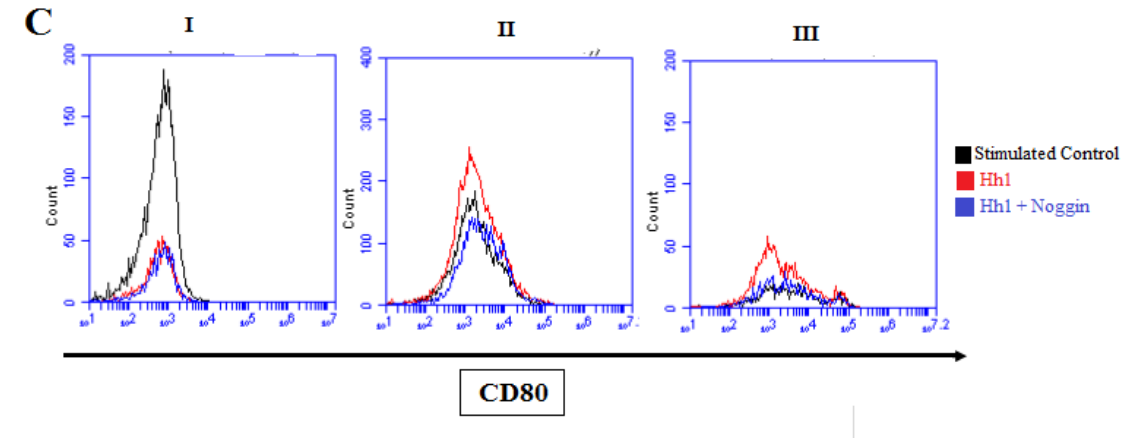


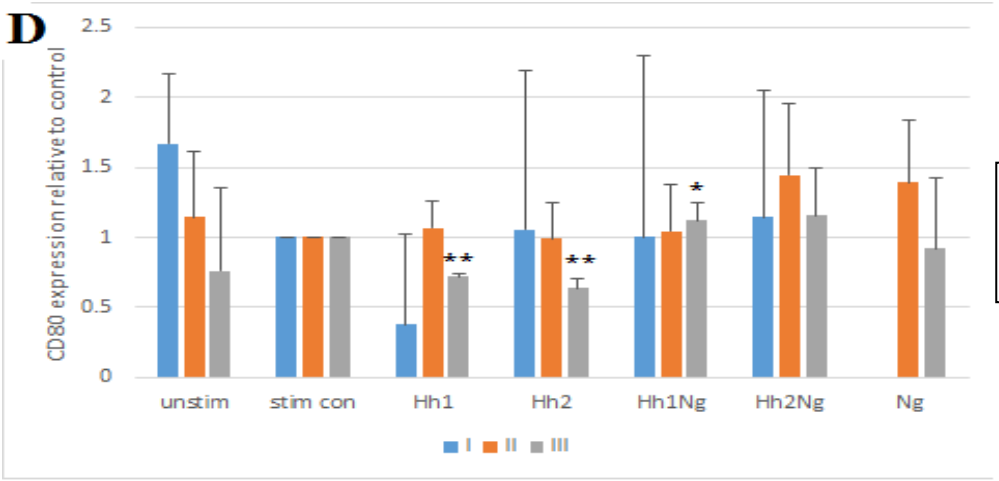


Subset I: CD23+CD25-

Subset II: CD23+CD25+

Subset III: CD23-CD25+





Subset I: CD23+CD25-

Subset II: CD23+CD25+

Subset III: CD23-CD25+

Figure 3.3. Expression of CD23 and CD25 at 40 hours within a mixed cell population.

Splenocytes from Balb/c female mice were stimulated with anti-IgM and anti-CD40, and co-cultured for 40 hours in the presence or absence of rShh and Noggin. Increasing doses of rShh were used: Hh1 = 0.005ug/ul and Hh2 0.05ug/ul. Scale representative of expression normalised to the stimulated control. No rShh was added to wells labelled unstim, stim and Ng. Error bars represent +/- standard deviation (SD). N=3. **A)** Dot plots represent cells stained for CD23 and CD25 relative to control. Left = Stimulated control, Mid = Hh1 and Right = Hh1+Noggin. **B)** Bar chart depicting expression of CD23 and CD25. **C)** Cells were stained for CD80 at 40 hours, analysed by flow cytometry and expression depicted as histograms. CD80 was analysed within three subsets gated as I) CD23+CD25- II) CD23+CD25+ III) CD23-CD25+. **D)** Bar chart showing levels of CD80 within previously defined subsets.

At 40 hours subset II (CD23+CD25+) increased significantly (p=0.05) and III (CD23-CD25+) increased and was nearing significance, in the presence of 0.005µg/ml rShh, from 35.6% to 57.9% and 5.6% to 13.2%, respectively (figure 3.3A and figure 3.3B). Identical effects were seen in cells co-cultured with 0.05µg/ml rShh, however, only the CD23+, subset I population, was of any significance (p=0.05). The addition of Noggin led to a significant decrease (p=0.01) in expression of CD25 in terms of subset II whilst significantly increasing CD23 expression in subset I (p=0.05) (figure 3.3B). These results indicate that Hh and BMP signalling are required for B cells in order to increase their maturation status and consequently, reduce the levels of activated effector cells. Surprisingly, CD80 expression, at 40 hours, decreased in CD23-CD25+ subsets (III) in cultures containing 0.005µg/ml and 0.05µg/ml rShh and this was highly significant at p=0.01 (figure 3.3C and figure 3.3D). Although a small increase was observed in our CD23+CD25+ subset (no significance), in the presence of 0.005µg/ml rShh, and CD23+CD25- in samples containing 0.05µg/ml rShh (no significance), variability between samples was high and may provide a possible reason for skewed results. As CD80 is associated with a more mature B cell phenotype, primarily memory cells, we expected levels of CD80 to increase particularly within a CD23-CD25+ subset.

Analysis of induction of apoptosis in these B cells following 18 hours in culture revealed that the presence of rShh in cultures had little effect on the induction of apoptosis. However, in the presence of Noggin, there was an apparent decrease in levels of Annexin-V staining indicating a pro-survival effect being mediated by the presence of Noggin. This would suggest that BMP signalling is inducing apoptosis in B cells early in the activation response, a finding that is consistent with previously published data whilst Hh signalling plays little role in the induction and maintenance of survival in at this early point in the immune response

Consistent with data obtained by Umukoro et al, we observed that levels of apoptosis increased, at 40 hours, in B cells treated with rShh as compared to a stimulated control (Figure 3.4A and Figure 3.4B) and this level of apoptosis decreases with the addition of Noggin. This data suggests that Hh signalling does play a role in inducing apoptosis of B cells later in the immune response and that this effect is mediated by downstream BMP signalling. The fact that Hh signalling may induce apoptosis in B cells is a novel finding, and is not consistent with previously published data suggesting that Hh signalling delivers a pro-survival signal (Sacedon *et al*., 2005). However we believe that this finding reflects a role for Hh signalling later in the immune response that has not previously been investigated. This is consistent with previous reports that indicate that BMP signalling may be pro-apoptotic and suggests that the later pro—apoptotic events induced by Hh signalling are in fact attributable to BMP signalling.

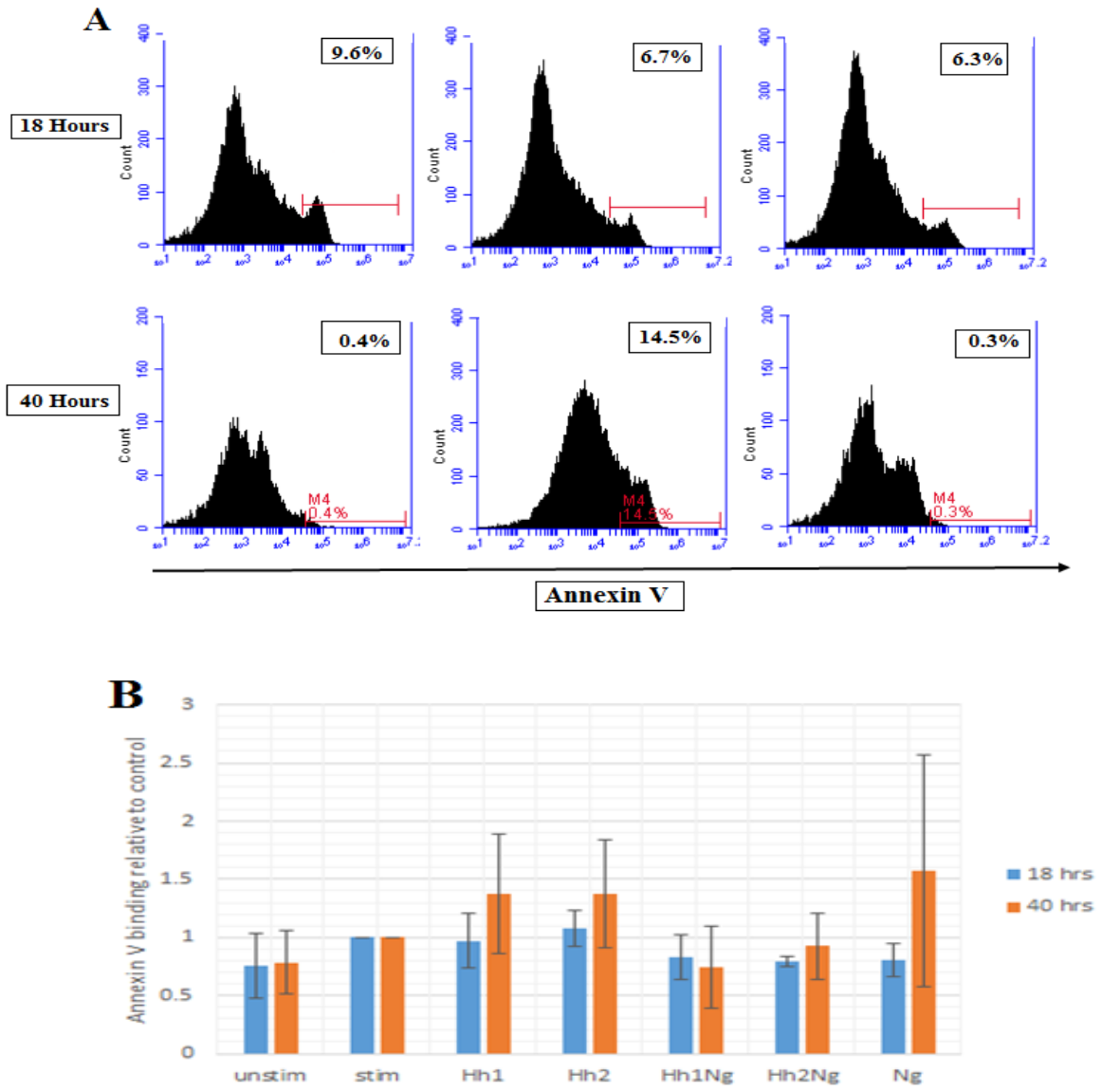


Figure 3.4. Annexin V binding at 18 and 40 hours.

A) Histograms representing Annexin V binding levels, at 18 and 40 hours post stimulus, in BALB/c female mice. Left=Stimulated control, mid=Hh1 and right=Hh1+Noggin. **B)** Bar chart depicting levels of Annexin V binding at 18 and 40 hours. Data represents three experiments and error bars represent +/- SD. Hh1 = 0.005µg/ml and Hh2 = 0.05µg/ml.

Surprisingly, when analysing expression of a marker of early plasma cell differentiation (plasmablasts), CD138, we could not detect expression in any B-cell subset (data not shown). Interestingly, a previous study noted that CD138 was expressed to a greater level in B220 negative cells and therefore we may not have observed it in our study due to the use of the expression of B220 to gate our B cell population. Furthermore, problems may have been encountered due to poorly optimised staining concentrations, alternatively, culture times were not prolonged enough to see the appearance of plasmablasts in our cultures.

3.2 The effects mediated by Hh and BMP signalling are intrinsic to B cells

To determine whether observations seen earlier when analysing mixed cell cultures are a result of B cell intrinsic responses, splenic B cells were isolated using magnetic bead enrichment techniques and purified B cells were subjected to identical culture conditions as for previous experiments. C57BL/6 male mice were utilised as our future studies using ***BMPR1a*** KO mice were on a C57BL/6 male background. Interestingly, figure 3.5B shows that there was a generally higher expression of CD25 observed in B cells isolated from this strain and gender of mouse at this early time point in B cell activation. Thus, the presence of a CD23+ CD25+ population was significantly increased ($p = <0.05$) when compared to the control, in samples cultured for 18 hours with 0.005µg/ml Hh (Figure 3.5A and 3.5B). The large presence of this CD23+CD25+ subset was somewhat unexpected in an early immune response. However, we suggest that this subset represents a heterogeneous population of B cells which display increased plasticity and ability to form a Follicular I and II cells, and T2-MZP cells and the enhanced appearance of this population may be a characteristic of the male immune response which is not seen to any great degree in the female immune response at this time-point. Alternatively, the presence of CD25+CD23- cells, which may represent a

mature memory cell population, decreased significantly at 18 hours in the presence of 0.05μg/ml of rShh ($p = < 0.05$) whilst the addition of Noggin led to a 2.4 fold increase ($p = < 0.001$) in the presence of this CD23-CD25+ subset. Remarkably, there appeared to be no difference in the relative expression of CD25+ CD23- cells co-cultured with Noggin (no significance) and samples containing 0.005μg/ml rShh plus Noggin when compared to cells cultured with 0.005μg/ml rShh exclusively (figure 3.5B) This data may suggest that BMP signalling plays a more dominant role in driving the emergence of the CD23-CD25+ subset which we believe to be a memory type B cell whilst Hh and BMP signalling act synergistically to modulate the newly activated CD23+CD25- B cells. Furthermore, this is consistent with studies suggesting that BMP signalling induces the inhibitor of differentiation family of proteins (ID) mediated by the Smad transcription factors resulting in a loss of the possibly more mature CD23-CD25+ B cells (Kersten *et al*., 2005).

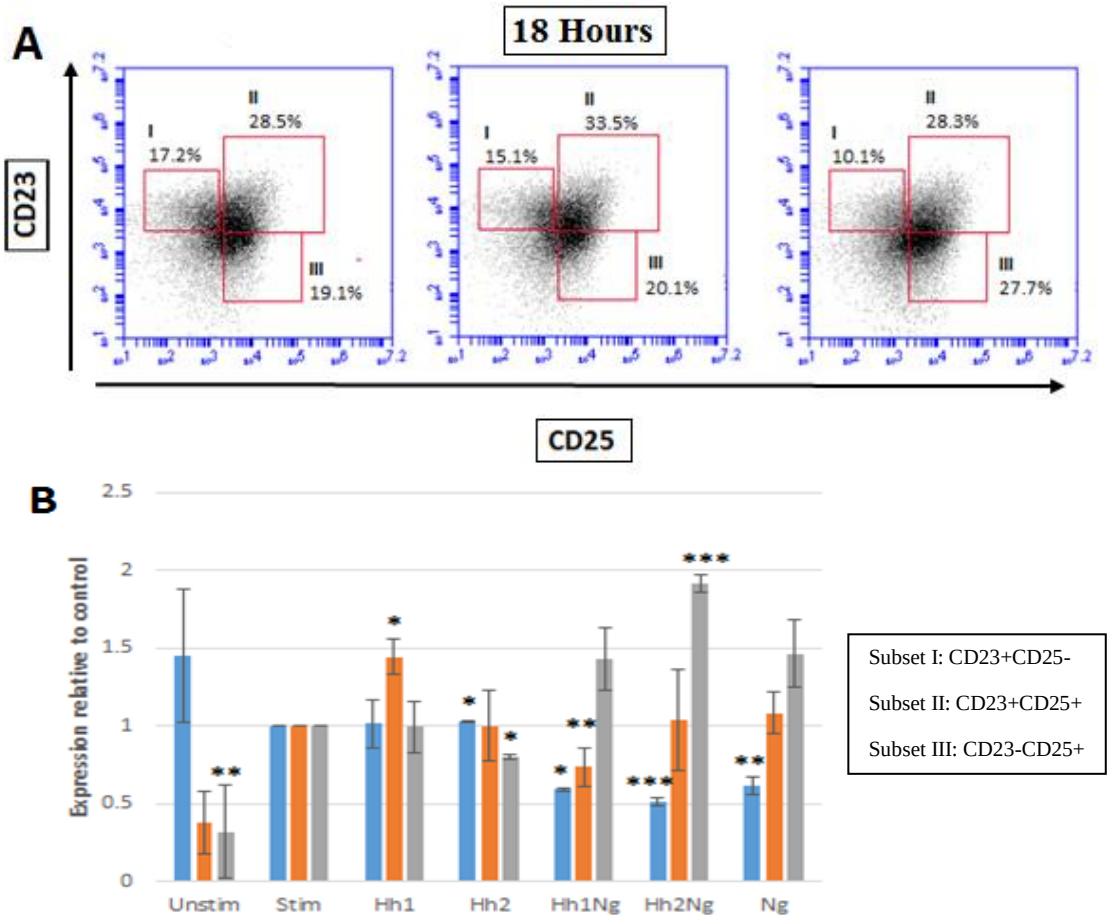


Figure 3.5. Expression of CD23 and CD25 at 18 hours in purified B cells.

B cells purified from the spleens of C57BL/6 male mice, stimulated as before, were co-cultured with rShh at varying doses. Hh1=0.005µg/ml and Hh2=0.05µg/ml. Cells were stained for CD23 and CD25 at 18 hours post stimulus. **A)** Dots plots representing changes in expression of markers. From left to right dot plots represent: stimulated control, Hh1 and Hh1 + Noggin. **B)** Bar chart depicting expression levels of CD23 and CD25. Scale represents expression normalised to stimulated control. Error bars represent +/-. N=3.

Furthermore, we show that Hh signalling reduced levels of expression of the co-stimulatory marker CD80 on all B cell subsets at 18 hours in the presence of 0.005µg/ml rShh (Figure 3.6A), particularly in subsets expressing CD23, and this expression was restored and upregulated in all subsets in the presence of Noggin, this data is consistent with the data obtained using the higher dose of rShh in mixed cultures and may reflect the fact that cells, other than B cells, have mopped up some of the rShh protein thereby

lowering the dose seen by the B cells. However, in the presence of 0.05 μg/ml rShh, CD80 expression was only lowered in subset I but this expression was apparently restored in the presence of noggin although these data are not yet significant but approaching significance. Thus, we show that B cell phenotypes vary in the presence of different doses of rShh, a characteristic that is typical of a morphogen and this may represent the natural events, in vivo, with the spatial arrangement of cells located at a particular place within a morphogen gradient thereby receiving differing dose of that morphogen.

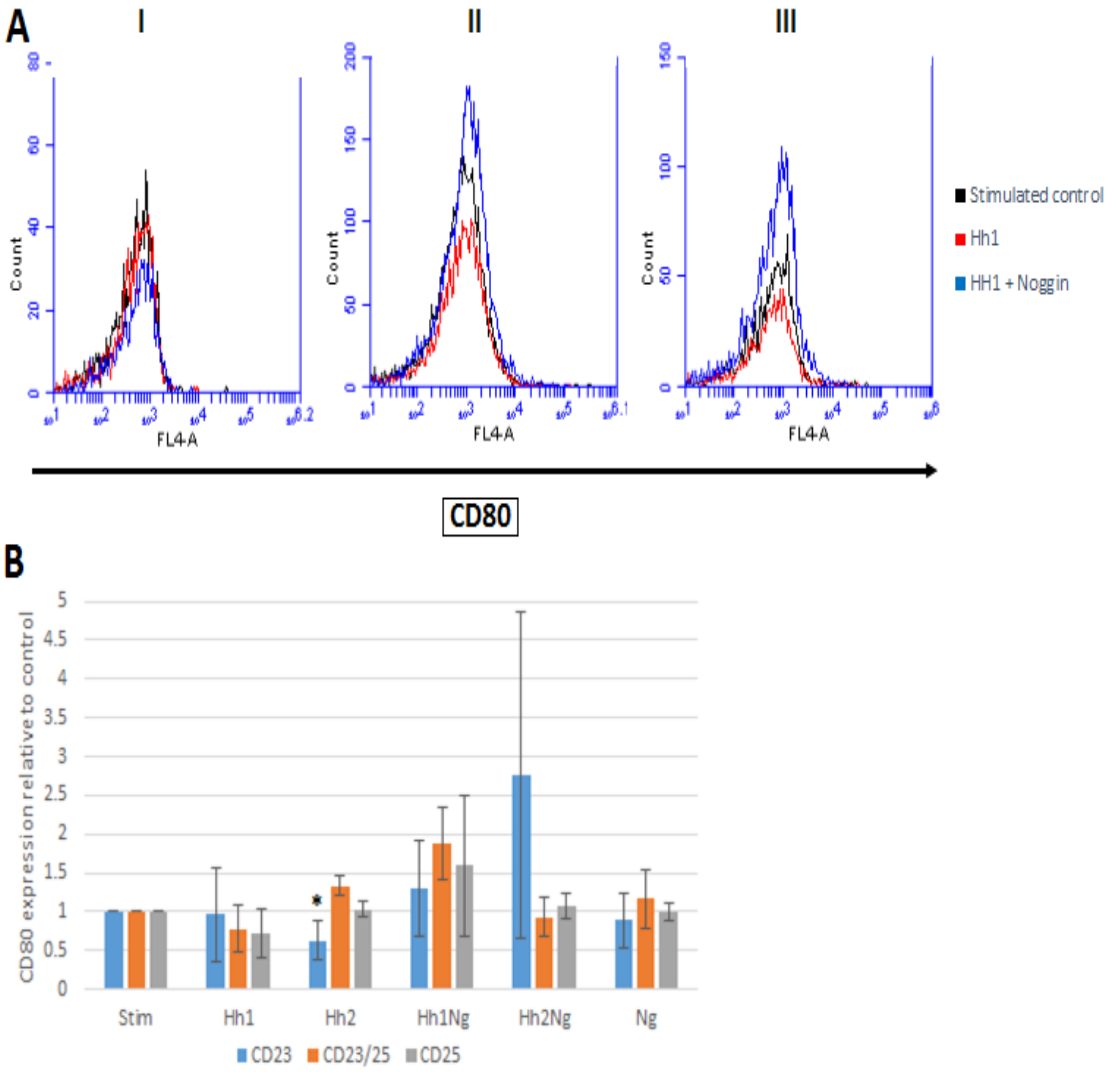


Figure 3.6. CD80 expression in C57BL/6 male B cells.

A) Histograms represent levels of CD80 expression in subsets: I) CD23+CD25- II) CD23+CD25+ III) CD23-CD25+, as compared to the stimulated control, 18 hours post stimulus. **B)** Bar chart depicting relative expression of CD80 in noted subset. Cells cultured in varying doses of rShh: Hh1=0.005µg/ml and Hh2=0.05µg/ml. Scale represents expression normalised to control. Error bar represents +/- SD. N=3.

3.3 BMP signalling may drive the development of a Follicular B cell subset

For an effective humoral immune response to occur, B cells must undergo a maturation process from T2 cells to FOI in order to develop specialised germinal centres in preparation for affinity maturation and memory formation. It is important therefore to identify how Hh and BMP signalling may influence this developmental process. In order to identify these particular subsets, cells were stained for markers for which the expression levels alter with increasing maturity. The markers selected for use were IgD, IgM and CD23 and their differential expression is indicated below.

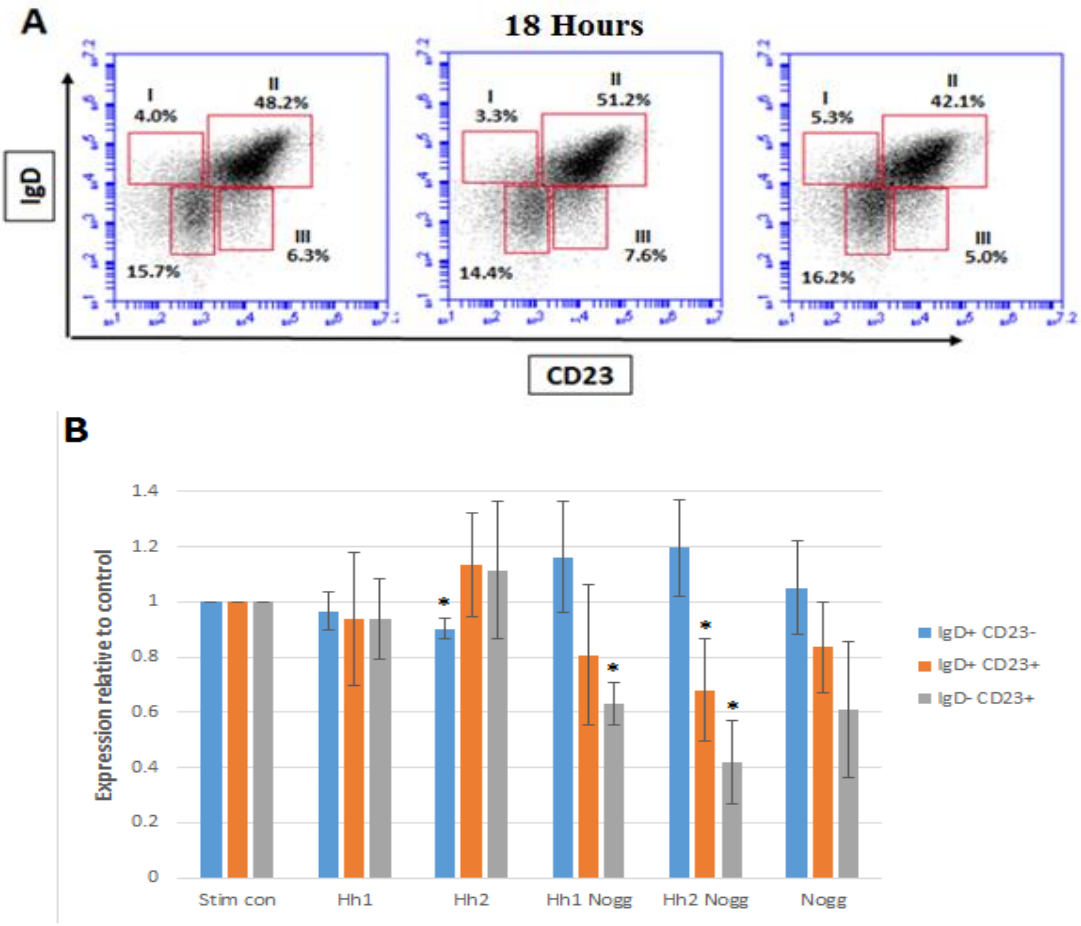


Figure 3.7. IgD and CD23 expression at 18 hours.

A) Flow cytometry dot plots depicting changes in IgD and CD23 expression in response to Hh and Hh+Noggin presence. From left to right: Left = stimulated control, Mid = Hh2 and Right = Hh2 + Noggin. Hh1=0.005µg/ml and Hh2=0.05µg/ml. Subsets are I) IgD+CD23- II) IgD+CD23+ III) IgD-CD23+ **B)** Bar chart depicting expression of IgD and CD23 at 18 hours post stimulus. B cells isolated for C57BL/6 male mice. Scale represents expression normalised to

In order to determine which B cell subsets are emerging in response to the presence of rShh and Noggin, we analysed expression of IgD and CD23 and once again determined the presence of three distinct subsets of cells developing following 18 hours in culture. These are subset I: IgD+CD23-, subset II: IgD++CD23+ and subset III: IgD-CD23+. Whilst still controversial, it is generally felt that IgD and CD23 expression increases as IgM expression decreases on a splenic B cell in response to antigen, with IgD and CD23 expression ultimately being switched off in the more mature class switched memory B cell. The data shown in Figure 3.7 indicates that in the presence of 0.005μg/ml rShh there is an increase in the presence of the more mature subsets II and III at the expense of subset I and this trend is reversed in the presence of 0.005μg/ml rShh together with Noggin.

We then went on to look at the expression of IgM in all three subsets. At 18 hours post stimulus Hh signalling appears to drive down the expression of IgM in subsets I and II (Figure 3.8A) in the presence of 0.005μg/ml rShh, (figure 3.8A). Conversely, the presence of noggin in these cultures caused an upregulation of expression of IgM in all three subsets although the data was not yet significant. The significance of these findings remains to be fully understood and may just represent an anomaly caused by the fact that the B cells are themselves cultured in the presence of anti-IgM in order to become activated, and therefore the presence of IgM in the cultures may have affected the subsequent patterns of IgM staining.

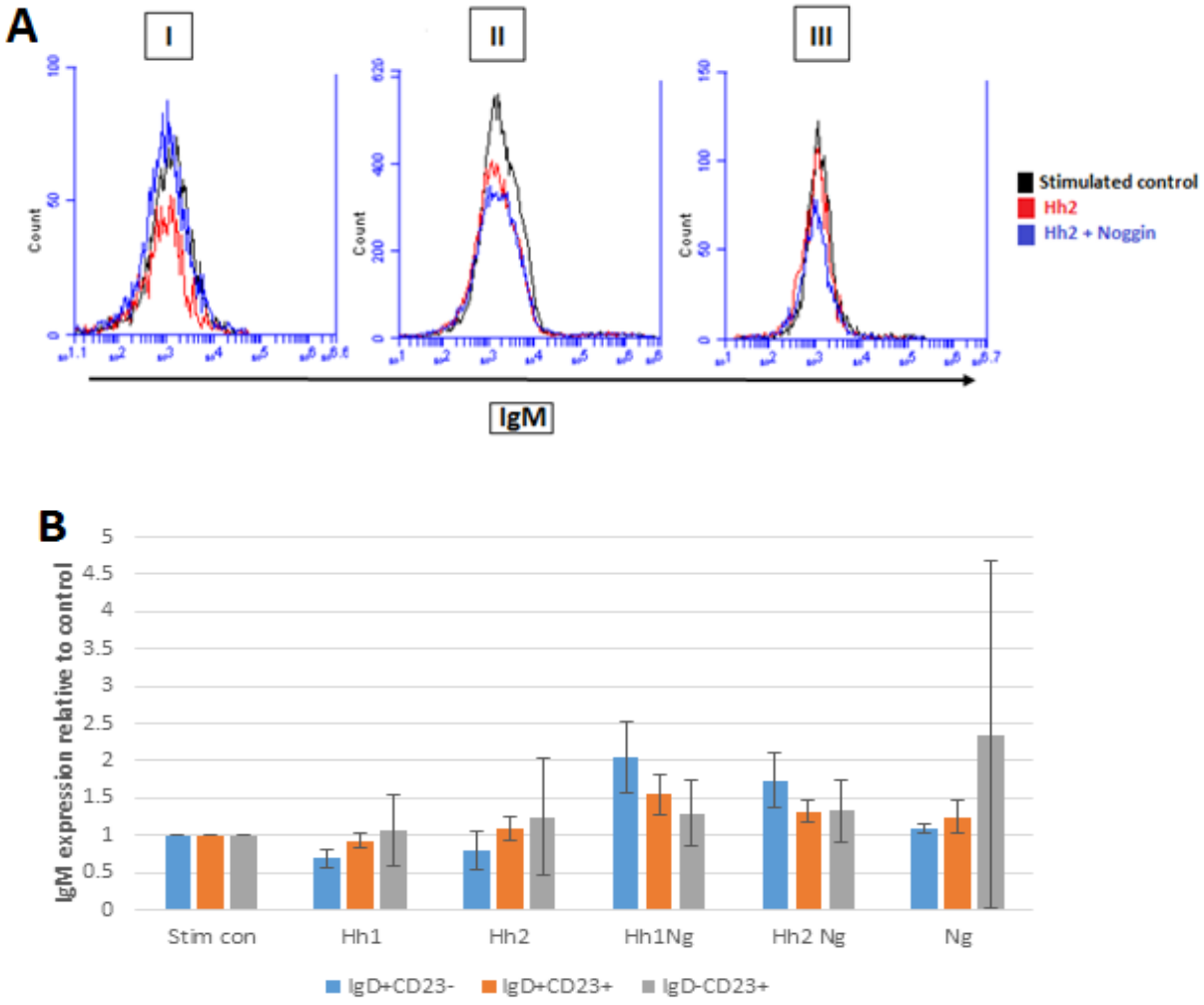


Figure 3.8. IgM expression at 18 hours.

A) Histogram showing expression of IgM within subsets, I) IgD+CD23- II) IgD+ CD23+ III) IgD-CD23+, at 18 hours post stimulus. Cells co-cultured with varying doses of rShh: Hh1=0.005ug/ml and Hh2=0.05ug/ul. B cells isolated from C57BL/6 male mice. **B)** Bar chart depicting relative expression of IgM in subsets. Scale represents expression normalised to the stimulated control. Error bars represents +/- SD. N=3.

Pillai and Cariappa (2009) suggest that the development of an IgD^{mid} IgM^{low} $CD23^{high}$ FOI subset from a T2 cell (IgM^{high} IgD^{high} $CD23^{mid}$) is dependent on a strong BCR-BTK stimulus and therefore it is important to analyse the effect of Hh and BMP synergy on the strength of BCR-BTK signalling. Our results suggest that Hh and, to a larger extent, BMP signalling regulate early BCR-dependent development of B cells.

3.4 Dampening down of the immune response is a result of BMP expression.

To elucidate whether the synergy observed between Hh and BMP signalling at 18 hours post-stimulus also influenced the status of the immune response at 40 hours, expression of these same cell surface markers were analysed at 40 hours to determine any differences in expression.

We observed, that at 40 hours, 0.005μg/ml rShh increases the presence of the IgD+CD23+ subset significantly ($p=<0.005$) and this decreased in the presence of Noggin. These results were largely unexpected; during a later immune response we would expect levels of IgD and CD23 to decrease as expression of both markers have been shown to be inversely proportional to numbers of mature cells such as plasma and memory B cells. Both markers are associated with immature or early activated B cells. However, as can be seen in figure 3.9B, data varied greatly from the mean and may provide a possible explanation for unusual results. Furthermore, figure 3.10 shows cells stained for IgM displayed similar variability and non-significant data. Nonetheless, a clear trend can be seen, as with 18 hours data, in that the addition of Noggin to samples containing rShh led to a large increase in IgM expression.

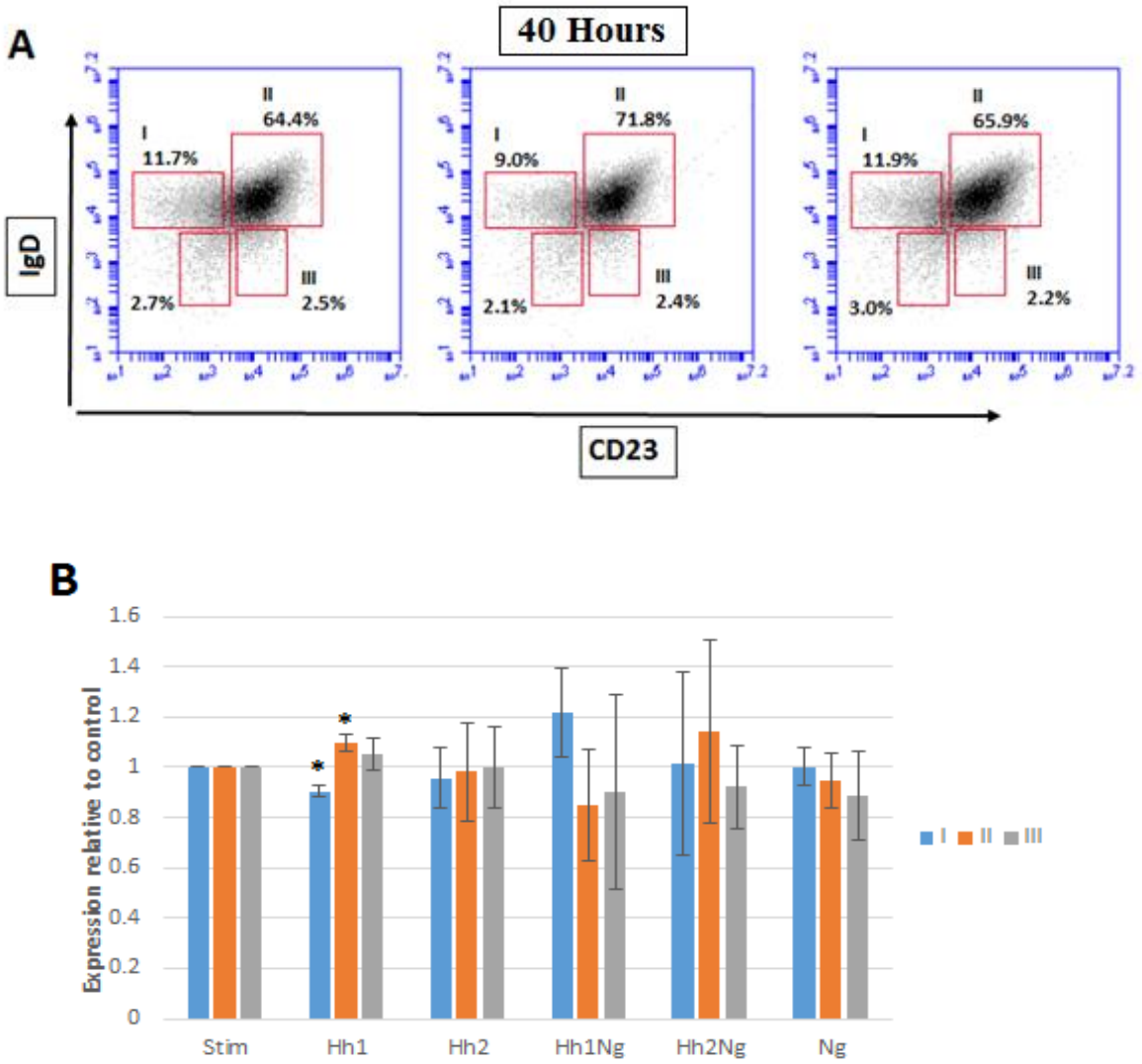


Figure 3.9. IgD and CD23 expression at 40 hours.

A) Flow cytometry dot plots depicting changes in IgD and CD23 expression in response to Hh and Hh+Noggin presence. Left = stimulated control, Mid = Hh1 and Right = Hh1 + Noggin. Hh1=0.005µg/ml and Hh2=0.05µg/ml. Subsets are I) IgD+CD23- II) IgD+CD23+ III) IgD-CD23+ **B)** Bar chart depicting expression of IgD and CD23 at 18 hours post stimulus. B cells isolated for C57BL/6 male mice. Scale represents expression normalised to stimulated control. Error bars

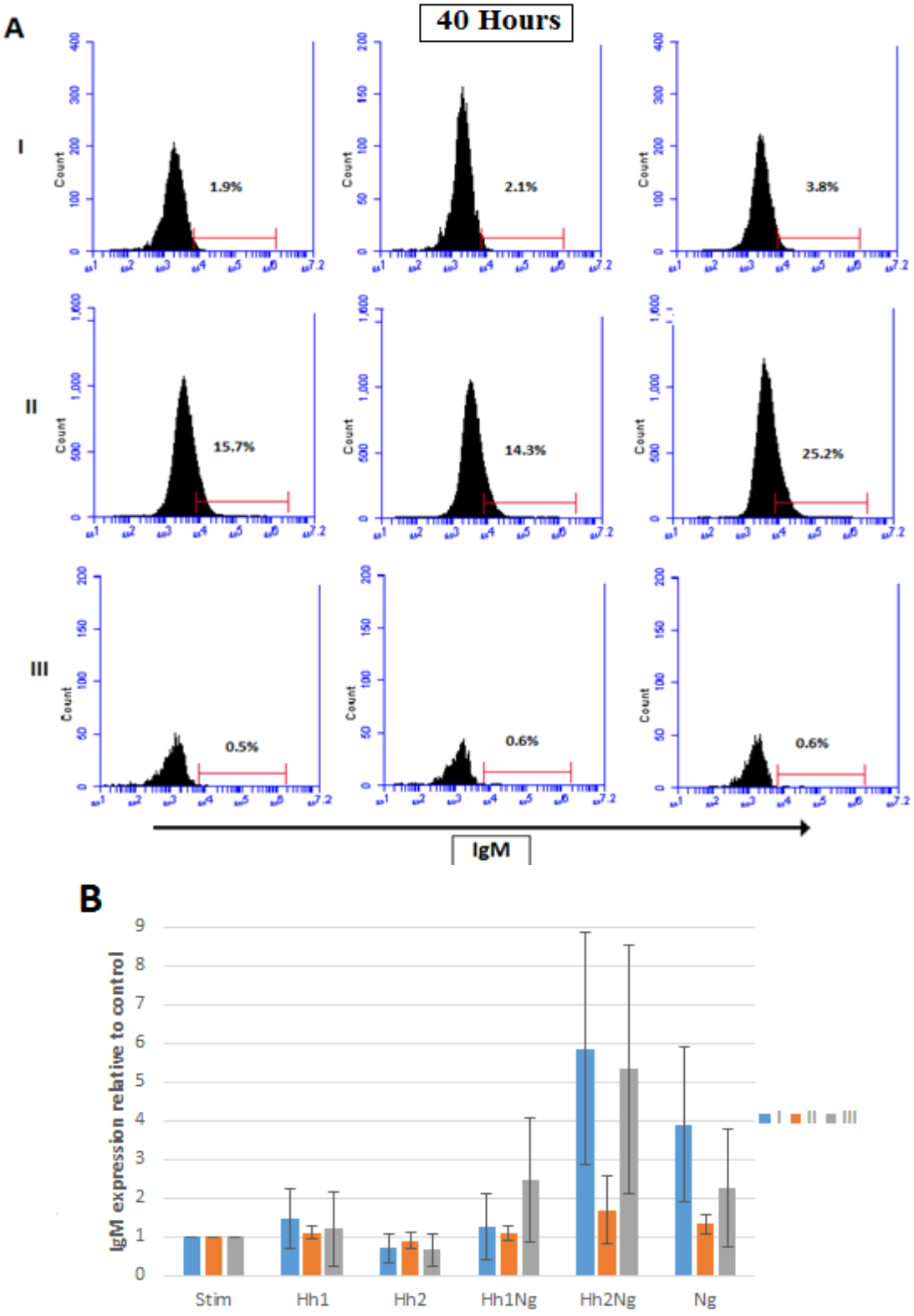


Figure 3.10. IgM expression at 40 hours.

A) Histogram showing expression of IgM within subsets, I) IgD+CD23- II) IgD+ CD23+ III) IgD-CD23+, at 40 hours post stimulus. Histograms are: Left = stimulated control, Mid = Hh1 and Right = Hh1 + Noggin. Cells co-cultured with varying doses of rShh: Hh1=0.005µg/ml and Hh2=0.05µg/ul. B cells isolated from C57BL/6 male mice. **B)** Bar chart depicting relative expression of IgM in subsets. Scale represents expression normalised to the stimulated control.

We observed that following 40 hours in culture, the presence of CD23+CD25+ and a CD23-CD25+ subsets increased when cultured in the presence of rShh (figure 3.11) consistent with the data of Umukoro et al. In the presence of Noggin, a significantly decreased presence of both subsets was observed (p=0.01). This data suggests that BMP signalling is required at 40 hours to induce and maintain the effects previously seen in the presence of rShh signalling which was the differentiation of the splenic B cells to the more mature subset of B cells.

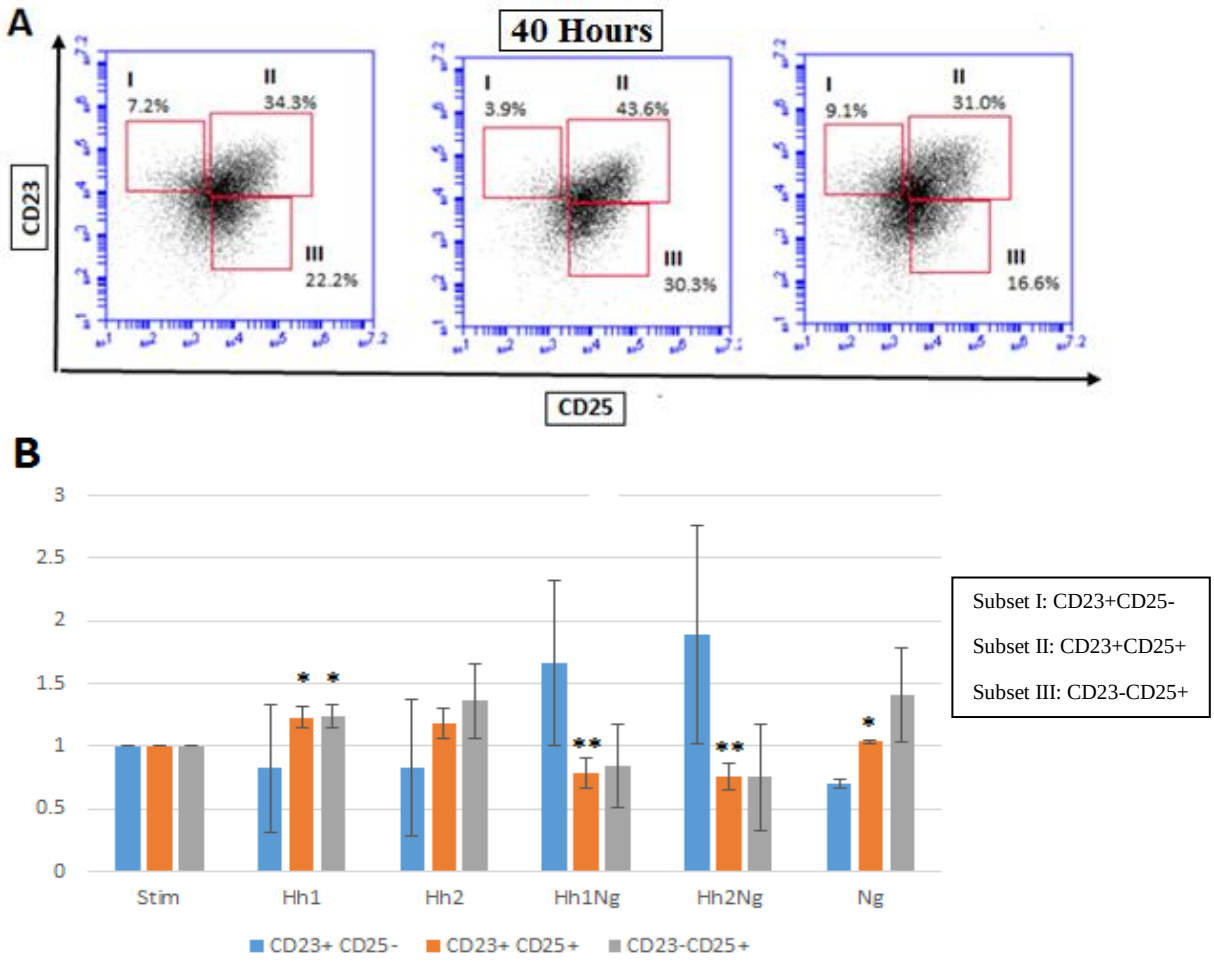


Figure 3.11. Expression of CD23 and CD25 at 40 hours in purified B cells.

B cells purified from the spleens of C57BL/6 male mice, stimulated as before, were co-cultured with rShh at varying doses. Hh1=0.005µg/ml and Hh2=0.05µg/ml. Cells were stained for CD23 and CD25 at 40 hours post stimulus. **A)** Dots plots representing changes in expression of markers. From left to right dot plots represent: stimulated control, Hh1 and Hh1 + Noggin. **B)** Bar chart depicting expression levels of CD23 and CD25. Scale represents expression normalised to stimulated control. Error bars represent +/-. N=3.

Furthermore, BMP signalling promotes memory B cell induction at 40 hours and this can be seen in figure 3.12 in which CD80 increases significantly (p=<0.005) in a CD25+ population in the presence of 0.005µg/ml rShh whilst decreasing when Noggin is added (no significance). These results are consistent with published works by Tomayko et al (2008) who found that memory B cells expressed BMPR1a at a 15-fold increase compared to naive cells.

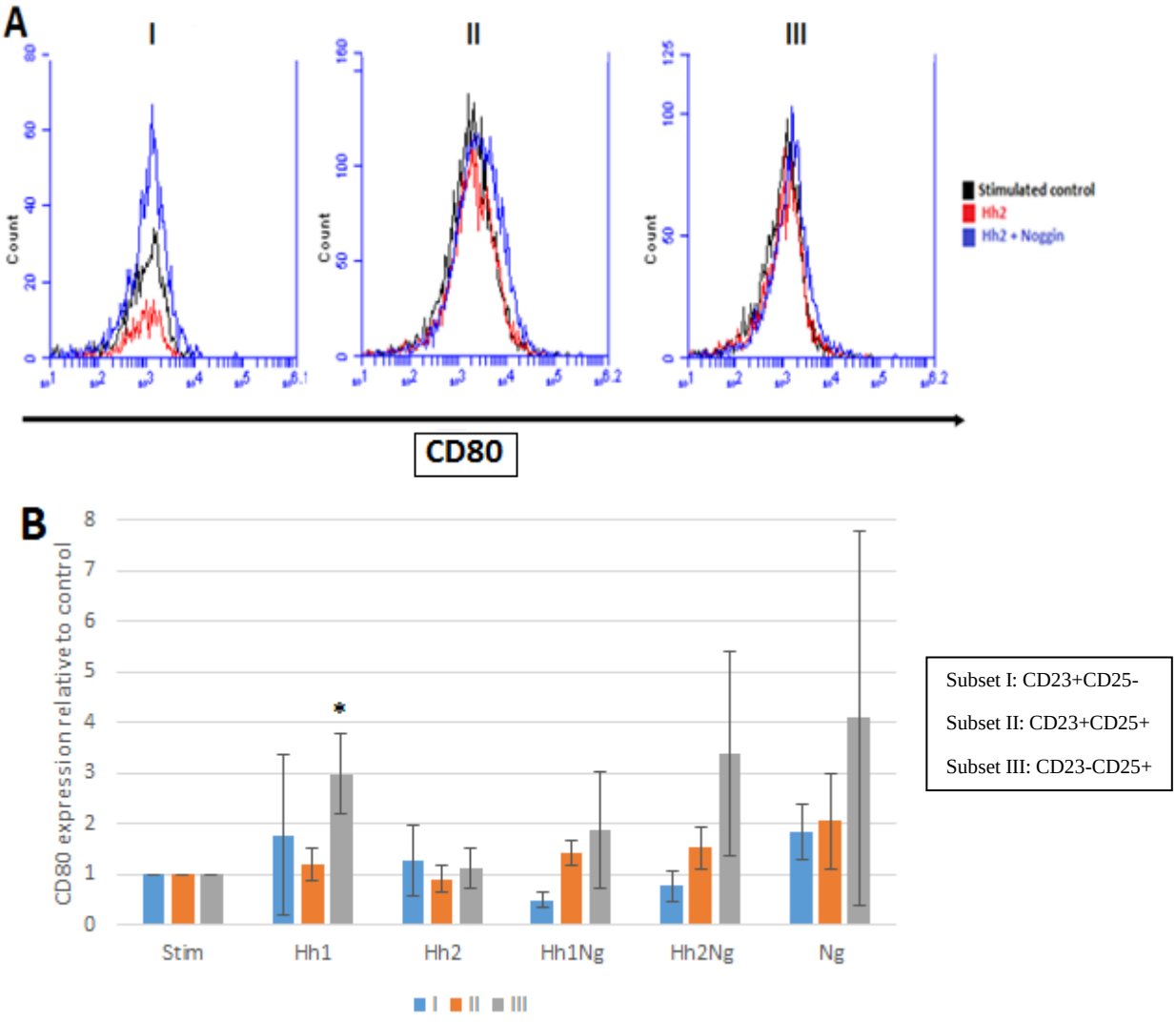


Figure 3.12. CD80 expression in C57BL/6 male B cells.

A) Histograms represent levels of CD80 expression in subsets: I) CD23+CD25- II) CD23+CD25+ III) CD23-CD25+, as compared to the stimulated control, 40 hours post stimulus. **B)** Bar chart depicting relative expression of CD80 in noted subset. Cells cultured in varying doses of rShh: Hh1=0.005µg/ml and Hh2=0.05µg/ml. Scale represents expression normalised to control. Error bar represents +/- SD. N=3.

As we expected BMP signalling to regulate later immune responses we looked to observe differences within Annexin-V binding at 18 and 40 hours in the presence of both rShh and Noggin. Figure 3.13A shows that, at 18 hours, rShh and Noggin had no significant effect on apoptosis and we suggest that this is a result of BMP absence. This figure also shows that at 40 hours, apoptosis increases in the presence of 0.005μg/ml rShh compared to the control ($p=<0.05$) from 12.9% to 28.9%. The addition of Noggin significantly abrogated apoptosis and increased survival at 40 hours ($p=<0.05$). In cells co-cultured with Noggin alone there was no significant decrease in apoptosis, thus indicating that Hh signalling upregulates BMP in a later immune response in order to increase cell death and dampen down immune responses.

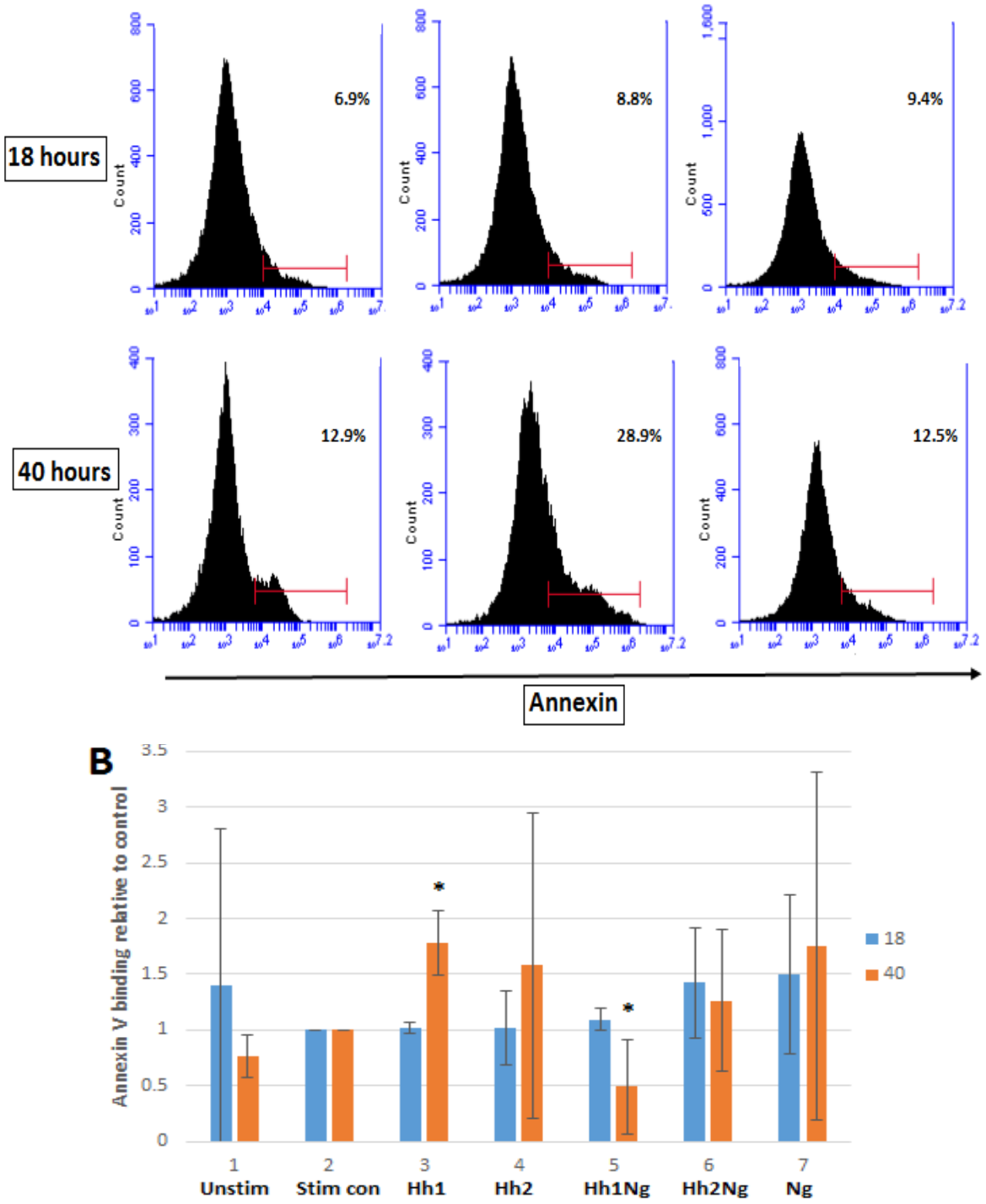


Figure 3.13. Annexin V binding at 18 and 40 hours.

A) Histograms representing Annexin V binding levels, at 18 and 40 hours post stimulus, in C57BL/6 male mice. Left=Stimulated control, mid=Hh1 and right=Hh1+Noggin. **B)** Bar chart depicting levels of Annexin V binding at 18 and 40 hours. Data represents three experiments and error bars represent +/- SD. Hh1 = 0.005µg/ml and Hh2 = 0.05µg/ml.

Our findings suggest that BMP at 40 hours is, firstly, upregulated by Hh and secondly, pro-apoptotic and verifies results from a previous study which revealed that BMPs differentially inhibit Ig production and B cell differentiation (Huse *et al.,* 2011). Therefore, we propose that BMP negatively regulates the immune response as a result of Hh signalling.

3.5. BMPR1α knockout mice display a skewed phenotype

As Hh and BMP have been shown previously, in our study, to interact synergistically in both an early and late immune response we decided to analyse ***BMPR1α*** KO and ***BMPR1α*** WT mice in order to confirm and validate the previous findings observed using Noggin as an inhibitor of BMP signalling. As expression of the ***BMPR1α*** receptor is an absolute requirement for effective BMP signalling to take place, this mouse model allows us to investigate the effect of absence of BMP signalling in B cells isolated from these mice as well as to determine if the observed effects mediated by Hh signalling require the ability of a cell to transduce a BMP signal.

We first analysed the presence of T1, T2/FO, MZ and M (mature) cells in fresh, uncultured splenic B cell populations derived from the ***BMPR1α*** KO and ***BMPR1α*** WT mice (Figure 3.14A). Using expression of CD23 and IgD, we could observe these subsets in freshly isolated tissue in order to observe the effects of lack of BMP signalling on B cell development in vivo. In ***BMPR1α*** KO mice a decrease was observed in the presence of the T2/FO cell subset when compared to the wild-type mice of 48.4% and 53.1%, respectively (Figure 3.14A).

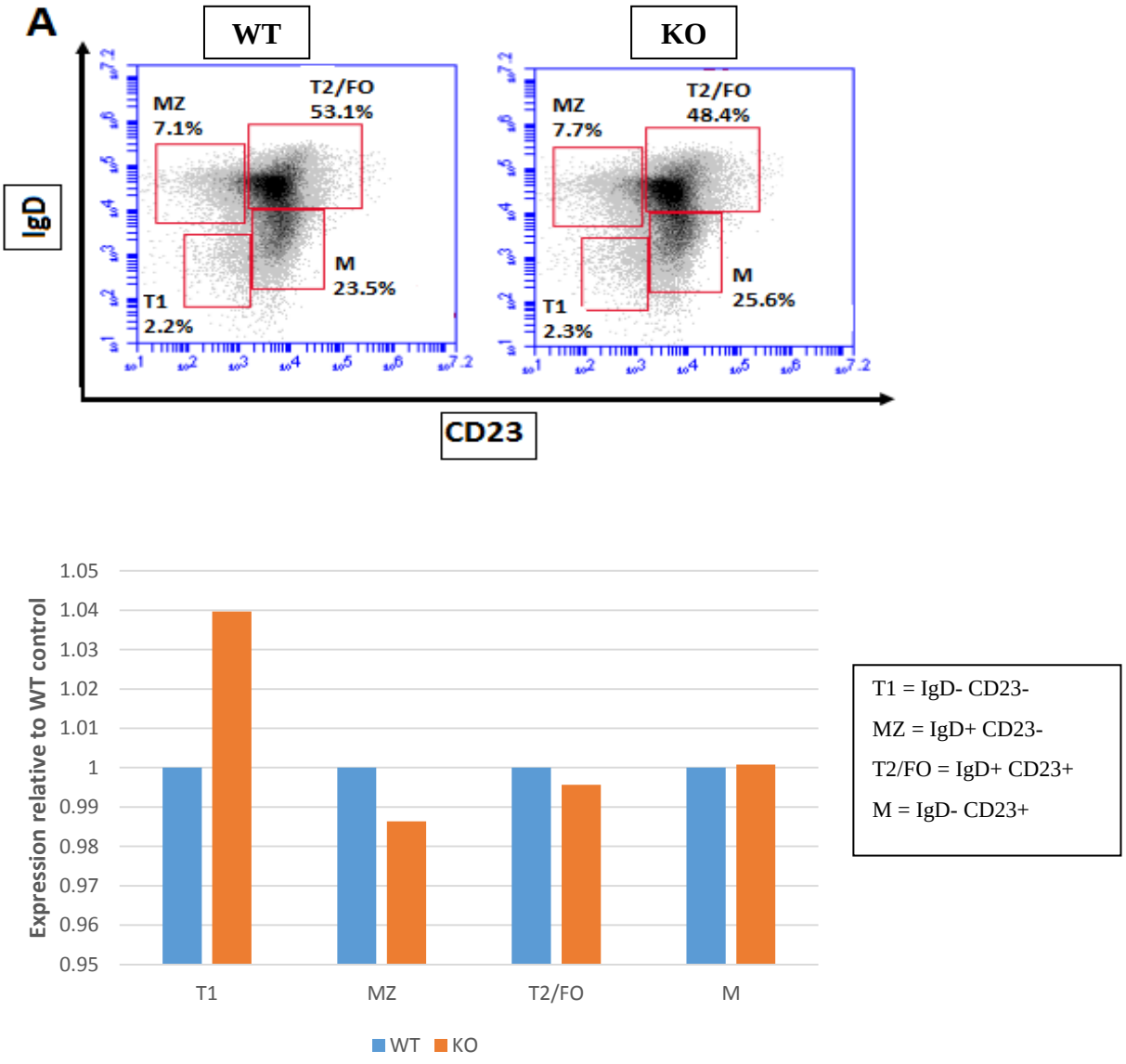


Figure 3.14. B cell subsets in *BMPR1α KO* and *BMPR1α WT* mice in fresh tissue.

A) Dot plot representation of B cell subsets denoted: T1 (IgD-CD23-), MZ (IgD+CD23-), T2/FO (IgD+CD23+) and M (IgD-CD23+). B) Expression of IgD and CD23 within aforementioned subsets as compared to the WT control. Error bars represent +/- SD. Data represents two experiments.

Once the B cells had been isolated and stimulated as described previously, we observed that analysis of B cells isolated from ***BMPR1α*** KO mice display a higher representation of cells with an immature phenotype (IgD+ CD23-) when cultured in the presence of rShh at 18 hours relative to the control (Figure 3.15A). This effect was not altered by dose. Analysis of the presence of the more mature IgD+CD23+ subset revealed that there were less cells present in the B cell cultures derived from ***BMPR1α*** KO mice as compared to WT mice (Figure 3.15A). Our results confirm that BMP signalling is required to regulate Hh-dependent B cell development, however, the specificities of BMP homologues remain to be revealed. Moreover, no differences could be observed between the subsets isolated from ***BMPR1α*** KO and ***BMPR1α*** WT at 40 hours indicating that a recovery process had taken place between 18 and 40 hours.

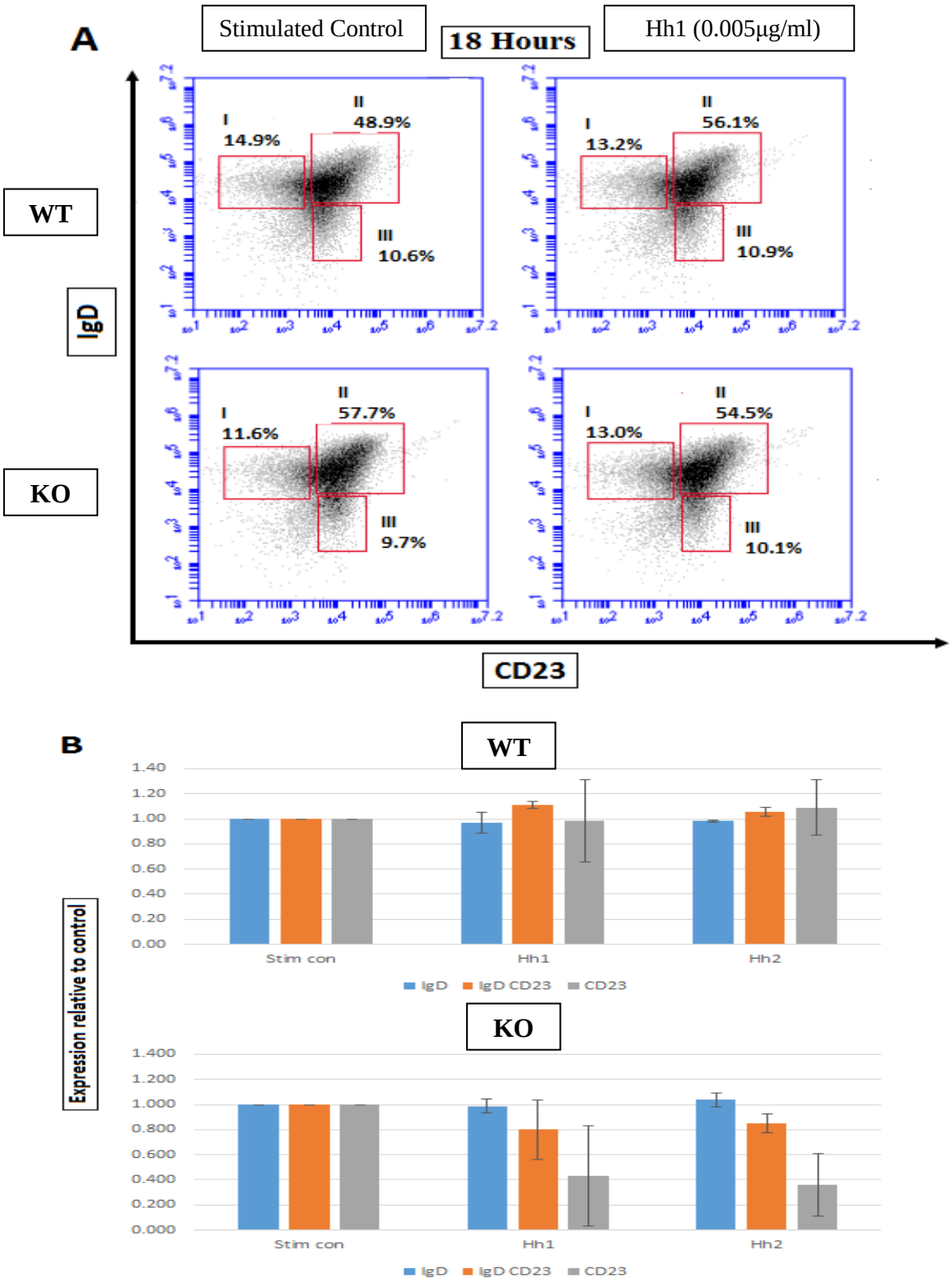


Figure 3.15. Bar chart depicting expression of IgD and CD23 in *BMPR1α KO* and *BMPR1α WT* mice.

B cells purified from ***BMPR1α KO*** and ***WT*** mice were subject to identical conditions as previous experiments. **A)** Dot plot representation of cell subsets, at 18 hours post stimulus, in female mice. Subsets characterised as I) IgD+CD23- II) IgD+CD23+ III) IgD-CD23+. **B)** Bar chart depicting relative expression of IgD and CD23 at 18 hours in female mice. Error bars represent +/- SD. Significance could not be obtained due to limited sample size as data is representative of two experiments.

In order to confirm whether previous results suggesting BMP's role as a negative regulator of the immune response was also the case using our system, we used Annexin-V staining to reveal any effect of the absence of the ***BMPR1a*** gene on the survival of B cells following the activation stimulus in the presence and absence of rShh protein. We show, in figure 3.16, that BMP signalling is crucial for the observed induction of apoptosis in the presence of rShh protein at 40 hours, as analysis of B cells isolated from ***BMPR1α KO*** mice show a decrease in the Hh signalling mediated induction of apoptosis as judged by Annexin-V staining as compared to the wild-type control. No notable difference in induction of apoptosis was detected at 18 hours. This confirms our earlier studies which indicated that BMP signalling is required to mediate the Hh regulated termination of the immune response.

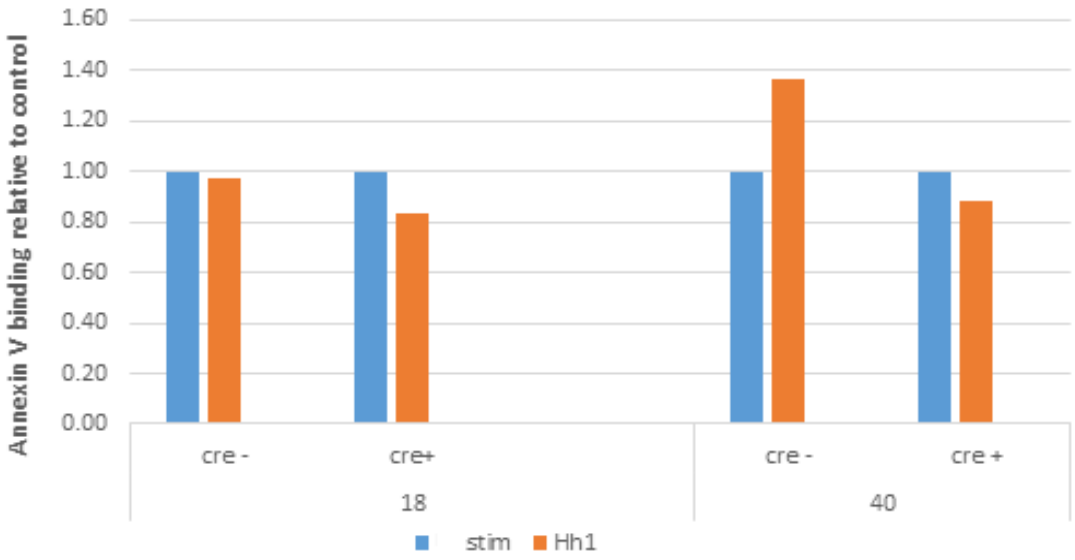


Figure 3.16. Annexin V binding, at 18 and 40 hours, in *BMPR1α KO* and *WT* female mice.

Bar chart depicting levels of Annexin V binding at 18 and 40 hours. Data represents two experiments and error bars represent +/- SD. Hh1 = 0.005ug/ml and Hh2 = 0.05ug/ml. Significance could not be obtained due to limited sample size.

During a normal early immune response, secretion of IL-10, a regulatory cytokine, is repressed in order to permit efficient cell activation whilst in later stages, expression is increased significantly in order to inhibit hyper-activity of immune effector cells. We show, in figure 3.17, that IL-10 secretion from B cells isolated from ***BMPR1α KO*** mice is increased considerably in the presence of a Hh signal following 18 hours in culture relative to the wildtype control, which, conversely, drives down expression of IL-10. This data suggests that the interplay between Hh and BMP during the B cell response to antigen is critical for the appropriate regulation of the early immune response creating not only an activatory environment essential for a rapid response to antigen but also the means by which this response may be dampened down. Surprisingly, analysis of IL-10 expression at 40 hours revealed that expression appeared identical in both WT and KO mice (not shown) mirroring the previous data obtained suggesting that there is some sort of recovery process happening in ***BMPR1a KO*** mice between 18 and 40 hours.

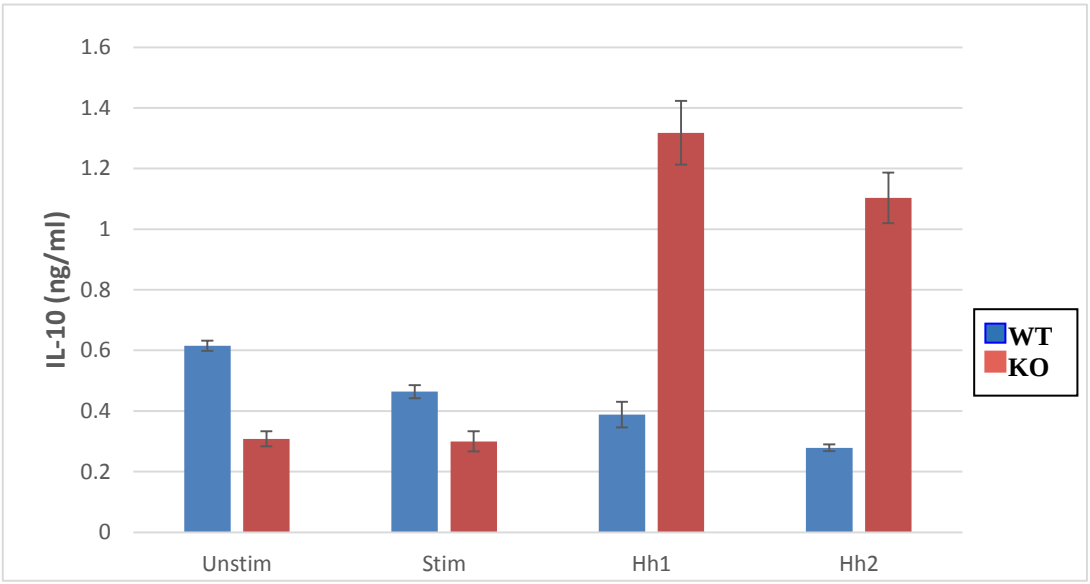
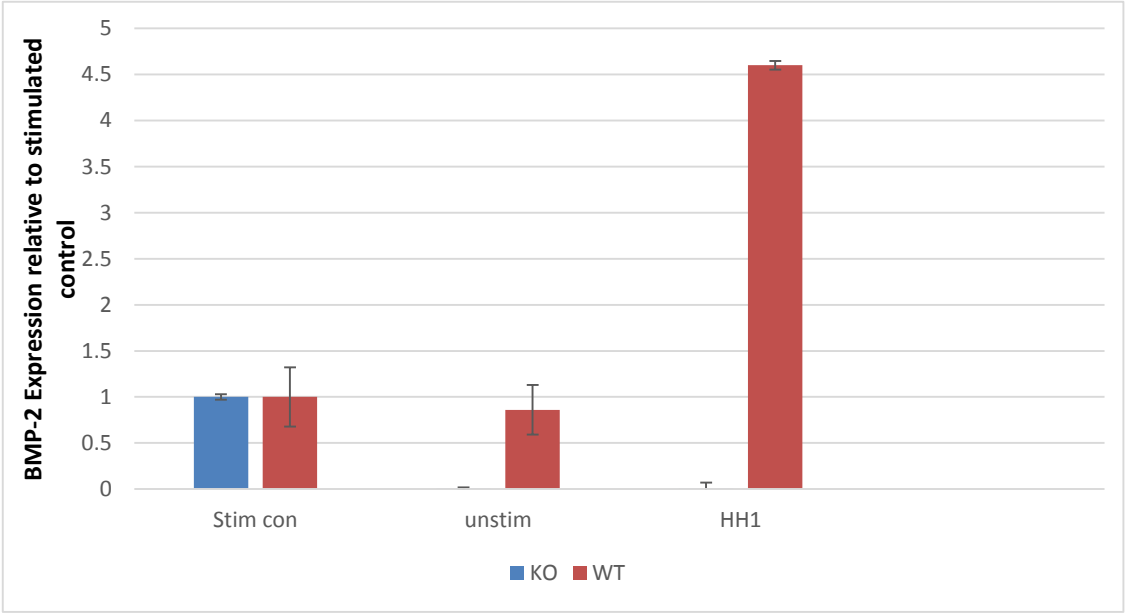


Figure 3.17. IL-10 secretion at 18 hours in *BMPR1α KO* and *WT* mice.

IL-10 secretion, measured using ELISA, at 18 hours post-stimulus in KO and WT mice. Error bars represent +/- SD. Significance could not be obtained due to limited sample size. N=2.

To confirm that the Hh mediated effects on the B cell response could be mediated by upregulation of expression of BMP we analysed BMP expression by RT-PCR (figure 3.18). Consistent with this hypothesis, an upregulation of expression of BMP-2 was observed when B cells isolated from ***BMPR1a*** WT were activated in the presence of Hh. Interestingly, when this same experiment was performed on B cells isolated from ***BMPR1a*** KO mice there was no increase in BMP-2 expression observed in the presence of a Hh signal indicating that a signal mediated by the presence of ***BMPR1a*** receptor is needed in order to upregulate expression of BMP-2 by B cells suggesting complex levels of regulation of these two pathways.



3.18. BMP-2 expression at 18 hours in *BMPR1α* KO and WT mice.

BMP-2 expression measured using RT-PCR at 18 hours post stimulus. Results are representative of two experiments thus significance could not be obtained. Error bars represent +/- SD.

4. Discussion

In this study, we examined the possibility that, Hh and BMP signalling, play a synergistic role in the antigen-dependent development of peripheral B lymphocytes and this discussion will focus on our results which define a role for Hh and BMP as a critical regulators of TD B cell development. It has previously been shown that Hh signalling delivers a positive signal for B cell proliferation, antibody secretion and survival and that Hh proteins were shown to be expressed by the splenic stroma and components of the Hh signalling pathway expressed in germinal centre B cells. Interestingly, these authors showed that, the expression of Smoothened, the Hh signal transducing receptor, is rapidly up-regulated upon GC B cell activation using anti-IgM and anti-CD40 (Sacedon *et al*., 2005). Conversely, Huse *et al* (2011) revealed a negative regulatory role for BMP signalling in the development of human CD27+ memory B cells with the finding that BMP 2, 4, 6 and 7 were all capable of inhibiting Ig production. With this in mind, we explored the concept of interplay between Hh and BMP signalling in peripheral B-cell development.

We have shown that BMP signalling is an essential requirement for exogenously added rShh to give a positive activation signal at 18 hours and that inhibition of BMP signalling leads to skewing of development of specific B cell subsets. Additionally, we show that BMP signalling is essential for dampening down the immune response at 40 hours by increasing apoptosis and inhibiting differentiation. Overall, our results suggest that, whilst BMP is a key mediator of Hh signalling, both Hh and BMP are essential regulators of B cell mediated immune responses.

4.1. Hh and BMP signalling regulate early activation and survival of peripheral B cells

Hh has previously been described as a positive regulator of T cell development (Crompton *et al*., 2007; Varas *et al.*, 2003) and a potent inducer of a Th2 transcriptional profile (Furmanski *et al*., 2013) as well as being capable of regulating B cell development (Sacedon *et al*., 2005). Our analysis of B cells isolated from BALB/c and C57BL/6 mice suggest that Hh signalling drives B cell development in an accelerated fashion at 18 hours in response to BCR ligation and co-stimulation. We demonstrate, that purified B cells cultured with rShh, display an increased level of co-expression of CD23 and CD25; markers associated with activation and differentiation. Interestingly, in B-cells analysed within a heterogenous splenocyte population, Hh signalling appeared to increase activation whilst reducing differentiation in a dose-dependent manner. To a certain extent this may be explained by the presence of surrounding cells already expressing Hh ligands, thus, exogenously added rShh is exacerbating B cell development. Additionally, the possible presence of B220+ dendritic cells, in our flow cytometry gating strategy may have skewed results (Segura *et al*., 2009). Alternatively, variability may be due to gender differences between the mice analysed in this study

When Noggin was added to cultures already containing rShh to determine whether BMP signalling is required for Hh-mediated development we observed a significant skewing of B cell subsets; namely that cells appeared to increase expression of CD25 whilst downregulating expression of CD23. Our data suggests a novel finding that BMP is an absolute requirement to prevent aberrant Hh signalling in splenic B cells.

4.2. *Hh and BMP synergistically induce a T2 to follicular B cell transition*

Knowledge of how B cell subsets develop, in response to antigen stimulation in the mouse, is lacking. In recent times, studies investigating human B cell subsets within the spleen have suggested that, dependent on the strength and type of antigenic stimulation, naïve resting transitional B cells develop into FOI, FOII,T2-MZP and MZ B cells (Pillai and Cariappa, 2009); and that these cells will go on to form plasma, memory or regulatory B-cells. Interestingly, preliminary data suggest that Hh signalling upregulates expression of two factors involved in transmitting the BCR signal into the B cell, Btk and NFATC1 (Umukuro, unpublished). Consequently, it is important that the factors involved in regulating the development of B cell subsets in response to differing levels of BCR stimulation are elucidated. Therefore, we investigated the role of Hh and BMP signalling on the polarisation of specific B cell subsets.

We found at 18 hours, in the presence of rShh, there was an increase in the levels of representation of a B cell population co-expressing IgD and CD23 and the addition of Noggin significantly decreased the presence of this population. However, in samples containing cells co-cultured with Noggin alone, we noted a similar decrease in expression within the same subset. These results may indicate that BMP signalling alone is responsible for driving development of particular B cell subsets, however, we suggest that Hh ligands expressed by surrounding cells upregulate BMP expression *in vivo.*

To more clearly identify B cell subsets, we examined the differential expression of IgM within three subsets: I) IgD+ CD23-, 2) IgD+ CD23+ and 3) IgD- CD23+. Our results showed that IgM expression is reduced following Hh signalling on all subsets at 18 hours and that this dependent on the concentration of exogenously added rShh. This would suggest that Hh signalling increases the maturation status of B cells from an

IgD++CD23+IgM+ T2/FOII cell line to an IgD+CD23+IgM- FOI subset. Moreover, the presence of Noggin led to a noteworthy increase in IgM expression in all subsets indicating that Hh and BMP signalling interrelate to modulate the development of a T2 to FOI cell line. The data represented here corroborates previous data suggesting that the emergence of T2 and the intermediary FOII B cells, which are defined only by the expression of AA4.1, develop independently of antigen; and that FOII cells, upon Btk signalling, develop into FOI B cells (Pillai and Cariappa, 2007). Furthermore, we show that Hh and BMP signalling regulate this developmental process in a dose-independent manner which is typical of morphogen gradient dynamics.

Of interest is that, the BMP inhibitor, Noggin, appeared to have a greater inhibitory effect on BMP signalling than observed using the BMPR1α KO model which will be discussed below. However, Lavery *et al* (2008) observed that BMP 2 and 4 preferentially signal through BMPR1α whilst BMP 6 and 7, despite signalling canonically through BMPR1a, may also utilise ACVR1a and ACVR2, both canonical TGF-β receptors, as an alternative route.

4.3. BMP signalling dampens down later immune responses

A role for BMP as a negative regulator of immune responses has previously been described (Huse *et al*., 2011; Kersten *et al*., 2005), however, little is known regarding how interactions between Hh and BMP influence this process. As the induction of apoptosis and inhibition of cellular differentiation has been shown to be a critical factor in dampening down immune responses we investigated whether a survival signal previously observed to be provided by Hh at 18 hours (Umukuro., 2012) becomes a signal for death at 40 hours as a result of BMP expression upregulation. We found that at 40 hours the presence of rShh led to an increase in apoptosis and the emergence of a mature B cell phenotype (CD23-CD25+). The addition of Noggin to cultures containing rShh led to a significant increase in cell survival and increased activation.

We observed increased numbers of a FOI B cell subset and suspect that these represent a population of cells which have the capacity to form long-lived plasma and memory cells (Pillai and Cariappa., 2007). To confirm whether Hh and BMP signalling can influence memory cell formation we analysed the expression of CD80, a co-stimulatory protein and a marker of memory B cells, within subsets labelled, I) CD23+ CD25- II) CD23+ CD25+ III) CD23- CD25+ as we suspect that memory cells are retained within the CD23-CD25+ population. We show that exogenously added rShh to samples at 40 hours significantly increased the expression of CD80 in a CD23-CD25+ population in a dose-independent fashion and this effect was abrogated by the addition of Noggin in samples co-cultured with rShh. As previous studies have noted that expression of BMPR1α is significantly upregulated in memory B cells (Huse *et al*., 2007; Tomayko *et al*., 2008), we suggest that these results, taken together with our data, imply that Hh and BMP signalling may indeed regulate memory cell formation.

As BMP signalling has previously been documented to induce apoptosis (Huse *et al.,* 2011), we investigated whether interplay between Hh and BMP influenced cell death at 18 and 40 hours after stimulation. We show that in the presence of Hh signalling, at 40 hours, apoptosis increased significantly and, that, when Noggin was added to cultures containing rShh this effect is completely reversed. There are several possible mechanisms by which BMP may induce cell death: the first is most likely to be induction of a caspase pathway (Mogi and Togari., 2003); secondly, BMP may be upregulating inhibitory ligands such as PD-L1 and OX-40L (Martinez *et al.*, 2014) and lastly, BMP may increase expression of Fas ligands leading to apoptosis. Our data, presented together with published works by Huse *et al* (2011) and Kersten *et al* (2005), which imply that BMP is a potent inducer of ID1 and ID3 whist inhibiting the expression of XBP-1, indicate that BMP negatively regulates the later stages of an immune response by regulating the degree of apoptosis.

4.4. BMPR1a KO *mice display impaired B cell development*

We have shown, in this study, that BMP signalling is required to modulate Hh signalling during B cell development using a highly specific BMP inhibitor, Noggin. To further validate these findings, we utilised a ***BMPR1a*** knockout model in order to confirm that BMP signalling is an essential requirement for Hh signalling to provide a positive signal to B cell development at 18 hours and a negative signal at 40 hours. When co-cultured with 0.005μg/ul rShh we observed that, both male and female, ***BMPR1a*** KO B cells displayed an impaired capacity to develop from a T2 to FOI subset indicating that Hh and BMP signalling are critical modulators of this transition. Hh signalling has previously been shown to modulate the signal strength of TCR signalling in mature T cells (Rowbotham *et al.*, 2007) and preliminary data suggest that Hh signalling regulates the expression of key mediators of BCR signalling, thus, we propose that Hh and BMP signalling may interact to modulate BCR signalling in B cell development. Furthermore, as the development of FOI B cells are dependent exclusively on BCR ligation and signalling (Pillai and Cariappa., 2007), we suggest that BMP is indispensable in peripheral Hh mediated B cell development.

In order to maintain peripheral immune homeostasis, the duration and strength of the immune response to antigen must be tightly regulated and, although a complex series of events, it is well established that order is restored via two distinct mechanism: induction of apoptosis and secretion of IL-10. The molecular events involved in regulation of these two processes within B cells, are largely unresolved but may involve interplay between Hh and BMP signalling. Our findings demonstrate that Hh signalling regulates BMP signalling in order to induce apoptosis as, in our BMPR1α KO model, Hh signalling induced apoptosis is largely reduced at 40 hours in comparison to BMPR1α WT mice. Our study confirms previous findings obtained at UEL and those

from a previous study which determined that BMP signalling negatively regulates B cell responses (Huse *et al*., 2011).

Our study also demonstrates that Hh and BMP are regulators of IL-10 secretion in splenic B lymphocytes. At 18 hours B cells isolated from BMPR1α KO mice displayed a substantial increase in IL-10 expression, in the presence of rShh, as compared to B cells isolated from BMPR1α WT mice. Although controversial, it is understood that IL-10 acts on cells to reduce antigen presentation via MHC molecules, increase expression of inhibitory ligands and inhibit pro-inflammatory cytokines. Therefore, our data indicates that Hh and BMP signalling are required in early responses to keep levels of IL-10 expression low in order to enhance the activatory environment required for efficient TD B cell responses.

4.5. Hh and BMP signalling interact to induce BMP-2 expression

As we have shown in this study Hh and BMP interplay influences TD B cell development, however, whether BMP is transcriptionally activated in B cells by Hh signalling remained to be elucidated. Using RT-PCR analysis in ***BMPR1α*** KO mice we show that, at 18 hours, B cells isolated from WT mice upregulate expression of BMP-2 greatly in response to Hh whilst B cells isolated from ***BMPR1α*** KO mice exhibit an impaired capacity for BMP-2 expression as no visible expression could be distinguished. This would suggest that Hh signalling and signalling through ***BMPR1a*** is required in order to upregulate BMP-2 in response to antigenic stimulation making the interplay between these two molecules far more complex than first expected. Although some studies have implied that Gli and Smad proteins may interact at the transcriptional level and this may go some way to explaining our phenomenon (Nye *et al*., 2014). In addition, RT-PCR analysis of BMP-4 expression showed no notable difference between samples stimulated with anti-IgM/anti-CD40 and Hh (Not shown).

Therefore, we show that BMP-2, specifically, regulates early immune responses in a Hh-driven mode.

For the first time, our data demonstrates that BMP is a critical regulator of Hh signalling in B cells and that both Hh and BMP signalling is essential in order for splenic B cells to develop in response to antigenic stimulation. The role of Hh and BMP on peripheral B cell development is summarised in figure 4.1.

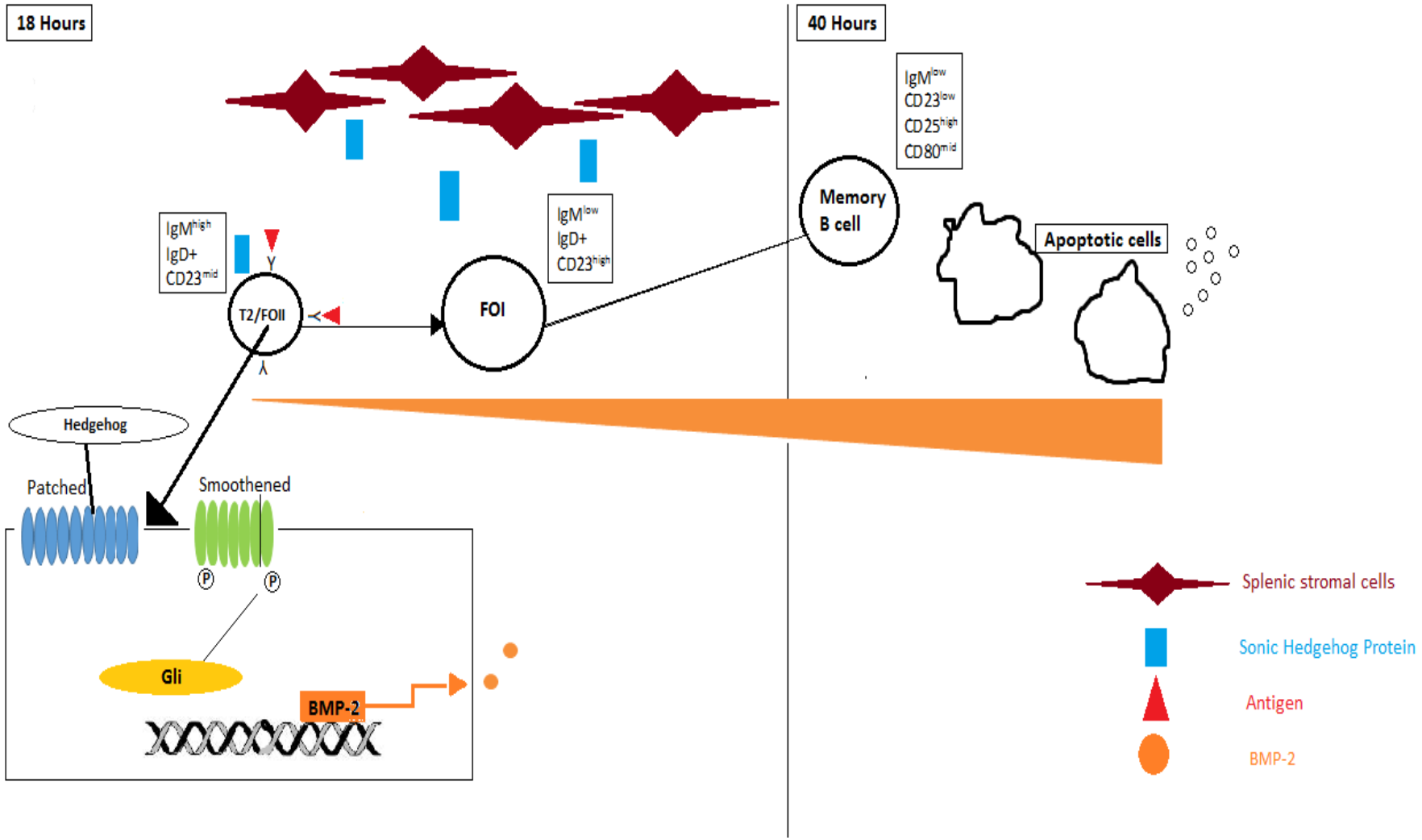


Figure 4.1. TD B cell development in response to Hh signalling. At 18 hours post-stimulus, Hh ligands secreted by splenic stromal cells, lead to an increase in BMP-2 expression. Hh and BMP elicit a positive signal, decreasing IL-10 secretion, in order to induce B cell development, particularly a T2/FOII to FOI transition. Following this, at 40 hours, BMP negatively regulates B cell development by inducing differentiation into a mature memory B cell subset and increasing apoptosis. Here we show that BMP modulates Hh signalling and that both Hh and BMP are required for efficient TD B cell development.

4.6. Future work

B-cell driven adaptive immune responses require an intricate balance between activation and termination to prevent unnecessary or inadequate host tissue damage. Currently, research into the regulation of B cell development in the mouse is limited with the majority of studies focussing on the role of B cells in disease, as such inadequate definitive research defining the normal splenic B cell developmental process exists. Splenic B cell development is a multifaceted process involving complex interactions between many secreted factors including Hh and BMP and future studies will need to determine which specific BMPs are responsible for the regulation of Hh-driven responses. Additionally, it is highly likely that BMPs themselves interact to modulate Hh signalling thus closing the circle of regulation. BMP expression is regulated by a number of secreted proteins within the extracellular space including Noggin and Twisted Gastrulation, thus, the interplay between regulators of BMP in directing B cell development also remains to be properly characterised. Furthermore, it is a possibility that Hh and BMP signalling pathways interact or converge at key checkpoints in B cell development and this is likely to play a crucial role in B cell development. Understanding the mechanisms that contribute to peripheral B cell development will influence future studies and may provide scope for novel therapeutic targets in B cell mediated immune disorders and malignancies.

5. Concluding remarks

In conclusion**,** our data revealed a novel role for BMP, as a modulator, of Hh signalling in the activation of splenic B cells in response to TD antigenic stimulation. Use of a highly specific BMP inhibitor, Noggin, results in a skewed phenotype in an early immune response. Moreover, Hh and BMP signalling together generate an

environment for activation by inhibiting IL-10 secretion at 18 hours. We show that loss of BMPR1α leads to an anti-apoptotic and activated response at 40 hours post stimulation. Thus, we propose that Hh and BMP interplay is an essential requirement for Hh driven B cell development.

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