Piperine Modulates B cell Activation and Function

by

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Piperine Modulates B cell Activation and Function" by David A. Soutar in partial fulfillment of the requirements for the degree of Master of Science.

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Signature of Author

DEDICATION

I would like to dedicate this thesis to my parents and two sisters,
who have always been wonderfully supportive.

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ABSTRACT

Piperine, the major alkaloid derived from black pepper corns, has played an important role in traditional medicine worldwide. Current research has demonstrated piperine to have several anti-inflammatory properties, however, little is known concerning the effect of piperine on B cells. Spleen-derived murine B cells were cultured in the presence or absence of piperine during T-dependent or T-independent activation. Piperine reversibly inhibited B cell proliferation in a dose-dependent manner. This was due to a $G_{0/1}$-phase cell cycle arrest, and was associated with a reduction in phospho-ERK, phospho-AKT, and Cyclin D1, D2, and D3. Piperine also inhibited antibody and cytokine production. Furthermore, piperine treatment diminished B cell-mediated antigen presentation determined by measuring OT-II transgenic T cell proliferation in response to OVA, which was attributed to the decreased MHC-II ad co-stimulatory molecule expression observed. This *in vitro* study shows that piperine has potent immuno-suppressive effects on B cell activation and effector function.

LIST OF ABBREVIATIONS USED

Abbreviations

Ab	Antibody
α-CD40	purified anti-CD40 antibody
α-BCR	purified anti-immunoglobulin/BCR antibody
APC	Antigen Presenting Cell
Akt	v-Akt Murine thymoma viral oncogene homolog; also known as Protein Kinase B (PKB).
Bcl-xL	B cell lymphoma-extra large
Bcl-2	B cell lymphoma-2
BCR	B cell Receptor
BLINK	B cell linker protein
BSA	Bovine Serum Albumin
Btk	Bruton's tyrosine kinase
°C	Celsius
Cbl	Casitas B-lineage Lymphoma
CD	Cluster of Differentiation: CD(19/40/80/81/86/154)
CI of Diff.	95 % Confidence Interval of the Difference between two means
CYP	Cytochrome P
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
E-selectin	Endothelial Selectin
ERK	Extracellular Signal-regulated Kinase
FCS	Fetal Calf Serum
FOXO	Forkhead box protein factors
g	Gravity
Grb2	Growth factor receptor-bound protein 2 adaptor protein
h	Hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
ICAM	Intracellular Cell Adhesion Molecule
IκB	Inhibitor of κB
IKK	Inhibitor of IκB
IFN	Interferon
Ig	Immunoglobulin: Ig(α/β/A/D/E/G/M)
IL	Interleukin
JNK	c-Jun N-terminal kinase
KCl	Potassium Chloride
LPS	Lipopolysaccharide
MAPK	Mitogen-activated Protein Kinase
Mean Diff.	Mean difference/average absolute difference

mg	Milligram
mL	Millilitre
MHC	Major Histocompatibility Complex
min	Minutes
mRNA	Messenger RNA
MTT	(3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
MyD88	Myeloid Differentiation Primary Response Gene 88
NaCl	Sodium Chloride
NF-κB	Nuclear Factor- κB
ng	Nanogram
NS	Not Significant
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3-kinase
PLC-γ2	Phospholipase Cγ2
PMA	Phorbol 12-Myristate 13-Acetate
PMSF	Phenylmethylsulfonyl Fluoride
PRR	Pattern Recognition Receptor
PTK	Protein tyrosine kinases
p38	p38 mitogen-activated protein kinase
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
sec	Seconds
SEM	Standard Error of the Mean
SH2	Src homology 2
Syk	Spleen tyrosine kinase
Sos	Son of sevenless, guanine nucleotide exchange factor
TAK-1	Transforming growth factor β-activated kinase
TCR	T Cell Receptor
Td	T-dependent activation
T_H	T Helper
Ti	T-independent activation
TLR	Toll like Receptor
TNF	Tumour Necrosis Factor
Tpl2	Tumour progression locus 2
TRAF	TNF receptor associated factor
TRPV1	Transient Receptor Potential Vanilloid 1
wt	Wild Type
µL	Microlitre
µm	Micrometre
µM	Micromolar
Vav	A guanine nucleotide exchange factor
vs.	Versus
[^{3}H]-TdR	Tritiated Thymidine

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If I waited until it was perfect, it would never be done (Chinese proverb). I would like to thank those who were patient and helpful.

CHAPTER 1. INTRODUCTION

1.1 THE IMMUNE RESPONSE.

Protection from the plethora of pathogens encountered during ones life is dependent on two arms of the immune response: the innate, and the adaptive immune response. The interaction between the innate and adaptive immune responses depends on intrinsically linked cellular and intracellular cross-talk. Because of this coordinate interaction, the innate and adaptive immune responses are able to survey, recognize, and eliminate potential pathogens (Janeway, Jr. and Medzhitov, 2002; Minguet et al., 2008).

1.1.1 THE INNATE IMMUNE RESPONSE.

The innate immune response is the initial response of the immune system, which provides early non-specific host defence to pathogens. There are many cells involved in the innate immune response, such as dendritic cells (DC), macrophages, and neutrophils, as well as many molecules such as anti-microbial peptides, acute-phase proteins, and complement proteins (Janeway, Jr. and Medzhitov, 2002; DeFranco et al., 2007; Travers and Walport, 2007). Important in the innate immune response is the early recognition of pathogens by pattern recognition receptors (PRR). There are many PRR classes, such as the C-type lectins, scavenger receptors, complement receptors, nucleotide-binding oligomerization domain (NOD)-like receptors and caspase-recruiting domain (CARD) helicases; however, toll-like receptors (TLR) are arguably the major signal-generating PRR (Lee and Kim, 2007). Specifically, and pertinent to this investigation, the germ-line-encoded TLR subset, TLR4, recognizes the prominent and important Gram-negative bacterial pathogen associated molecular pattern (PAMP), lipopolysaccharide (LPS).

Engagement of TLR4 promotes innate immune cells, such as DC, to phagocytose antigen and present antigen peptide in the context of MHC II. Engagement of TLR4 also promotes pro-inflammatory cytokine production and an increase in the expression of co-stimulatory molecules, all of which link the innate immune system to the adaptive immune response. Furthermore, the innate system is intrinsically linked to the adaptive system in that TLR4 expression is also present on adaptive immune cells, such as the B lymphocyte. Engagement of LPS with TLR4 on B cells promotes antigen presentation as well as B cell activation and proliferation, which are integral to the adaptive immune response (Ogata et al., 2000; Palm and Medzhitov, 2009; Pasare and Medzhitov, 2005).

1.1.2 THE ADAPTIVE IMMUNE RESPONSE.

Adaptive immunity evolved after the innate immune system and is oriented for specific targeting of pathogens (Laird et al., 2000). Rather than the germ-line-encoded receptors used in innate immunity, the adaptive immune system relies on gene segment rearrangements to express highly antigen-specific receptors. These receptors are found on both B and T lymphocytes, and recognition of antigen by these receptors initiates the adaptive immune response (Laird et al., 2000; Jung et al., 2006). This response includes antibody and cytokine production, which facilitates immune cell mediated clearance of the pathogen (Dogan et al., 2009; Lund, 2008; Nimmerjahn and Ravetch, 2010; Wojciechowski et al., 2009). Unlike PRR, B and T lymphocyte receptors are antigen-specific, and antigen binding causes membrane-associated and intracellular molecules to come into proximity to initiate cell signalling. Only highly antigen-specific lymphocytes are activated to proliferate and differentiate, thereby mounting an antigen-directed

effector immune cell response. The adaptive arm of the immune system also enables a rapid long lasting immune response against recurrent pathogen, as activated B and T lymphocytes can differentiate into memory cells. This is referred to as immunological memory (Burnet, 1976; Depoil et al., 2008).

1.2. THE B LYMPHOCYTE

Lymphocytes are the cellular component of our immune system that are responsible for the highly specific adaptive immune responses. Lymphocytes are hematopoietic cells, which can be divided into two major groups, the T and B lymphocytes. The B lymphocyte (B cell) is so named after the "*bursa* of Fabricius in chickens," which is the equivalent of the mammalian bone marrow where B cells mature (Glick and Whatley, 1967), whereas the T lymphocyte (T cell) is named after its maturation site in the thymus. Mature naïve B cells display a highly specific receptor for a particular antigen. This B cell receptor (BCR) is a monomeric membrane-bound immunoglobulin–M or –D molecule (IgM or IgD antibody), which consists of two domains, the constant region and the antigen binding region. The constant region defines the immunoglobulin isotype, the ability to interact with Fc-receptors, and complement binding capacity. The antigen binding region can be subdivided into the variable (V), diversity (D) and joining (J) regions (Casadevall and Pirofski, 2004). The antigenic diversity of the BCR arises from immunoglobulin gene recombination events where non-contiguous V, D and J immunoglobulin gene segments are recombined into functional VDJ genes during B cell development within the bone marrow (Jung et al., 2006). Once in circulation, mature naïve B cells become activated upon BCR engagement of antigen,

which initiates activation, proliferation, and differentiation of naïve B cells (Nimmerjahn and Ravetch, 2010; van Zelm et al., 2007). B cells initially produce IgM, but upon antigen binding and activation B cells undergo class-switch DNA recombination events, in which the isotype of the antibody being produced switches from IgM to IgG, IgA or IgE. Throughout B cell proliferation, the BCR variable coding region undergoes somatic hypermutation events that culminate in enhanced or diminished antibody binding of antigen. Competition for antigen selects for B cells that will produce mutant antibodies with the highest affinity for the antigen. This process is called affinity maturation, and results in the differentiation of B cells into effector plasma cells and memory B cells (Nimmerjahn and Ravetch, 2010; Stavnezer and Amemiya, 2004). The short lived B effector plasma cells are involved in many specific functions; whereas the memory B cell population is long lived, and the persistence of this population provides lifelong humoral immunity towards pathogen re-challenge (Dogan et al., 2009). The B effector plasma cell is most notably known for its tremendous antibody producing capacity; however, B effector cells can also produce cytokines that shape and direct the adaptive immune response, as well as stimulate T cells through antigen presentation (Dogan et al., 2009; Lund, 2008; Wojciechowski et al., 2009).

Activated B cells are important professional antigen presenting cells (APC). This occurs as BCR engagement of antigen results in antigen internalization and processing for peptide presentation in the context of major histocompatibility complex class-II (MHC-II) proteins. Mature T cells recognize and are stimulated by B cell antigen presentation through their peptide-specific T cell receptor (TCR). However, unlike the BCR which binds free antigen, the TCR only recognizes small peptides presented in the

context of the MHC II complex (Clark et al., 2003). During MHC II−TCR interaction, co-stimulatory molecules, CD86 and CD40 on the B cell interact with the $CD4^+$ T cell CD28 and CD40L, respectively, which stimulates the T cell to proliferate and become an effector cell as well. The $CD4^+$ T cells that respond to B cell antigen presentation are helper T cells (T_H cell), which proliferate, differentiate, and secrete large amounts of cytokines resulting in a directed adaptive immune response. In this manner, it becomes apparent that B cells play an integral role in the adaptive immune response specific to infecting pathogens (Rivera et al., 2001; Vascotto et al., 2007).

It should be noted that B cells are not homogeneous in their biological functions. There are three major subsets of mature naïve B cells: follicular B cells, marginal zone B cells, and B-1 B cells, with the latter being subdivided into either B-1a or B-1b B cells. Each B cell subset carries particular characteristics addressing their location, ability to migrate, and method of activation. Follicular B cells are the predominant B cell found within the spleen and circulatory system (Allman and Pillai, 2008), and the focus of this study; whereas, other B cell subsets are outside of the scope of this introduction.

1.3 T-DEPENDENT AND T-INDEPENDENT B LYMPHOCYTE ACTIVATION

Observations of humans and mice born without a thymus, led to the discovery of two different manners of B cell activation: activation occurring without thymus-derived T cells (T-independent activation), and activation requiring thymus-derived T cells (T-dependent activation). T-dependent activation requires direct protein receptor binding between a T_H cell and the B cell, whereas, T-independent activation does not require T_H cell interaction (Vos et al., 2000). T-dependent activation requires BCR engagement

(signal one) and CD40 co-receptor binding (signal two). MHC II−TCR interaction is required for CD40 binding CD40L on T cells, and provides survival signals necessary for full B cell activation and survival (Parry et al., 1994). T-independent activation requires large polymeric/repeating antigenic determinants. These can be subdivided into two groups, Ti-1 and Ti-2 antigens, based on their method of activation. Ti-2 antigens are not B cell mitogens, and therefore, do not activate B cells in a polyclonal fashion. Ti-2 antigens mediate their activation by cross-linking the BCR, and are usually polymeric proteins or bacterial cell wall components with repeating polysaccharide units (DeFranco et al., 2007; Travers and Walport, 2007; Coutinho and Moller, 1975; Vos et al., 2000). Conversely, Ti-1 antigens are mitogenic polyclonal activators of B cells. One of the most important Ti-1 antigens is LPS, a strong activator of the innate system. LPS not only interacts with the murine BCR, but also binds TLR4 on B cells. LPS can either indiscriminately or specifically cross-link the BCR, instigating either a polyclonal antibody response or an anti-LPS driven response, respectively. Moreover, LPS binding of TLR4 also initiates polyclonal production of antibody with diverse specificity. Interestingly, although T-dependent activation requires a second signal for B cell activation, T-independent activation via TLR4 is strong enough to not require a second signal. Therefore, both adaptive and innate immune signals can activate B cells, and be integrated within the same cell. Further commonality between T-dependent and T-independent activation is the resultant cell growth, up-regulation of molecules necessary for antigen presentation, proliferation and differentiation into plasma cells (Bone and Williams, 2001; Minguet et al., 2008; Vos et al., 2000).

1.4 CELL CYCLE PROGRESSION IN B LYMPHOCYTES

T-dependent or T-independent activation of mature naïve B cells results in clonal expansion or proliferation. At the heart of cellular proliferation is the cell cycle. The cell cycle is a tightly coordinated series of events leading to cellular growth, nucleic acid synthesis, and mitosis. Non-dividing quiescent cells remain in a gap 0 (G0)-phase. Transition from G0-to gap 1 (G1)-phase involves cellular growth and progression towards the restriction (R1)-checkpoint. Transition beyond the R1-checkpoint is necessary to enter the synthesis (S)-phase in which DNA synthesis takes place and transition into a short growth gap 2 (G2)-phase occurs. Furthermore, beyond R1-checkpoint, mitogenic signalling is not required. The second restriction (R2)-checkpoint inhibits G2-phase to mitotic (M)-phase transition. Cellular division or mitosis occurs in M-phase (Pardee, 1989; Vermeulen et al., 2003).

Progression through the cell cycle is cooperatively regulated by heterodimeric complexes of Ser/Thr cyclin-dependent kinases (CDK) and specific regulatory subunits called cyclins. Moreover, proper cell cycle progression requires activation of the appropriate cyclin−CDK complexes at the appropriate times (Sherr and Roberts, 1999). CDK expression levels are relatively constant throughout the cell cycle, whereas cyclin expression fluctuates during the cell cycle. Cell cycle progression depends on cyclin availability, based on rates of transcription and proteolysis, and determines the subsequent substrates of CDK-cyclin heterodimers throughout the cell cycle (Morgan, 1995). The role of cyclins, therefore, is to control CDK-mediated cell cycle progression through their phosphorylation of proteins at specific cell cycle stages.

Cell cycle progression commences with early progression from G0- to G1-phase. Cyclin Ds are involved in this early phase transition, and are mediators of extracellular signals. An increase in D-type cyclins is observed during mitogenic activation, which then complexes with CDK4 and CDK6. This results in the hyper-phosphorylation of the retinoblastoma protein (pRb). The pRb family are tumour suppressor proteins that, when hypo-phosphorylated, are bound to the E2F transcription factors that are essential for cell cycle progression from G0- to S-phase. Cyclin-binding of CDK allows the enzyme to hyper-phosphorylate pRb, disassociating it from E2F transcription factors, and allows E2F to translocate to the nucleus and induce the transcription of key cell cycle progression factors (Harbour and Dean, 2000). Once released from pRb, the E2F transcription factors are free to promote cyclin E expression, as well as many other factors involved in DNA replication (Massague, 2004). Cyclin proteins complexed with CDK are subject to CDK inhibitors (CKI), which modulate or constrain cell cycle progression in response to various stimuli (Sherr and Roberts, 1999). The most important CKIs in G1- to S-phase transition are $p27^{KIP1}$ and $p21^{WAF1}$, which inhibit cyclin E/A/CDK2 complexes from recruiting DNA helicases, primases, and polymerases necessary for DNA replication. Furthermore, $p27^{KIP1}$ can bind the cyclin D−CDK4/6 complex, blocking cell cycle progression at the G0/1-phase. However, during mitogenic stimulation, the previous increase in cyclin D/CDK4/6 complexes inhibits the essential cell cycle break, $p27^{KIP1}$, by sequestration, and encourages hyper-phosphorylation of the pRb protein. As mentioned earlier, the hyper-phosphorylation of pRb permits further transcription of cyclins E/A, which tips the activation balance in favour of CDK2 activation; moreover, CDK2 activation is able to phosphorylate $p27^{KIP1}$, and mark it for

ubiquitination (Massague, 2004). If the cell incurs replication errors or DNA damage by outside factors, the defect may induce a G1 or G2 stall through many differing sensory proteins. One major protein involved in this surveillance is p53, which can induce the expression of $p21^{WAF1}$ and thus inhibits cell cycle progression in G1. A similar restriction checkpoint at G2 (R2) can inhibit the phosphatase CDC25 by causing its nuclear exclusion or degradation, which in turn prevents cyclin A/B/CDK1 complex-mediated transitioning of the cell from G2- to M-phase. Although not reviewed here, other CKI, such as $p15^{INK4B}$, $p16^{INK4}$, $p57^{KIP2}$, also play an important role in cytostatic signalling (Blain et al., 2003; Massague, 2004).

1.5 SIGNAL TRANSDUCTION IN B LYMPHOCYTE ACTIVATION

B cell proliferation and differentiation into effector plasma or memory cell function depends upon signal transduction that occurs during activation (Genestier et al., 2007; Ogata et al., 2000; Omori et al., 2006; Pasare and Medzhitov, 2005). Signal transduction in B cells can occur in a T-dependent or T-independent manner; however, intrinsic cross-talk between aspects of T-dependent and T-independent activation signalling can occur (Bone and Williams, 2001; Minguet et al., 2008). Also of note, the absence of CD40 ligation or LPS stimulation during BCR engagement can lead to activation-induced B cell death; this process ensures that self antigen, in the absence of disease, will not induce autoimmunity (Donjerkovic and Scott, 2000). For the purpose of this study, three major mechanisms of B cell activation are discussed: BCR engagement, CD40 ligation, and LPS stimulation. All signalling pathways discussed are integral to B cell survival, activation, and differentiation (Al-Alwan et al., 2007; Banerjee et al., 2006;

Banerji et al., 2001; Purkerson and Parker, 1998; Ruprecht and Lanzavecchia, 2006; Schauer et al., 1996; Siebelt et al., 1997; Sutherland et al., 1996; Vivarelli et al., 2004; Zhu et al., 2002).

1.5.1 SIGNAL TRANSDUCTION BY BCR ENGAGEMENT

The BCR is essential to maturation, survival, activation, and differentiation. The BCR is composed of a membrane bound immunoglobulin flanked by a two Igα and Igβ heterodimers containing cytoplasmic tails. Antigen binding of the BCR causes BCR cross-linking and association with the CD19 complex (Depoil et al., 2008). The membrane bound B cell receptor moiety is highly specific for a particular antigen, and binding transmits a signal through the adjacent Igα and Igβ chains (Figure 1, A-B). The Igα and Igβ chains contain the immuno-receptor tyrosine-based activation motifs (ITAM), and are considered the signal transduction moiety (DeFranco, 1997). BCR Antigen binding causes receptor-associated protein tyrosine kinases (PTK) such as the resident Src kinase, Lyn, to phosphorylate ITAMs; however, antigen-mediated-aggregation of BCR molecules is necessary for sufficient PTK activation to initiate signal transduction (Dal Porto et al., 2004). Initial ITAM phosphorylation is mediated by Src kinases, such as Lyn, which creates specific spatially-defined tandem Src homology 2 (SH2) domain biding sites for the spleen tyrosine kinase (Syk). Syk couples BCR signal transduction to downstream signalling elements, such as through B cell linker protein (BLINK) (Rowley et al., 1995), and B cell adaptor for PI3K (BCAP), which themselves act as adaptor molecules for distal signalling elements (Figure 1, A) (Fu et al., 1998; Okada el al., 2000). These adaptor molecules, activated by Syk, are able to bind Bruton's

tyrosine kinase (Btk), phospholipase Cγ2 (PLC-γ2), growth factor receptor-bound protein 2 (Grb2), and the guanine nucleotide exchange factor, Vav, all of which contain and are recruited by their SH2 domains. Syk is then able to activate these molecules for further downstream signalling cascades. However, the limiting step in the activation of these SH2 domain-containing signalling elements is their recruitment through phosphatidylinositol-3-kinase (PI3K) activity (Dal Porto et al., 2004; Kurosaki, 2011).

The PI3K pathway is predominantly activated through the CD19 complex (Figure 1, A). The CD19 complex is composed of CD19, 21 and 81, and is activated by CD21 binding antigen tagged with the complement protein, C3d, which cross-links the BCR with the CD19 complex. CD19 contains the intracellular signalling region of the complex and is phosphorylated by Lyn, which creates binding sites for the SH2 domains of PI3K (Depoil et al., 2008). Recruitment of PI3K to CD19 localizes it to the cell membrane, where it is in proximity to its lipid substrate, phosphatidylinositol-4,5-bisphosphate (PIP2). PI3K phosphorylates the hydroxyl group of PIP2 to produce phsphatidylinositol-3,4,5-triphosphate (PIP3) (Dal Porto et al., 2004). The phosphatidylinositol, PIP3, enhances activation by recruiting intracellular enzymes having pleckstrin homology (PH) domains, such as Btk, PLC-γ, Vav, and Akt, which are all important in downstream signalling cascades (Okkenhaug and Vanhaesebroeck, 2003; Gold et al., 2000; Aiba et al., 2008).

However, PI3K can also be activated independently from CD19 signalling by BCR engagement, although to date, only demonstrated in avian B cells (Figure 1 A). BCAP bridges BCR-associated kinases to the PI3K pathway by regulating PI3K localization. BCAP, like BLNK, is phosphorylated by Syk, which presumably opens

SH2 binding domains, and is also activated by Btk. BCAP then binds PI3K, localizing it to the cell membrane where PI3K exerts its enzymatic activity (Okada et al., 2000; Kurosaki, 2011).

Perhaps one of the more important enzymes recruited by PI3K activity is Akt, a Ser/Thr kinase that plays an important role in cell growth, metabolism, proliferation, and survival (Figure 1, A). For full activation, Akt must be phosphorylated at both Ser^{473} and Thr^{308} sites by the Ser/Thr kinase phosphoinositide-dependent kinase (PDK)-1, and another poorly characterized enzyme (Vanhaesebroeck and Alessi, 2000; Mao et al., 2008; Yan el al., 2008). Activated Akt is able to inactivate several pro-apoptotic molecules, including Bad, Bax, and, forkhead transcription factors (FOXO), while promoting IκB kinase (IKK) activation and proteosome-mediated p53 degradation (Shin et al., 2002; Vanhaesebroeck and Alessi, 2000).

Other important intracellular enzymes recruited by PIP3 via PI3K activity are Btk and PLC-γ2 (Figure 1, A-B). Syk activates BLNK (Lindvall et al., 2005) exposing SH2-binding domains, which allows the PIP3-recruited Btk and PLC-γ2 to bind in close proximity. This provides PLC-γ2 access to its substrate, PIP2, and allows Syk to phosphorylate Btk, which by proxy is able to phosphorylate PLC-γ2 (Kurosaki and Hikida, 2007). The now activated PLC-γ2 hydrolyzes PIP2 to IP3 resulting calcium mobilization and producing second messenger diacylglycerol (DAG), which activates protein kinase C (PKCβ) (Lindvall et al., 2005). Although there are many isoforms of PKC, most have been demonstrated to be recruited and activated downstream of BCR engagement. As expected, PKC knockout mice have pronounced B cell deficits, including reduced numbers of B cells and antibody production. The defect is thought to

originate from an inability to activate NF-κB signalling by Btk/PLCγ signal transduction. During BCR engagement, activated PKC recruits IKK which allows NF-κB translocation (Figure 1, A) by phosphorylation-mediated degradation of inhibitor of κB (IκB), resulting in up-regulated expression of B cell lymphoma-extra large (Bcl-xL), a pro-survival member of the B cell lymphoma-2 (Bcl-2) family (Guo et al., 2004; Leitges et al., 1996; Moscat et al., 2003; Kurosaki, 2011).

Interestingly, BCAP has also been implicating in participating in PLC-γ2 activation; furthermore, BCAP-deficient avian B cells demonstrated a reduction in c-Rel/NF-κB expression, which may be a result of decreased PLC-γ2 activity. This is important as c-Rel/NF-κB activity is important in Bcl-2, cyclin D and CDK4 expression, and is necessary for cell survival and proliferation (Okada, 2000). Moreover, PI3K is known to play an important role in early B cell activation, and is involved in many cellular functions from cell growth or metabolism, to proliferation, differentiation and survival (Al-Alwan et al., 2007). Impaired PI3K signalling leads to extreme defects in B cell immunity, whereas up-regulated PI3K signalling can lead to malignancy (Fruman, 2004; Okkenhaug and Vanhaesebroeck, 2003).

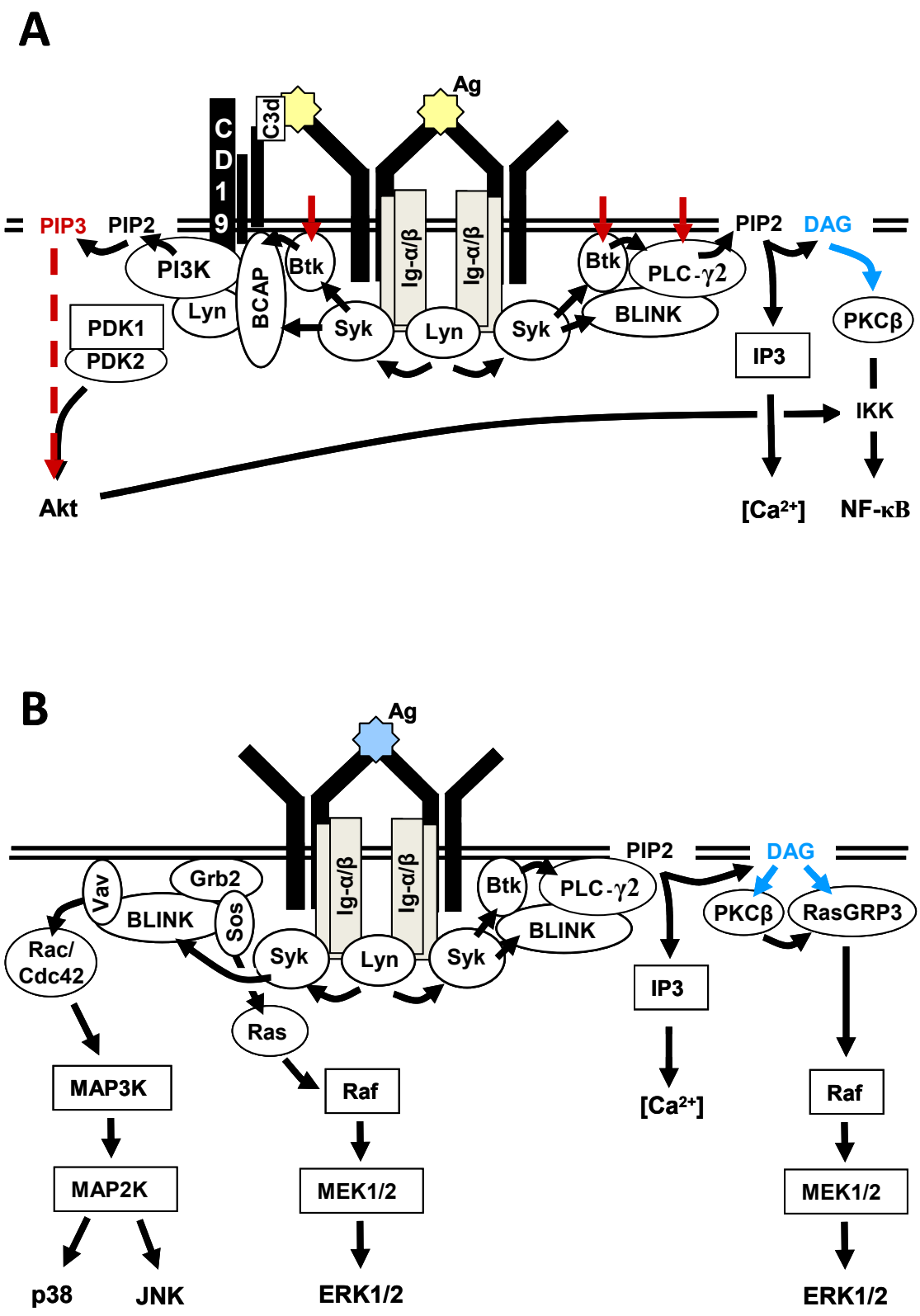
Another arm of BCR signalling consists of the mitogen activated protein kinase (MAPK) pathways (Figure 1 B). The MAPKs are a family of serine-threonine protein kinases, with three distinct subsets: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinases (JNK), and the p38 MAP kinases (Roux and Blenis, 2004). Both JNK and p38 are generalized as being, although not limited to, activated in the setting of environmental stresses such as UV light, whereas ERK1/2 activation is associated with growth factor stimulation (Sutherland et al., 1996). During BCR engagement, MAPK

signal transduction occurs through differing members of the Ras superfamily, including the Rho- and Ras-family of GTPases. These GTPases can activate multiple and distinct signalling pathways during BCR engagement. The Rho-family of GTPases, Rac, Rho and Cdc42, are recruited to the BCR by BLNK and activated by Vav, which is phosphorylated by Syk. In B cells, Rac and Cdc42 are assumed to be JNK- and p38-directed, whereas ERK1/2 is associated with Ras GTPase activity (DeFranco, 1997; Fu et al., 1998; Ishiai et al., 1999). These GTPases induce signal transduction by activating serine-threonine MAPK-Kinase-Kinases (MAP3K), which then activate MAPK-Kinases (MAP2K), leading to directed JNK, p38 or ERK1/2 phosphorylation (Sutherland et al., 1996).

The ubiquitously expressed ERK1/2 are 44 and 42 kDa Ser/Thr kinases, respectively (Pages et al., 1999; Chong et al., 2003). ERK1/2 downstream signalling is important in regulation of cell proliferation and substrate activation, for example, through c-myc phosphorylation (Roux and Blenis, 2004). ERK1/2 signal transduction commences with BCR engagement, and transduces through the guanine nucleotide exchange factor, son of sevenless (Sos) and its recruitment of growth factor receptor-bound protein 2 (Grb2) adaptor protein, a guanine nucleotide exchanger (Figure 1, B). Grb2/Sos bind RasGDP, which induces RasGTP generation (McKay and Morrison, 2007). RasGTP binds the MAP3K Raf, which phosphorylates the MAP2K, MEK1/2, which subsequently phosphorylates ERK1/2 kinases at Thr^{202} and Tyr^{204} residues (Chong et al., 2003; Pages et al., 1999). However, emerging data has implicated an alternative and dominant ERK1/2 activation pathway, which involves RasGRP3, another Ras exchange factor (Figure 1 B). Specifically, RasGRP3 is thought to be recruited by the

PLC-γ2 product DAG, and activated by PLC-γ2 product PKCβ. This results in increased RasGRP3 enzymatic activity that leads to Raf activation, which eventuates in ERK1/2 phosphorylation (Kurosaki, 2010). This pathway has been implicated as being the dominant ERK1/2 pathway, as Sos knockout avian B cells demonstrated normal Ras activation, whereas a dramatic reduction in Ras activation occurred in RasGRP3 knockout B cells (Oh-hora et al., 2003). Moreover, it has been demonstrated that PLC-γ2 is required for BCR-ERK1/2 signalling in murine B cells (Bell et al., 2004).

Figure 1. Signal transduction by BCR engagement. BCR engagement (A) with antigen causes protein tyrosine kinase, Lyn, to phosphorylate the ITAM motifs on Igα and Igβ. This results in Syk recruitment and activation, which phosphorylates BLINK and BCAP adaptor molecules. During BCR engagement, the CD19 complex is also activated by Lyn, resulting in PI3K activity. PI3K also activated by BCAP, links BCR and CD19-mediated signalling pathways. Activated PI3K produces PIP3, which recruits (red arrows) Akt, Btk and PLC-γ2. Recruited Akt is then activated by PDK1/2, whereas recruited Btk and PLC-γ2 are bound by the activated BLNK, promoting PLC-γ2 catalytic conversion of PIP2 to DAG and IP3 second messengers. Production of IP3 results in Ca^{2+} influx, whereas production of DAG results in the recruitment (blue arrows) of PKCβ, which activates of IKK. Furthermore, production of DAG recruits RasGRP3 (B) to become activated by PKCβ leading to ERK1/2 activation. Alternatively, the Grb2/Sos complex which is bound by BLINK results in ERK1/2 activation. Similarly, Vav bound to BLINK activates p38 and JNK MAPK.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C3d
CD19
PIP3
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BCAP
Btk
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Ig-α/β
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DAG
Vav
Grb2
BLINK
Sos
Rac/
Cdc42
Syk
Lyn
Syk
Btk
PLC-γ2
BLINK
PKCβ
RasGRP3
IP3
Ras
MAP3K
Raf
[Ca2+]
Raf
MAP2K
MEK1/2
MEK1/2
p38
JNK
ERK1/2
ERK1/2

Figure 1.

1.5.2 SIGNAL TRANSDUCTION BY CD40 LIGATION.

The BCR is undoubtedly vital to B cell activation and differentiation; however, other necessary receptors can positively or negatively influence the functionality of B cell signalling by modulating downstream signal thresholds of the BCR. Activation-induced cell death occurs in mature naïve B cells that bind self-antigen in the absence of T cell help (Harnett, 2004; Donjerkovic and Scott, 2000). This clonal deletion is necessary to prevent autoimmunity. However, during appropriate non-self antigen engagement, causing the BCR to cross-link, a second survival signal is provided by CD40 binding CD40-Ligand (CD154) on T cells (Parry et al., 1994). Trimeric CD40L exerts its potent biological activity by binding and causing receptor aggregation of cell surface CD40 (Bishop, 2004). Activation of CD40 results in full B cell activation, survival, germinal center formation, immunoglobulin class switch, affinity maturation and differentiation into effector plasma or memory cells (Bishop and Hostager, 2003; Clatza et al., 2003).

CD40 (Figure 2, A-B) is a member of the TNF-receptor superfamily which signals through TNF-receptor associated factors (TRAF) 1, 2, 3, 5 and 6 (Hanissian and Geha, 1997; Pullen et al., 1999; Wajant et al., 2004). Activated CD40 can bind directly TRAF2, 3, and 6, and can indirectly bind TRAF1 through its heterodimerization with TRAF2 (Brown et al., 2001), similarly, TRAF5 is indirectly bound to CD40 in a TRAF3-dependent manner (Leo et al., 1999). Interestingly, although TRAF6 can bind CD40 directly, it is believed that TRAF2 recruits and binds TRAF6, which allows CD40-mediated TRAF6 signalling (Hostager, 2007). It is generally understood that TRAF proteins act as adapter proteins binding necessary molecules for directed CD40-driven downstream signalling events (Bishop and Hostager, 2003).

Specifically, TRAF1 appears to decrease TRAF2 degradation, which augments TRAF2-mediated JNK, p38 activation (Hostager et al., 2003; van and Bancherear, 2000; Arcipowski et al., 2001; Xie et al., 2006), IgM production (Brown et al., 2001), class switch recombination (Jabara et al., 2009), as well as TRAF2-mediated NF-κB activation (Qing et al., 2005; Oeckinghaus et al., 2011; Wajant et al., 2001). Furthermore, TRAF2 induces TRAF3 degradation (Brown et al., 2001; Brown et al., 2002; Bishop, 2004), which plays a negative role in decreasing JNK activation, IgM production, co-stimulatory molecule up-regulation, as well as inhibiting CD40-BCR synergy (Ying et al., 2011; Haxhinasto and Bishop 2004; Bishop, 2004; Grammer et al., 1998). TRAF5 however, is involved in increasing co-stimulatory molecule expression, immunoglobulin production as well as proliferation (Arcipowski et al., 2011; Nakano et al., 1999). TRAF6 is involved in JNK and ERK activation, increasing NF-κB activation, IL-6 production, class switch recombination, and increasing co-stimulatory molecule up-regulation (Kashiwada et al., 1998; van and Banchereau, 2000; Wajant et al., 2001; Bishop, 2004).

Interestingly, CD40 signalling through a TRAF6-dependent or -independent pathway, results in a Ras-independent or -dependent activation of ERK1/2 (Figure 2, B), respectively (Kashiwada et al., 1998; van and Banchereau, 2000). However, this pathway was elucidated in the human embryonic kidney 293 cell line, and has yet to be demonstrated in murine B cells. TRAF6-mediated ERK1/2 signal transduction is thought to occur through the MAP2K, MEK1, converging on the BCR−ERK1/2 signalling pathway (Purkerson and Parker, 1998). BCR engagement, without CD40 ligation, results in strong ERK1 and weak ERK2 activation, as well as weak JNK and P38 activation. Conversely, CD40 ligation results in strong JNK and p38 activation, and weak ERK1/2

activation. This MAPK signalling cascade relationship may provide insight into the process by which both receptors synergise to regulate B cell activation and differentiation (Sutherland et al., 1996). Such as signalling by the BCR in the absence of CD40 ligation abrogates sustained ERK1/2 activation; however, BCR and CD40 ligation promotes prolonged ERK1/2 activation and rescues cell cycle arrest (Gauld et al., 2002), although it has been noted that CD40 may play a minimal role in ERK1/2 activation (Pearson et al., 2001).

Another example of BCR-CD40 cross-talk between BCR and CD40 signalling also exists (Figure 2, B). During BCR engagement, Btk activation promotes TRAF2 activity, which is assumed to counteract TRAF3 negative signalling events (Ying et al., 2011; Haxhinasto and Bishop 2004). Conversely, CD40 signalling strongly augments BCR-induced activation of Syk, which promotes activation of Btk, BLNK and PLC-γ2 (Ying et al., 2011; Harnett, 2004). This presumably results in IP3-mediated Ca^{2+} mobilization and the formation of second messenger DAG, leading to translocation of NF-κB and ERK1/2 phosphorylation (Oh-hora et al., 2003; Bell et al., 2004; Kurosake, 2010).

Investigation into CD40-mediated PI3K signalling has delineated two distinct pathways (Figure 2, A). The first describes a TRAF3 association with PI3K through the recruitment of PI3K subunit p85 (Ha and Lee, 2004), bringing PI3K into proximity with its substrate. The second apparent CD40-mediated PI3K signalling pathway occurs through the proto-onco Casitas B-lineage Lymphoma (Cbl) family of proteins (Figure 2A). Although Cbl family of proteins have been held to play inhibitory roles in PTK-signalling, work has also demonstrated Cbl is required for CD40-mediated Akt activation

indicating a Cbl-positive relationship with PI3K. This may be a temporal arrangement as Cbl may act as a temporary scaffold for the assembly of PI3K signalling complexes by associating with the PI3K p85 subunit and Src family of kinases before Cbl shortens the duration of signalling by acting as an E3 ubiquitin ligase (Arron et al., 2001; Hostager, 2007). Furthermore, CD40-mediated PI3K signalling supports Akt degradation of IκB. This promotes NF-κB activation, which is required for B cell proliferation and pro-survival Bcl-xL gene expression (Andjelic et al., 2000).

Tight regulation of TNF-Receptor-family signalling is necessary, as aberrant regulation is implicated in the development of certain autoimmune diseases and malignancies (Aggarwal, 2003). One mechanism involved in this regulation is through receptor-induced degradation of TRAFs. Indeed, this receptor-mediated regulation has been demonstrated in B cells in CD40-mediated degradation of TRAF3 by TRAF2 (Brown et al., 2001; Brown et al., 2002; Bishop, 2004). For example, under resting conditions TRAF3 negatively regulates CD40 signalling (Arcipowski et al., 2011) by inhibiting non-canonical NF-κB activation through promoting proteosomal degradation of NF-κB inducing kinase (NIK). However, upon CD40 ligation TRAF2 mediates TRAF3 degradation, which permits NIK activation (Figure 2, B). Subsequently, NIK is then able to phosphorylate NF-κB precursor protein p100 for proteosomal processing into the p52 subunit allowing NF-κB translocation to the nucleus (Qing et al., 2005; Oeckinghaus et al., 2011; Vallabhapurapu et al., 2008). Although TRAF2 is important in the non-canonical NF-κB pathway, TRAF1, 2, 5, and 6 are all known to interact with NIK to some degree (Wajant et al., 2001).

TRAF2 and 6 are also known to be strong canonical NF-κB activators through CD40-mediated activation of TAK1 (Bishop, 2004; Wajant et al., 2001), which implies overlapping functions for these TRAF molecules. TAK-1 is crucial for proliferation on CD40- and BCR-stimulated B cells (Figure 2, B), which is likely a result of downstream IKK, JNK and p38 activation (Chen et al., 2006). Moreover, CD40-induced NF-κB activation has been associated with increased survival and proliferation (Zhu et al., 2002), which corresponds to the maintenance of the transcription factor, c-myc. This is associated with mitigating activation-induced cell arrest caused by BCR engagement alone (Schauer et al., 1996; Siebelt et al., 1997).

B cells also play an important role in antigen presentation and T cell stimulation. Following CD40 ligation, B cells increase their expression of CD80 and CD86 co-stimulatory molecules, as well as adhesion molecules that are necessary for efficient B cell−T cell interaction (Bishop and Hostager, 2003). B cell effector function is also augmented by CD40 ligation, as CD40 signalling is required for class switch recombination (Pasare and Medzhitov, 2005) and enhanced IL-10 production. However, further cytokine production, such as IL-6, requires synergistic signalling by other cytokines or BCR engagement (Burdin et al., 1997; Jeppson et al., 1998).

Figure 2. Signal transduction by CD40 ligation. CD40 ligation signals through TRAF1 through 6. Akt phosphorylation (A) is a consequence of TRAF3-mediated activation of PI3K, or TRAF6 signalling through Cbl. Subsequent signalling results in PI3K-mediated production of PIP3, which recruits (red dotted lines) and activates Akt. CD40-BCR cross-talk pathways are indicated by black dotted lines (B). CD40 activates Syk kinase which phosphorylates Btk protein. Conversely, BCR-mediated cross-talk occurs through Btk activation of TRAF2. CD40-BCR cross-talk activates many common pathways resulting in NF-κB translocation as well as EKR1/2, JNK and p38 activation. Both CD40 and BCR signalling converge in ERK1/2 phosphorylation, however, CD40-mediated ERK1/2 activation may occur through a TRAF6 Ras-independent pathway converging with BCR signalling at MEK1, or through CD40-mediated ERK1/2 activation in a Ras-dependent fashion. CD40-mediated NF-κB, JNK, and p38 pathways are activated through TRAF1,2,5 and 6, either independently (not shown), or through either the canonical (TAK-1) or non-canonical (NIK) pathways.

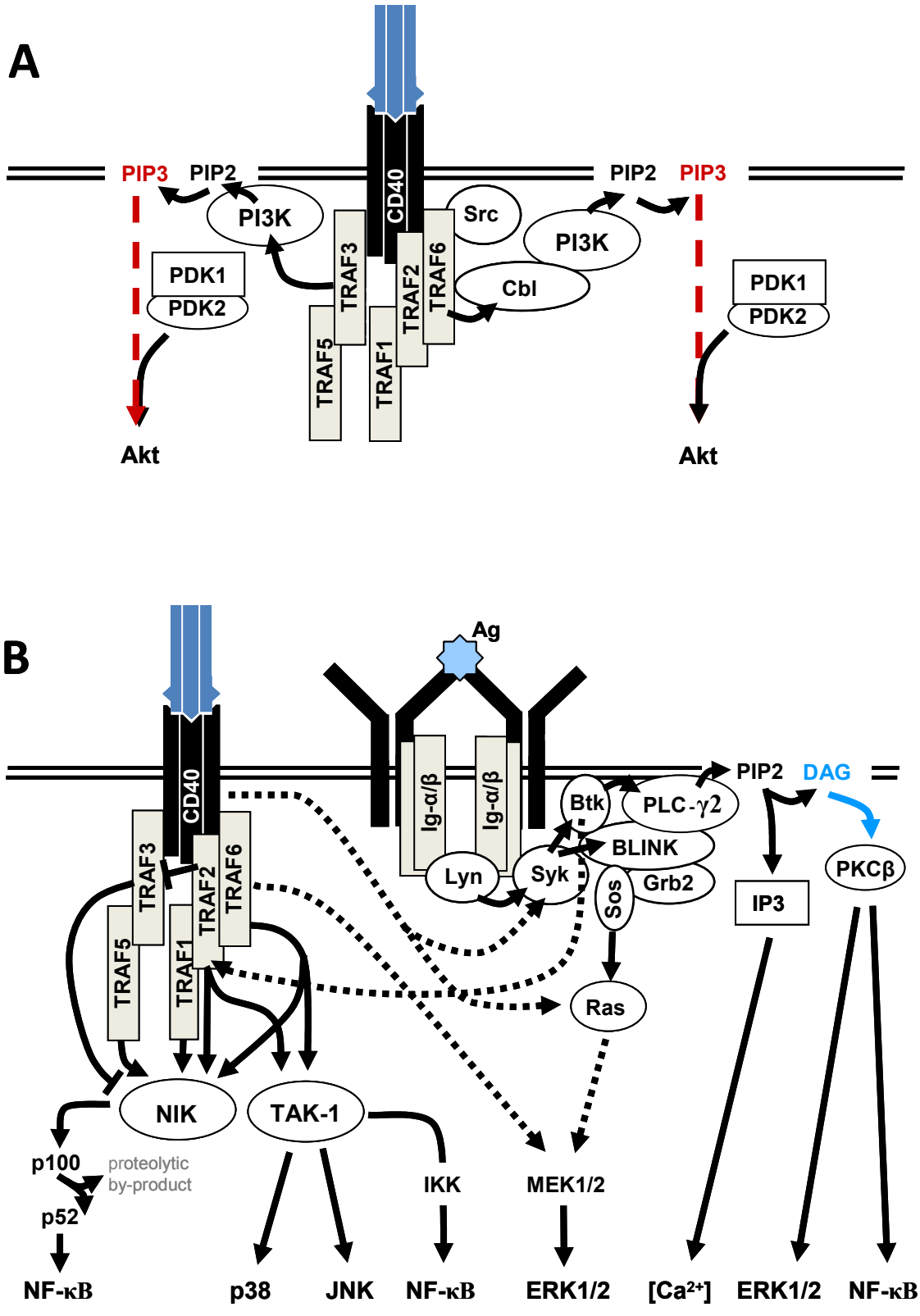

A
PIP3
PIP2
PI3K
CD40
Src
PI3K
PIP2
PIP3
PDK1
PDK2
TRAF3
TRAF2
TRAF6
Cbl
TRAF5
TRAF1
PDK1
PDK2
Akt
Akt
B
Ag
Ig-α/β
Ig-α/β
CD40
PIP2
DAG
Btk
PLC-γ2
BLINK
Lyn
Syk
Grb2
Sos
PKCβ
IP3
TRAF3
TRAF2
TRAF6
TRAF5
TRAF1
Ras
NIK
TAK-1
p100
proteolytic
by-product
p52
IKK
MEK1/2
NF-κB
p38
JNK
NF-κB
ERK1/2
[Ca2+]
ERK1/2
NF-κB

Figure 2.

1.5.3 SIGNAL TRANSDUCTION BY TLR4 LIGATION.

Toll-like receptors (TLR) are pattern recognition receptors that recognize common microbial products, PAMPs, which are known to initiate an innate immune response. TLRs consist of an extracellular leucine-rich repeat domain, a transmembrane region and a cytoplasmic domain homologous to the cytoplasmic region of the interleukin-I receptor (Ogata et al., 2000). It is widely accepted that TLR signalling promotes the activation and maturation of DC, which in turn stimulate T cell activation. However, there is now increasing evidence supporting the importance of TLR-mediated activation of B cells and elicitation of the humoral response (Gururajan et al., 2007; Pasare and Medzhitov, 2005).

There are many well described natural and synthetic TLR agonists including: peptidoglycan and Pam3CSK4 (TLR1/2), bacterial lipoproteins and MALP2 (TLR2/6), dsRNA and poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), ssRNA and imidazoquinolines (TLR7/8), unmethylated CpG oligodeozynucleotides (TLR9), and profiling-like molecule (TLR11) (Genestier et al., 2007) to name a few. To date, TLR1 through TLR13 are expressed in mice, however, TLR10 is non-functional due to a retroviral insertion (Kawai and Akira, 2010). With the possible exception of TLR5 and 8, all TLRs are known to be expressed on splenic murine B cells (Gururajan et al., 2007; Pasare and Medzhitov, 2005; Bekeredjian-Ding and Jego, 2009). Interestingly, the function of TLR-mediated signalling may be dependent on the B cell subset and activation status (Allman and Pillai, 2008; Gururajan et al., 2007), with respect to T-dependent or T-independent activation. Although marginal zone B cells are known to be important and predominant in T-independent immune responses (Gururajan et al., 2007),

follicular B cells are also able to respond in a T-independent fashion. Furthermore, signalling by BCR engagement of T-dependent antigen is thought to synergize with CD40 and TLR ligation by promoting plasmablast differentiation (Allman and Pillai, 2008). Interestingly, in the generation of T-dependent antigen-specific antibody responses, the necessity for TLR activation is controversial. Specifically, MyD88 adaptor protein, utilized in most TLR signalling events, may be dispensable to enhance a Td-antibody response; however, TLR signalling has shown to be important in germinal centre formation, IgG antibody response, and increasing B cell ability to discriminate between antigens based on TLR ligand properties (Palm and Medzhitov, 2009; Pasare and Medzhitov, 2005; Hou et al., 2011).

The antigenic lipid-A component of LPS is a fundamental component of a Gram-negative bacteria cell wall, and arguably one of the most important innate triggers in the initial stages of antimicrobial host defense (Kearney and Lawton, 1975; Ogata et al., 2000). LPS recognition occurs through cell surface CD14 receptor binding (Figure 3) and association with the signal-transducing TLR4 homodimer and MD-2 glycoprotein (Minguet et al., 2008). Binding of LPS can result in dual signal transduction by the myeloid differentiation factor 88 (MyD88) and the TIR domain-containing adaptor inducing interferon-β (TRIF) pathway (Yamamoto et al., 2003; Minguet et al., 2008).

Signal transduction through MyD88 adaptor molecule recruits IL-1 receptor associated kinases (IRAK) to TLR4, where IRAK then associates with TRAF6. TRAF6 binds MAP3K, transforming growth factor β-activated kinase (TAK-1), with aid of specific binding proteins, as well as the MAP3K, tumour progression locus 2 (Tpl2). The TAK-1 pathway further diverges to activate p38 and JNK, as well as the IKK pathway;

whereas, the Tpl2 pathway results in ERK1/2 activation (Barton and Medzhitov, 2003; Gerondakis et al., 2007), but not p38 or JNK activation (Banerjee et al., 2006). TRAF6-activated Tpl2 is bound by the NF-κB p50 precursor, p105. IKK activated by TAK-1 phosphorylates p105, targeting it for ubiquitination, and releasing Tpl2 to phosphorylate MAP2K that activate ERK1/2 (Gerondakis et al., 2007). IKK also phosphorylates I-κB, allowing NF-κB subunits to translocate into the nucleus. NF-κB plays a role in LPS-mediated B cell proliferation, cytokine production, as well as antibody production (Doi et al., 1997; Deenick et al., 2005; Gerondakis et al., 2007).

Conversely, the MyD88-independent pathway signals through TRIF, which utilizes TRAF3 to induce transcription of Interferon-regulator-factors (IRF) important in B cell development and immunoglobulin production, or through TRAF6 to activate NF-κB (Takeda and Akira, 2004; Honda and Taniguchi, 2006). TRIF-dependent TRAF6-mediated signalling, like MyD88-dependent signalling, also activates TAK-1 and associated downstream events (Honda and Taniguchi, 2006).

Further upstream, PI3K associates with MyD88 via its p85 subunit and becomes activated during TLR4 signalling in a TAK-1-dependent manner. Interestingly, while prolonged PI3K activity may have a negative feedback role in TLR4 signalling, immediate PI3K activity appears to be essential for cell survival in response to LPS stimulation (Laird et al., 2009; Vivarelli et al., 2004). As seen with CD40- and BCR-mediated signalling, Akt activation occurs as a result of PI3K signalling, and results in B cell growth, metabolism, proliferation, and survival (Vanhaesebroeck and Alessi, 2000).

Interestingly, although the non-canonical NIK-NF- κB pathway is important in CD40-mediated B cell signalling (Liao et al., 2004), it appears to play a minor role

compared to the canonical pathway which remains dominant in TLR4-mediated B cell signalling (Sun, 2011). However, research has demonstrated that TLR4 and CD40 signalling activates many overlapping biological functions, such as cell survival, proliferation, metabolism and class switch recombination (Doyle et al., 2002).

Figure 3. B lymphocyte TLR4 signal transduction. CD14 receptor binding and association with the signal-transducing TLR4 homodimer and MD-2 glycoprotein initiates signal transduction. Dual signalling occurs through MyD88 and TRIF pathways. Although TRIF signals through IRF by TRAF3, TRIF also signals through TRAF6 to activate TAK-1. The MyD88 pathway similarly signals through the TRAF6-TAK-1 pathway, which activates p38, JNK, and NF-κB directly, as well as through IKK-mediated phosphorylation of p105, releasing p50. Phosphorylation of p105 releases Tpl2, allowing it to activate ERK1/2. TAK-1 also augments PI3K activity, which promotes Akt activation.

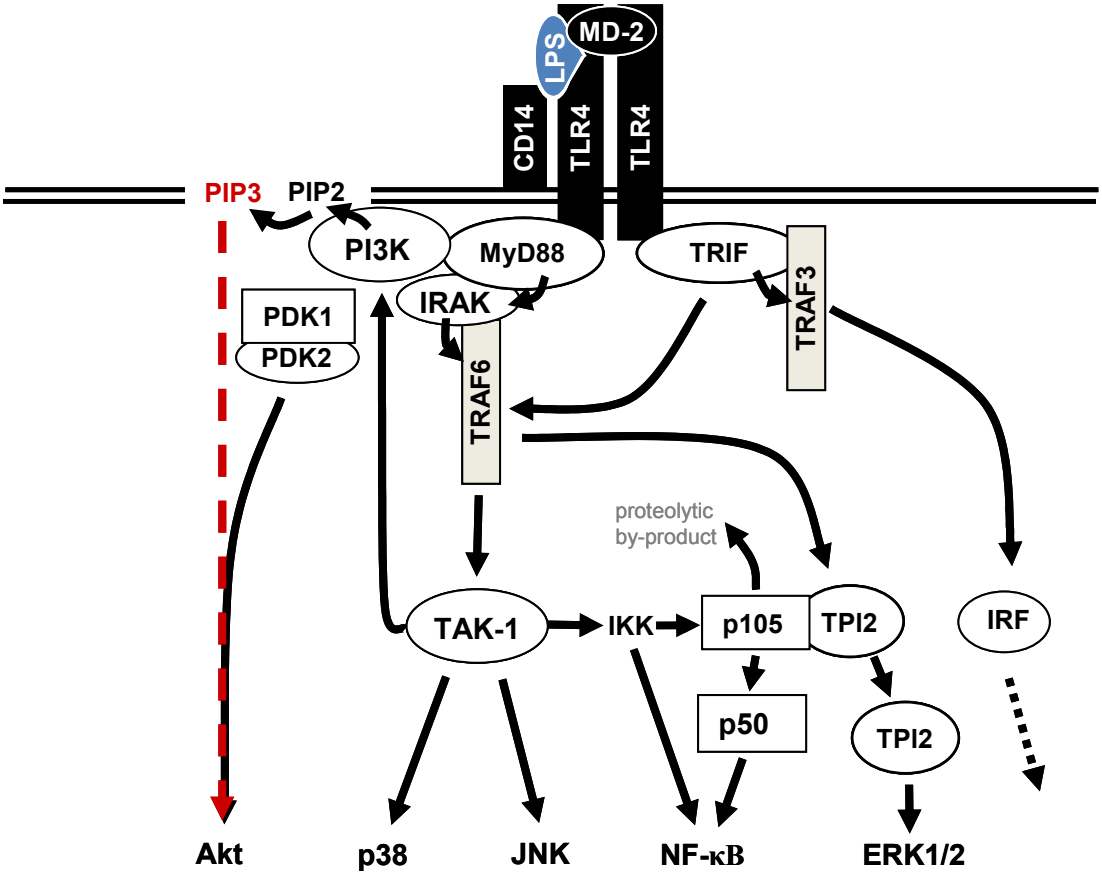

LPS
MD-2
CD14
TLR4
TLR4
PIP3
PIP2
PI3K
MyD88
TRIF
TRAF3
PDK1
PDK2
IRAK
TRAF6
proteolytic
by-product
TAK-1
IKK
p105
TPI2
IRF
p50
TPI2
Akt
p38
JNK
NF-κB
ERK1/2

Figure 3.

1.6 B LYMPHOCYTE EFFECTOR FUNCTION.

B cells possess effector functions beyond antibody production. Although antibody production is integral to the humoral system, there are many other important niches that B cells occupy. For example, B cells are important antigen presenting cells (APC), which help stimulate a strong T cell response (O'Neill et al., 2005). B cells have also been recently recognized as important players in cytokine production, and in directing T cell responses. Indeed, B cells are capable of not only contributing to a directed T cell response, but also in dampening an immune response as B regulatory cells (Harris et al., 2000; Mizoguchi and Bhan, 2006). Below, cytokine and antibody production, as well as B cell antigen presentation are discussed.

1.6.1 B LYMPHOCYTE CYTOKINE PRODUCTION.

It is well understood that T cells possess distinct subsets and roles in effector function, and that their cytokines play an important role in regulating the immune system. Current studies, however, have demonstrated that a reciprocal relationship exists between B and T cells, observed by distinct B cell subsets and polarized cytokine production (Harris et al., 2000). The study done by Harris et al., (2000) demonstrated *in vitro* that B cells can differentiate into subsets they refer to as B effector-1 (Be1) and B effector-2 (Be2) cells. These B effector subsets are able to produce cytokines with a comparable or directional profile similar to T_H1 and T_H2 T cell subsets. A reciprocal relationship with T cells became evident from the demonstration that cultured T cells in the presence of either Be1 or Be2 cells had a tenfold increase in IL-2 production over control cultured T cells, yet, importantly, the T cells cultured in the presence of Be1 cells had an additional

tenfold increase in IFN-γ production. A similar effect was observed on IL-4 production by T cells in the presence of Be2 cells. Moreover, Harris et al., (2000) went further by delineating Be1 cells as IFN-γ and IL-12-producing, and Be2 cells as IL-4, IL-6 and IL-10-producing effector cells. This directly shows that B cells not only augment T cell responses, but provide additional polarization of T cell responses *in vitro*. Although Harris et al., (2000) were unsuccessful in delineating whether B effector cell polarization of naïve T cells is valid within the *in vivo* immune response, a recent *in vivo* approach to assess the importance of effector B cell function was examined by Wojciechowsk et al. (2009), who looked at the contribution of B cells in generation of successful helper T memory cells against *Heligmosomoides polygyrus* infection (Wojciechowski et al., 2009). This study found that, although antibody generation was important to *H. polygyrus* immunity, it was not solely responsible for mediating protective immunity. Their results indicate that enhancement of primary and memory T_H cell response was in part a direct result of B cell antigen presentation and cytokine production (Wojciechowski et al., 2009). Of course, cytokine production also depends on the method of B cell activation and its setting.

For example, it has recently been shown that T_H1 cell-dependent activation of B cells increases B cell IFN-γ production via T_H1 cell IFN-γ production. Similarly, T-independent activated B cells increase B cell IFN-γ production in the presence of an IL-12 cytokine milieu (Harris et al., 2005). However, TLR4-activated B cells increase both IL-10 and IL-6 production (Barr et al., 2007). Our understanding of B cell cytokine function is in its infancy, although clearly it has important implications for immune activation.

1.6.2 B LYMPHOCYTE IMMUNOGLOBULIN PRODUCTION.

During B cell clonal expansion, affinity-matured antibody is produced on the order of thousands of molecules per second, which are targeted towards specific antigens and aid in the clearance of the B cell-stimulating pathogen (Ma and Hendershot, 2003). Antibodies can act directly by toxin or virus neutralization, or they can act as adaptor molecules bridging the pathogen to the immune system, such as by opsonization and complement activation (Casadevall and Pirofski, 2004).

During a primary infection, the first antibodies produced are of the IgM isotype. These antibodies have not undergone recombination or affinity maturation, and thus do not bind antigen with great affinity. However, as IgM is a pentameric structure, the avidity is relatively high, which promotes antigen coating during early stages of infection (Racine et al., 2011). In 1964, Nossel et al., experimentally observed for the first time that B cells undergo class-switch recombination (CSR) when activated to produce immunoglobulin of differing isotypes: IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE (Deenick et al., 2005). Isotype selection is controlled by two variables. One variable is cytokine-dependent, and results in specific transcription that determines isotype specificity. The second variable is the mode of B cell activation, whether that be T-dependent or T-independent activation. For T-dependent antigen-specific antibody class-switch to occur, the second signal emanates from CD40 binding CD40-Ligand on T_H cells, which activates machinery required for DNA recombination. For T-independent antibody class-switch to occur, the second signal emanates from TLR4 following LPS binding (Bacharier and Geha, 2000; Deenick et al., 2005). Key cytokines, such as IL-4, IFN-γ

and TGF-β play an important role in antibody class-switch. During T-dependent and T-independent activation, both IgE and IgG1 are produced in the presence of IL-4, and IgG2a in the presence of IFN-γ. However, in the presence of TGF-β, T-dependent activation induces little IgG2b and abundant IgA, while T-independent activation induces abundant IgG2b, and little IgA. Interestingly, only T-independent activation produces IgG3 (Deenick et al., 1999).

1.6.3 B LYMPHOCYTE FUNCTION IN ANTIGEN PRESENTATION.

Antigen presentation occurs as mature naïve B cells capture antigen by their highly specific BCR. BCR antigen engagement leads to receptor-mediated endocytosis, resulting in endocytic pathway-mediated processing of antigen, and peptide presentation on MHC II. Similar to the BCR, T_H cells possess a T cell receptor (TCR) that is highly specific to a particular peptide presented in the context of MHC II. Along with TCR recognition of peptide in the context of MHC II, expression of B cell co-receptors CD40, CD80 and CD86 is required to stimulate a robust T cell response (Vascotto et al., 2007; Rivera et al., 2001). B cells are understood as being essential in the generation of memory and effector T cells, and may be even more important than DC (Crawford et al., 2006). Furthermore, B cells are required for systemic T cell responses under limited antigen availability (Rivera et al., 2001). Both CD80 and CD86 are important in stimulating a T cell response by binding CD28, allowing sustained T cell proliferation and survival. Important to this study, CD86 is structurally similar to CD80, and equally important in B cell antigen presentation (Lenschow et al., 1996). CD86 expression is up-regulated in the presence of LPS, BCR cross-linking, and CD40 ligation (Hathcock et al.,

1994; Lenschow et al., 1996; Vivarelli et al., 2004). Interestingly, CD86 and MHC II can be up-regulated but not CD80, as observed in *N. meningitides*-activated B cells (MacLeod et al., 2008). Furthermore, the importance of CD86 is demonstrated by the observation that B cells from CD86 knockout mice have deficient isotype switching, as well as aberrant germinal center formation (Borriello et al., 1997). CD40 and MHC II have a common relationship, in that stimulation of one causes the up-regulation of the other. As well, candidate signals by TLR on B cells may up-regulate expression of both CD40 and MHC II (Clatza et al., 2003). Taken together, B cells are able to induce a strong T cell response to both T-dependent and T-independent activation, and up-regulate those molecules necessary for antigen presentation when activated.

1.7 PIPERINE

Piperine (1-peperoylpiperidine), the major alkaloid found in the fruit bodies of black and long pepper plants, *Piper nigrum* and *Piper longum*, respectively (family Piperaceae), is derived from one of the most widely used spices (Srinivasan, 2007). Piperine has played an important role in traditional medicine worldwide, stretching from Asia to the South Pacific (Singh, 1992). Piperine has been touted as having antidepressant, hepato-protective, anti-metastatic, anti-thyroid, and antitumor effects (Srinivasan, 2007). Although the above effects are beyond the scope for this study, significant research has gone into examining the anti-inflammatory effects of piperine, and is outlined below.

1.7.1 THE ANTI-INFLAMMATORY EFFECTS OF PIPERINE

Piperine has been reported to posses many anti-inflammatory properties. Bae et al., (2010) demonstrated that piperine reduced LPS-induced endotoxin shock through inhibition of type 1 interferon production in peritoneal macrophages, as well as inhibition of leukocyte accumulation. Piperine has also been shown to inhibit NF-κB signalling and pro-inflammatory cytokine gene expression in B16F-10 melanoma cells (Pradeep and Kuttan, 2004). Similarly, Kumar et al., (2007) demonstrated a piperine-dependent suppression of NF-κB and IκB kinase activation in endothelial cells, resulting in decreases in the TNF-α induced expression of ICAM-1, VCAM-1 and E-selectin, which required for leukocyte infiltration into inflammatory site. Eosinophil infiltration was inhibited in an ovalbumin-induced asthma murine model in the presence of piperine; furthermore, this study demonstrated that piperine inhibited airway hyper-responsiveness by suppressing T cell activity and T_H2 cytokine production (Kim and Lee, 2009). Piperine can decrease pro-inflammatory cytokine production in many other cell types: such as decreased MCP-1 by macrophages and adipocytes in an obesity-induced inflammatory response (Woo et al., 2007); decreased IL-1, IL-6, IL-12, TNF-α, and granulocyte monocyte-colony stimulating factor by malignant B16F-10 melanoma cells (Pradeep and Kuttan, 2004); and decreased IL-6 by IL-1β stimulated synoviocytes in a rat arthritis model (Bang et al., 2009). Furthermore, piperine can inhibit key inflammatory signalling pathways. Piperine was shown to inhibit IKK activation (Kumar et al., 2007; Pradeep and Kuttan, 2004) and translocation of NF-κB p50 and c-Rel subunits (Pradeep and Kuttan, 2004), as well as MAPK/ERK signalling (Bang et al., 2009; Pradeep and Kuttan, 2004). Although current research has demonstrated piperine to have several anti-

inflammatory properties, little is known concerning the effect of piperine on the B cell function.

1.7.2 TRPV1

Transient receptor potential vanilloid 1 (TRPV1) is believed to mediate the hot sensation that follows ingestion of piperine, and is also activated by heat and capsaicin (Hayes et al., 2000). TRPV1 is a member of the transient receptor potential family of non-selective cation channels. Many naturally occurring compounds are activators of TRPV1, such as the vanilloid compounds, cactus extract resinferatoxin and ginger extracts zingerone and gingerol (McNamara et al., 2005; Szallasi and Blumberg, 1999), as well as non-vanilloid compounds such as the fungal extract scutigeral and the black pepper extract piperine (Sterner and Szallasi, 1999; McNamara et al., 2005). TRPV1 is also known to be activated by injurious heat (>42ºC) and high proton concentrations (pH<6). TRPV1 is known to be expressed on sensory neurons involved in pain perception pathways (Hayes et al., 2000). Of interest, TRPV1 has been shown to be up-regulated in chronic conditions such as inflammatory and neuropathic pain, thereby contributing to the pathology of the disease (McNamara et al., 2005).

The expression of TRPV1 is not limited to sensory neurons. TRPV1 has been found on many cell types, including as keratinocytes, epithelial cells, peripheral cells, and mast cells, however, the presence of TRPV1 on B cells remains controversial. In one study by Mori et al., (2002) that using avian DT40 B cells, the authors found TRP1 to be present and functional. Whereas, in a more recent study by Inada et al., (2006) TRPV1 was not detected on murine splenocytes, and indeterminable on avian DT40 B cells. In

the latter study, reverse transcription-PCR was used to examine TRPV1 expression by B cells (Inada et al., 2006), whereas in the former study, Mori et al., (2002) used genetic disruption of TRP1 and measured Ca^{2+} release as a readout. Unfortunately, it is unclear as to whether the "TRP1" investigated by Mori et al., is in fact TRPV1; furthermore, the presence of TRPV1 mRNA does not ensure protein expression, nor does the lack of TRPV1 expression in avian cells preclude murine TRPV1 expression. B cell TRPV1 expression therefore remains contentious.

1.8 SUMMARY

Previous investigations have demonstrated the anti-inflammatory effects of piperine on many cells types, concerning inflammatory mediators and effector functions (Pradeep and Kuttan, 2004; Kumar et al., 2007; Bang et al., 2009). Many of these mediators are important to B cell activation and function (Gerondakis et al., 2007; Moscat et al., 2003; Schauer et al., 1996; Scheller et al., 2011; Sutherland et al., 1996); therefore, I examined how the anti-inflammatory effects of piperine may diminish T-dependent and T-independent activated B cells with respect to B cell activation, proliferation, as well as B cell effector function. Furthermore, investigation towards new anti-inflammatory agents which can be directed towards B cells is necessary as B cells are implicated in several human diseases, such as systemic lupus erythematosus (Mizoguchi and Bhan, 2006). Thus, this *in vitro* study aims to delineate piperine as a potent immuno-suppressive agent with respect to B cell activation and function.

CHAPTER 2. MATERIALS AND METHODS

2.1 MICE

C57BL/6 wild-type (wt) mice were obtained from Charles River Laboratories. Ova-specific transgenic OT II, mice, and C57BL/6-TRPV1$^{-/-}$ mice were obtained from Jackson Laboratories. All mice were housed in ventilated cages and fed ad libitum at the Carleton Animal Care Facility (Dalhousie University, Halifax, Nova Scotia). All animal work was done under the approval of Dalhousie University Committee on Laboratory Animals (UCLA) office of animal research oversight.

2.2 SOLUTIONS AND BUFFERS

All cells were maintained in complete media prepared by supplementing RPMI 1640 (Thermo Scientific, Ottawa, ON) with 5 mM 2-mercaptoethanol (Sigma-Aldrich, Oakville, ON), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES; Invitrogen, Burlington, ON), 200 U/mL penicillin/streptomycin (Invitrogen), and 10% [v/v] fetal calf serum (FCS; Wisent, St-Bruno, QC), at 37°C in a humidified incubator with 5% CO_2.

Flow cytometry buffer (FACS buffer) was prepared by supplementing phosphate buffered saline (PBS; Fisher Scientific, Ottawa, ON) with 0.1% [w/v] aqueous sodium azide (Sigma-Aldrich), 100 μM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), and 2% [v/v] FCS (Wisent) .

Red blood cell lysis buffer was prepared with 150 mM ammonium chloride (NH_4Cl), 10 mM potassium bicarbonate ($KHCO_3$), and 127 μM EDTA in deionized water (all purchased from Sigma-Aldrich).

Cell lysis buffer was prepared with 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM Na_2HPO_4, 0.25% [w/v] sodium deoxycholate, 0.1% [v/v] Nonidet P-40, 5 mM EDTA containing freshly added protease and phosphatase inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 mM NaF, 1 mM phenylmethyl sulfonyl fluoride (PMSF) (all purchased from Sigma-Aldrich), 1 mM dithiothreitol (DTT) (Bio-Shop Canada, Burlington, ON), 100 μM Sodium orthovanadate (Na_3VO_4) (EMD Chemicals, Gibbstown, NJ), 10 μM phenylarsine oxide, and 10 μg/ml aprotinin (all purchased from Sigma-Aldrich)

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer consisted of 200 mM Tris-HCl [pH 6.8] (Fisher Scientific), 30% [v/v] glycerol (VWR; Mississauga, ON), 6% [w/v] SDS (Bio-Shop Canada, Burlington, ON), 15% [v/v] β-mercaptoethanol (Sigma-Aldrich) and 0.01% [w/v] bromophenol blue (Sigma-Aldrich).

Tris−HCL 12% acrylamide resolving gels were prepared with 375 mM Tris−HCl [pH 8.8] (Bio-Shop Canada), 0.1% [w/v] SDS 0.1% [w/v] ammonium persulfate (APS;) and 0.15% [v/v] *N,N,N,N*-tetramethylethylenediamine (TEMED), with 4% acrylamide stacking gel containing 125 mM Tris−HCl [pH 6.8], 0.1% [w/v] SDS, 0.1% [v/v] APS, and 0.3% [v/v] TEMED (all purchased from Bio-Shop Canada).

SDS−PAGE running buffer consisted of 20 mM Tris−HCl [pH 8.3] (Bio-Shop Canada), 200 mM glycine and 0.1% [v/v] SDS (Bio-Shop Canada).

Blocking buffer solution contained 20 mM Tris−HCl [pH 7.6], 200 mM NaCl and 0.05% [v/v] Twee-20 (TBST) with 5% [w/v] fat-free milk (all purchased from Bio-Shop Canada).

Stripping buffer was prepared with 62.5 mM Tris, 2% SDS (Bio-Shop Canada), 100.1 mM β-mercaptoethanol, [pH 7] (Sigma-Aldrich).

2.3 SPLENOCYTE, B AND T LYMPHOCYTE ISOLATION

Splenocytes were isolated from C57BL/6, OT II, and C57BL/6-TRPV1$^{-/-}$ mouse spleens. To isolate B and T cell fractions, spleens were mashed using the blunt end of a sterile 3 ml syringe against a petri dish, red blood cells were then lysed, washed, and filtered to create a single cell suspension. The cells were then fractionated by MACS immuno-column negative isolation system (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. For flow cytometry gating and B cell purity was confirmed using α-CD19 mAb (APC labelled, clone:eBio1D3; eBioscience, San Diego, CA) and isotype control mAb (clone:eBR2a; eBioscience) by FACSCalibur (BD Bioscience, Mississauga, ON) flow cytometry.

2.4 B LYMPHOCYTE ACTIVATION AND PIPERINE TREATMENT

For T-dependent activation, B cells were stimulated with 10 μg/ml of purified α-CD40 mAb (clone: HM40-3; BD Pharmingen) and 4 μg/ml of purified α-immunoglobulin light chain (α-BCR) mAb (clone: 187.1; BD Pharmingen). For T-independent activation, B cells were stimulated with 5 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich). For sub-proliferative B cell activation, an LPS dose of 100 ng/ml was used. For each experiment, piperine (Fluka; Sigma Aldrich) was dissolved in 95% ethanol and was added to B cell cultures to create a final piperine concentration of 25, 50,

and 100 μM with a final vehicle concentration of 0.1% ethanol. Vehicle control cells were treated with 0.1% ethanol only.

2.5 PROLIFERATION ANALYSIS

B cell proliferation was analyzed using two different methods: 1) tritiated-thymidine incorporation assay, and 2) MTT colorimetric assay.

2.5.1 TRITIATED-THYMIDINE INCORPORATION ASSAY

B cells or whole spleen cells were plated in triplicate in 96-well round bottom plates in complete medium at 1×10^5 cells per well (5×10^5 cells/ml). Cells were activated in a T-dependent or T-independent manner in the presence or absence of increasing concentrations (25, 50 and 100 μM) of piperine or vehicle for 72 h. In some experiments, naïve B cells were pre-treated with piperine (25, 50 and 100 μM) or vehicle for 6 h, washed and replated in fresh medium prior to activation with T-dependent or T-independent stimuli. Cultures were pulsed with 0.5 μCi per well of tritiated-thymidine ([^{3}H]-TdR; MP Biomedical, Irvine, CA) for the last 18 h of the assay and were harvested onto fiberglass filter mats (Skatron Instruments, Sterling, VA) with Titertek Cell Harvester (Skatron Instruments). [^{3}H]-TdR incorporation was measured using Beckman LS6000IC liquid scintillation counter (Beckman Coulter Inc., Mississauga, ON). Proliferation was calculated as a percentage from the counts per-minute (CPM) read, normalized to the activated medium group.

2.5.2 MTT COLORIMETRIC ASSAY

(3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay, which utilizes the reduction of the yellow MTT (Sigma-Aldrich) reagent to the formation of a formazan crystal by mitochondrial succinate dehydrogenase activity (Mosmann, 1983), was used to demonstrate B cell metabolic activity. B cells were activated in a T-dependent or T-independent manner in the presence or absence of increasing concentrations (25, 50 and 100 μM) of piperine or vehicle for 72 h. During the last 2 h of the 72 h incubation period, 20 μl of MTT reagent (5 mg/ml in PBS) was added to each well, and incubated under standard culture conditions. Following the incubation, cells were centrifuged (200×g), supernatant discarded and remaining cells/formazan crystals solubilised in 100 μl of DMSO. Plates were shaken and optical densities were read using Expert 96 microplate reader (Asys, Biochrom, Montreal QC) at absorbance 490 nm. Optical density was plotted on 4 parameter curve using SoftMax Pro (Molecular Device, Sunnyvale, CA) microplate data acquisition and analysis software for data analysis. Percent absorbance normalized to activated B cells.

2.6 CELL VIABILITY/SURVIVAL ASSAY

To analyze the effect of piperine on activated B cell survival, B cells were activated in a T-dependent or T-independent manner in quadruplicate wells of a 96-well round bottom plate in complete medium at 1×10^5 cells/well (5×10^6 cells/ml), in the presence or absence of increasing concentrations (25, 50, and 100 μM) of piperine or vehicle. Quadruplicate wells were pooled, washed, and stained with 7-AAD exclusion dye (eBioscience) at 2.5 μg/mL for 20 min. Cells were washed and analyzed using a BD

FACSCalibur (Mississauga, ON) flow cytometer by gating on FL-3. The gate was set so that fewer than 2% of unstained cells were positive on the dot-plot. Percent viable cells were then counted and graphed as percent live/dead, ±SD.

2.7 CELL CYCLE ANALYSIS

To analyze the effect of piperine on activated B lymphocyte cell cycle progression, B cells were activated in a T-dependent or T-independent manner in triplicate wells of a 96-well round bottom plate, in complete medium at 1×10^5 cells/well (5×10^6 cells/ml), in the presence or absence of increasing concentrations (25, 50, and 100 μM) of piperine or vehicle. After 72 h incubation under standard culture conditions, cells were collected, washed and fixed drop-wise in 70% [v/v] ethanol in deionized water. The fixed cell suspension was stored at −20ºC for a minimum of 24 h prior to staining. Following storage, the fixed B cells were washed in PBS, centrifuged at 500g and re-suspended in propidium iodide (0.05% [v/v] Triton-X 100 (Sigma-Aldrich) in PBS (Fisher Scientific), 0.1 mg/ml DNase-free RNase A and 50 μg/ml propidium iodide) and incubated at 37ºC for 40 min, before washing. Analysis was performed by FACSCalibur flow cytometer by gating on FL2-A, and gating out cell aggregates with FL2-W, as well as gating out cellular debris associated with the sub-G0/1-phase peak. Data was graphed as percentage of total gated cells in G0/1-, S-, and G2/M-phase; cells with 2n were considered to be in G0/1-phase, >2n but <4n DNA was considered to be S-phase, and cells with 4n were considered to be in G2/M-phase.

2.8 CELL SIGNALING ANALYSIS BY WESTERN IMMUNOBLOT

B cells were pre-treated in the presence or absence of increasing concentrations (25, 50 and 100 μM) piperine for 1 h prior to activation. At the indicated time intervals, B cells were lysed with lysis buffer, incubated on ice for 15 min, and the lysate was clarified by centrifugation at 21 000 g for 10 min. Total protein was collected and quantified by colorimetric assay using Bio-Rad Protein Assay Dye Reagent (Bio−Rad Laboratories Inc., Mississauga, ON). Protein levels were equalized between samples using Bradford technique, denatured by the addition of SDS−PAGE loading buffer, heated to 95ºC for 5 min, and frozen at -80ºC until use. Pre-stained protein markers (Bio-Rad Laboratories) and protein samples were loaded onto a 4% acrylamide stacking gel, and resolved using Tris−HCL 12% acrylamide gels. The gels were electrophoresed at 200 V for 45 min in SDS−PAGE running buffer, and the proteins transferred to nitrocellulose membranes using the iBlot dye blotting system (Invitrogen). Nitrocellulose membranes were incubated in 5% milk blocking buffer solution for 1 h at room temperature, washed extensively with Tris-Buffered Saline and Tween 20 (TBST; chemicals purchased from Bio-Shop Canada), and then incubated with the appropriate primary antibody solution overnight at 4ºC. Stock antibodies were diluted in TBST containing either 5% milk or 5% BSA [w/v] as per manufacturer's instructions. Following extensive washing, TBST diluted HRP-conjugated secondary antibody containing either 5% milk or 5% BSA [w/v] was added for 1 h at room temperature. Membranes were reacted with chemiluminescence (ECL) reagents (GE Healthcare, Baie d'Urfe, Quebec, Canada) and exposed to X-ray film (Fuji, Sci-Med Inc., Truro, NS), and processed in a Kodak X-OMAT 1000A automated X-ray developer. Antibodies were then stripped using

stripping buffer, and re-probed for β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) expression or total target protein expression when phosphorylated proteins were examined. Antibodies used: total (clone K-23) and phospho-ERK1/2 (Tyr-204; clone E-4) through Santa Cruz Biotechnology, total (polyclonal) phospho-Akt (Ser-473; polyclonal), cyclin D1 (clone DCS6), cyclin D2 (clone D52F9), and cyclin D3 (clone DCS22) all purchased from Cell Signalling Technology (Danvers, MA).

2.9 CYTOKINE AND IMMUNOGLOBULIN PRODUCTION ANALYSIS

To test whether the observed effects of piperine on B cells extends to effector cytokine and antibody production, purified B cells were activated in a T-dependent manner with a concentration of LPS (100 ng/ml) that does not induce proliferation in 24-well plates at $2x10^5$ cells/well in 600 μl of complete medium. Transwell membrane inserts (0.3 μm pores; Corning Inc, NY) were placed in some wells and purified CD4+ T cells were added to the top chamber of the transwell system. T cells were activated with α-CD3 and α-CD28 antibody coated beads (Dynabead, Invitrogen) at a concentration of 4×10^4 beads/ 1×10^5 cells in 200 μl to provide initial cytokine help. After 48 h of incubation, B cells were removed, washed x 3 in 37ºC PBS, and added to the wells of 24 well plates in fresh complete medium. Cells were re-activated with α-BCR, α-CD40 and LPS (100 ng/ml) in the presence of 100 μM piperine or vehicle. Supernatant samples were taken at 1h (to control for residual cytokines/antibodies remaining post B and T cell co-culture) and 48h and stored at -70^{o}C for further testing. Levels of IL-4, IL-6, IL-10, IL-12 and IFN-γ in the supernatants were assessed by ELISA (DuoSet, R&D Systems, Minneapolis, MN), as directed by manufacturer. Similarly, levels of IgM, IgG1, IgG2a, IgG2b, IgG3

and IgA in the supernatants were measured by ELISA (Pierce Rapid ELISA Mouse mAb Isotyping Kit, Thermo Scientific) as directed by manufacturer.

2.10 B CELL EXPRESSION OF MHC CLASS II, CD40 AND CD86

To determine the effects of piperine on B cells expression of MHC class II, CD40 and CD86, purified B cells were activated in a T-dependent or T-independent manner in the presence or absence of 25, 50, and 100 μM piperine for 24 h. B cells were then washed, re-suspended in cold FACS buffer and stained with the following antibodies: α-MHC II-Alexa Fluor 488 mAb (clone:M5/114.15.2; Biolegend, San Diego, CA), α-CD40-PE mAb (clone:1C10; eBioscience) and α-CD86-APC mAb (clone:GL1; eBioscience). Cells were analyzed by using a FACSCalibur flow cytometer (BD Biosciences). Isotype antibody controls were included to control for each mAb. Purified α-CD16.1/32.1 (Fc Block) was used to eliminate non-specific binding.

2.11 B LYMPHOCYTE INDUCED T CELL PROLIFERATION

B cells were activated for 6 h in the presence or absence of piperine with biotin-labelled α-Ig/BCR mAb (clone:187.1; BD Pharmingen), secondary ovalbumin-labelled α-biotin (Ova Antigen Delivery Reagent; Miltenyi Biotec), α-CD40 mAb (clone: HM40-3; BD Pharmingen) as well as a sub-proliferative concentration of LPS (100 ng/ml, Sigma-Aldrich) to ensure a robust B cell activation. B cells were then washed x 3 in PBS and replated in 96 well plates at a concentration of 2×10^4 cells/well. Ova-specific transgenic OT-II CD^{4+} T cells were stained with 2 μM Oregon Green 488 (Invitrogen) in PBS at room temperature for 10 min before quenching with equal volume of FCS (Wisent).

Subsequently, the T cells were washed x 3 in PBS and co-cultured with the B cells at a concentration of 2×10^5 cells/well for 72 h. T cell proliferation was assessed by flow cytometry (FACSCalibur, BD Bioscience) using the Oregon Green dye dilution assay.

2.12 STATISTICAL ANALYSIS

Experiments were repeated as indicated, and error was expressed as either standard deviation (SD) or as standard error of the means (SEM). Statistical significance between groups was generated using the GraphPad Prism software. Non-grouped pairwise comparisons were assessed by one-way ANOVA with the Tukey-Kramer post-test. Grouped comparisons were analyzed by two-way ANOVA with the Bonferroni post-test. All observations being examined were independent with homogeneity of variance. Results were considered significant when $p<0.01$ (*), $p<0.005$ (**), $p<0.001$ (***), and considered to be not significant (ns) where $p>0.05$.

CHAPTER 3. RESULTS

3.1 THE EFFECTS OF PIPERINE ON B LYMPHOCYTE ACTIVATION

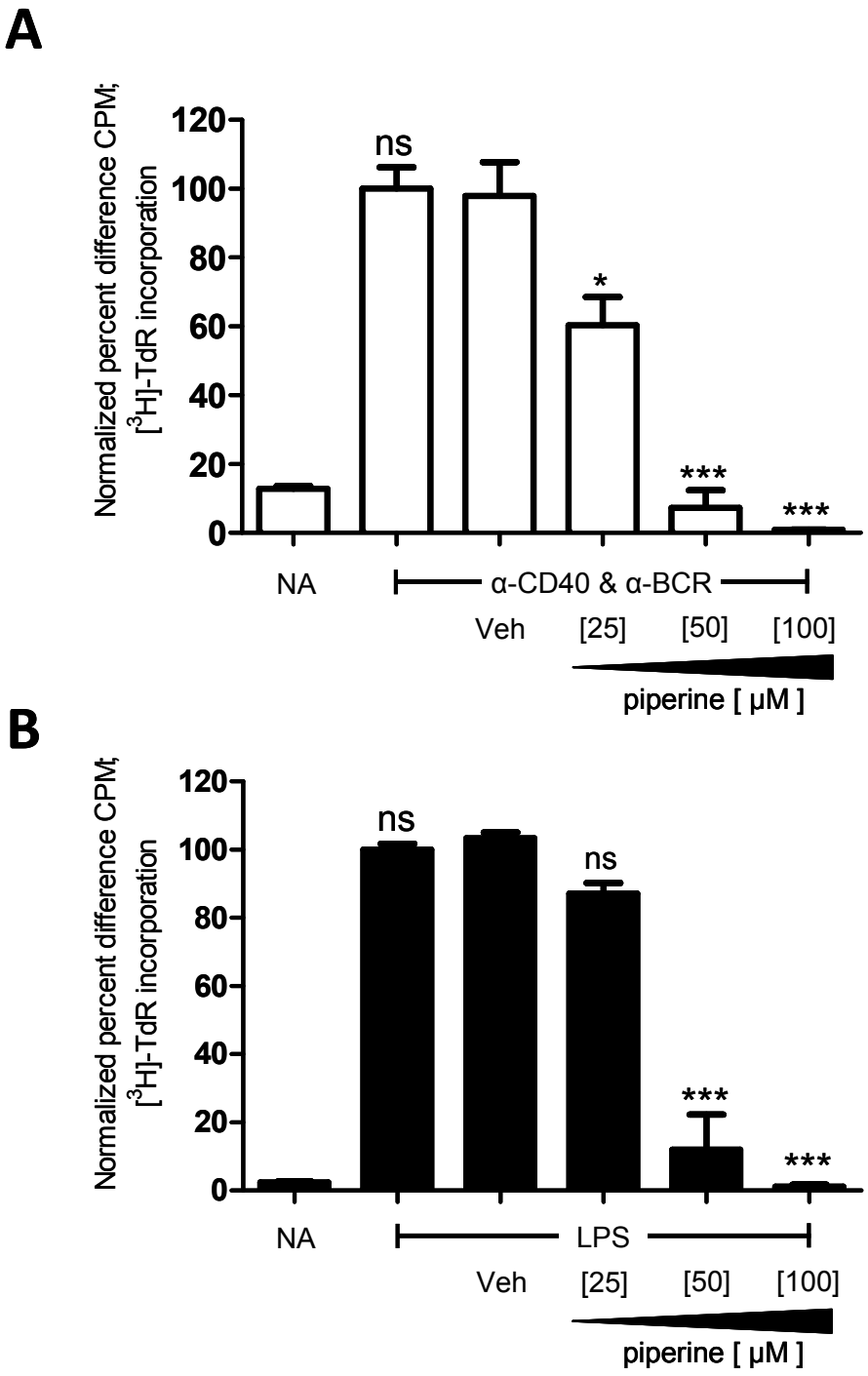
To understand the effect of piperine on B cells, this study began by examining B cell activation in the presence or absence of increasing concentrations of piperine. B cells were activated in either a T-dependent (α-CD40 and α-BCR Ab) or T-independent (LPS) manner for all assays. Specifically, B cell activation was measured by proliferation, metabolic activity, cell survival, cell cycle, and signal transduction. Unless otherwise stated, all experiments were done in triplicate (n=3, SEM) and analyzed by one-way ANOVA with Tukey's Multiple Comparison post test. There was no observed difference between activated medium and vehicle control groups for both methods of activation unless otherwise stated.

3.1.1 PIPERINE INHIBITS T-DEPENDENT AND T-INDEPENDENT B LYMPHOCYTE PROLIFERATION.

Initially, to demonstrate the effect of piperine on B cells, whole splenocytes were collected from C57BL/6 mice and activated in either a T-dependent or T-independent fashion in the presence or absence of increasing concentrations of piperine (25, 50 and 100 μM) for 72 h. [^{3}H]-TdR uptake demonstrated a dose dependent inhibition of proliferation by piperine (Figure 4 A-B). Although some variability in inhibition of proliferation was observed between T-dependent (Figure 4 A) and T-independent (Figure 4 B) activation at 25 μM piperine, significant inhibition was observed for T-dependent activation at 50 μM (Figure 4 A; $p<0.001$) and 100 μM ($p<0.001$) piperine compared to

untreated controls. Similar results were observed for T-independent activation at 50 μM (Figure 4 B; $p<0.001$) and 100 μM ($p<0.001$) piperine compared to vehicle controls.

Figure 4. Piperine inhibits T-dependent and T-independent splenocyte proliferation. Proliferation of splenocytes was induced by T-dependent activation with α-CD40 and α-BCR antibodies (A), or by T-independent activation with LPS (B), in the absence or presence of piperine (25, 50 and 100 μM) or vehicle control for 72 h. Splenocytes were pulsed with [^{3}H]-TdR 18 h prior to completion of the assay to quantify proliferation. Significance is indicated in comparison to the vehicle treated cells. NA = Non-activated; Veh = vehicle; (n=3, SEM ; ns $p>0.05$, *$p<0.05$, ***$p<0.001$).


A
Normalized percent difference CPM; [3H]-TdR incorporation
120
100
80
60
40
20
0
ns
*
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***
NA
α-CD40 & α-BCR
Veh
[25]
[50]
[100]
piperine [μM]
B
Normalized percent difference CPM; [3H]-TdR incorporation
120
100
80
60
40
20
0
ns
ns
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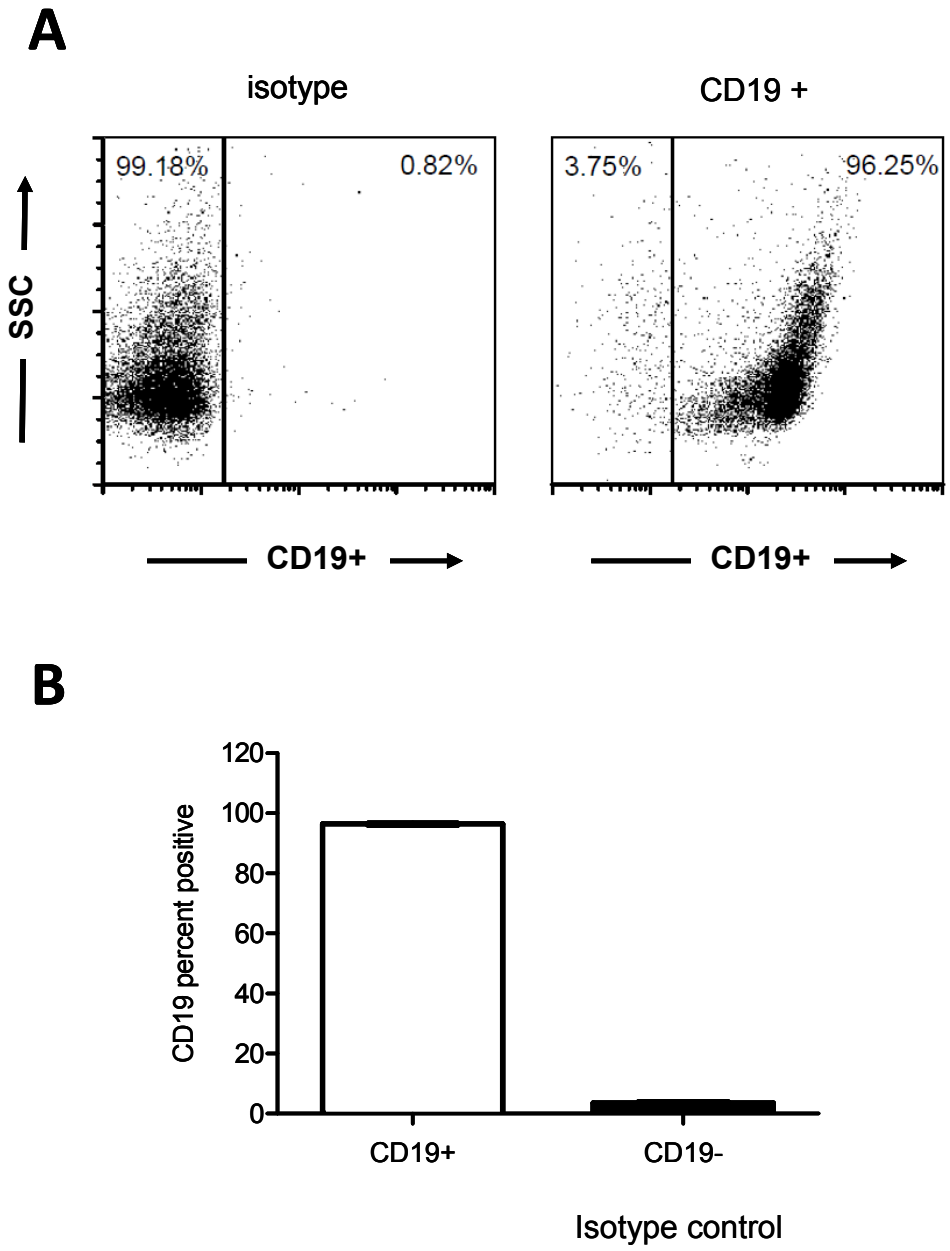
NA
LPS
Veh
[25]
[50]
[100]
piperine [μM]

Figure 4 A-B

Although piperine inhibited splenocyte proliferation in a dose-dependent manner, it remained unclear as to whether the effect of piperine on B cells was direct or indirect. Recent evidence has demonstrated that piperine directly inhibits T cell proliferation (Carolyn Doucette, personal communication). Furthermore, piperine has also been shown to lower DC recovery (Rodgers, 2010). Since, it was possible that the inhibitory effect of piperine seen in splenocyte proliferation assays was associated with an effect on either DC or T cells, B cells were enriched by negative selection using MACS immuno-columns and analyzed by flow cytometry for purity using anti-CD19 mAb (Figure 5 A). The resulting population was highly enriched for B cells showing 96% purity (Figure 5 B). The effect of piperine on this highly enriched B cell population was found to parallel the inhibitory effect observed with splenocytes. Specifically, the results show that piperine inhibited T-dependent B cell proliferation at both 50 μM ($p<0.001$) and 100 μM ($p<0.001$) concentrations compared to untreated controls (Figure 6 A). Similar results were observed for T-independent activation in the presence of 50 μM ($p<0.001$) and 100 μM ($p<0.001$) piperine compared to untreated controls (Figure 6 B). Variable inhibitory effects were observed with 25 μM piperine.

Figure 5. B lymphocyte isolation cell purity. Murine B cells were isolated from total splenocytes by negative selection using MACS immuno-columns and analyzed for purity by flow cytometry using anti-CD19 mAb. Representative dot plots (A) and cumulative results (B) are shown (n=3).


A
isotype
CD19 +
99.18%
0.82%
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96.25%
SSC
CD19+
CD19+
B
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100
80
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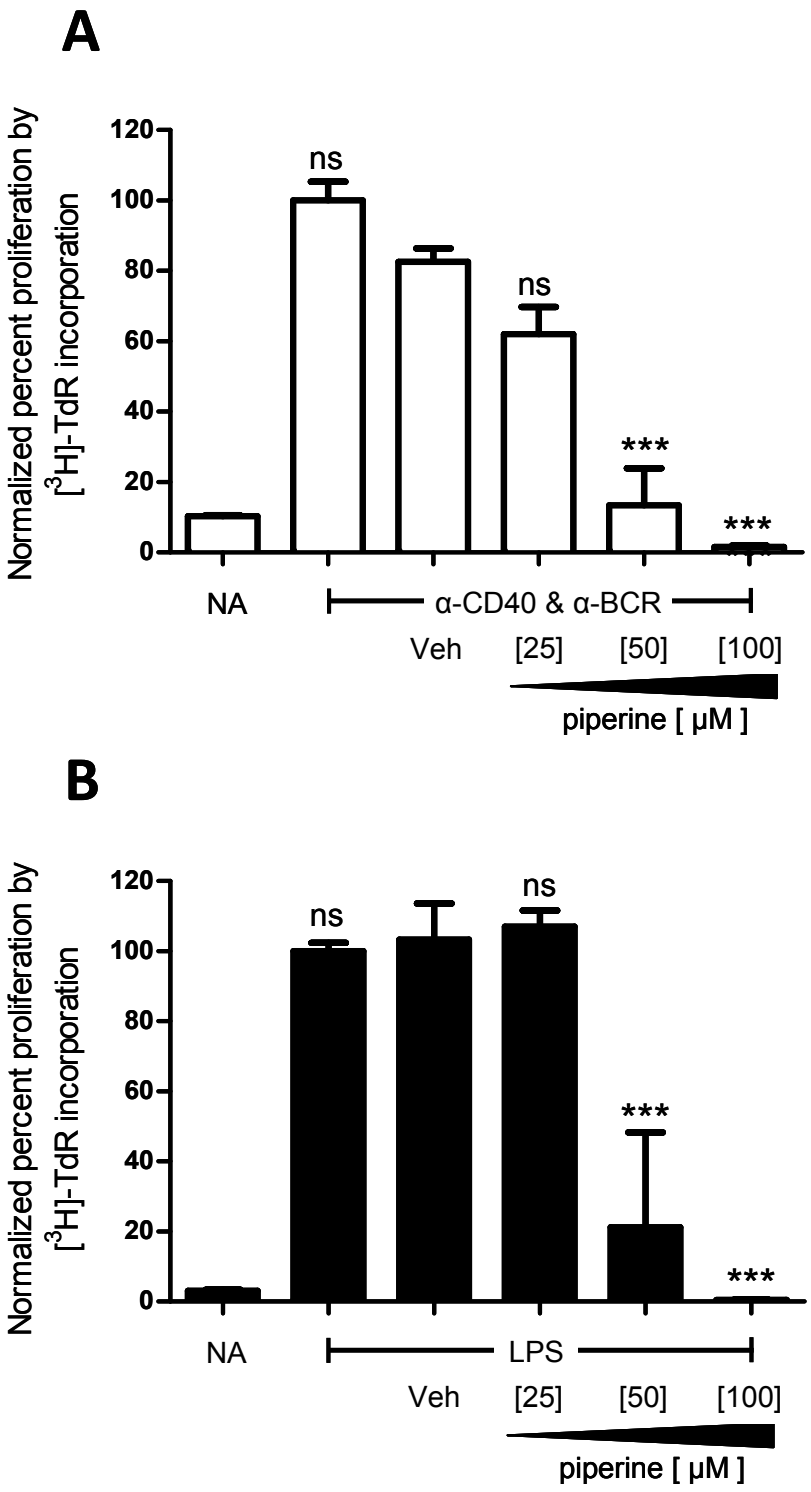
40
20
0
CD19 percent positive
CD19+
CD19-
Isotype control

Figure 5 A-B

Figure 6. Piperine inhibits T-dependent and T-independent activated B lymphocyte proliferation. Proliferation of B cells was induced by T-dependent activation with α-CD40 and α-BCR antibodies (A), or by T-independent activation with LPS (B), in the absence or presence of piperine (25, 50 and 100 μM) or vehicle control for 72 h. B cells were pulsed with [^{3}H]-TdR 18 h prior to assay completion of the assay to quantify proliferation. Significance is indicated in comparison to the vehicle treated cells. NA = Non-activated; Veh = vehicle; (n=3, SEM ; ns $p>0.05$, ***$p<0.001$).


A
Normalized percent proliferation by [3H]-TdR incorporation
120
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NA
α-CD40 & α-BCR
Veh
[25]
[50]
[100]
piperine [μM]
B
Normalized percent proliferation by [3H]-TdR incorporation
120
100
80
60
40
20
0
ns
ns
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NA
LPS
Veh
[25]
[50]
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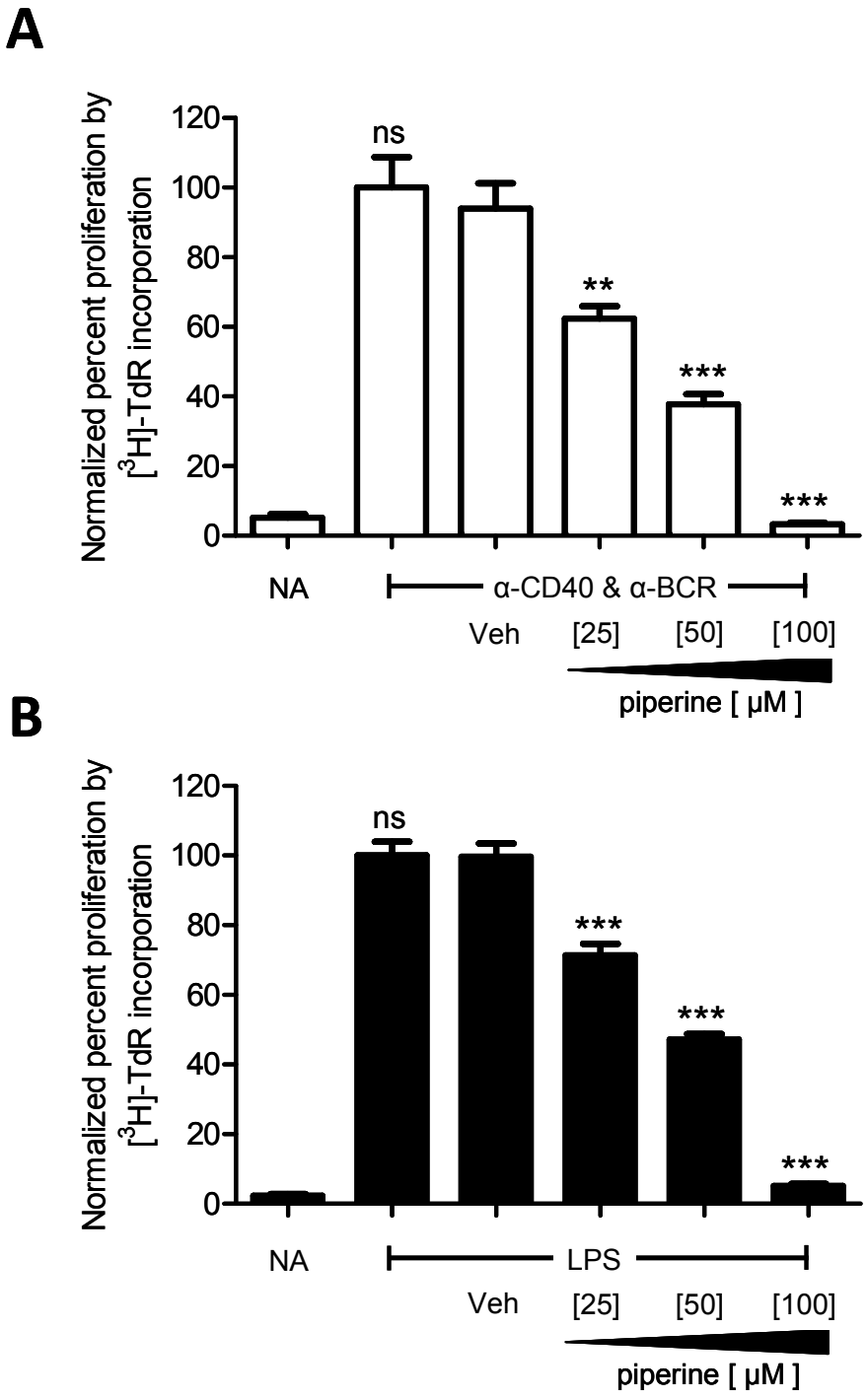
piperine [μM]

Figure 6 A-B

Although piperine inhibited naive B cell proliferation, it remained unclear as to whether piperine would similarly inhibit the proliferation of pre-activated B cells. Therefore, B cells were activated in a T-dependent or T-independent manner for 48 h prior to treatment with piperine (25, 50 and 100 μM). As observed with naïve B cells, B cells that were pre-activated in a T-dependent manner showed significantly decreased proliferation in the presence of 50 μM ($p<0.001$) and 100 μM ($p<0.001$) piperine when compared to vehicle treated cells (Figure 7 A). Similarly, B cells pre-activated in a T-independent manner showed decreased proliferation in the presence of 50 μM ($p<0.001$) and 100 μM ($p<0.001$) piperine when compared to the vehicle treated cells (Figure 7 B). Furthermore, 25 μM piperine treatment significantly reduced proliferation of B cells that were pre-activated in a T-dependent (Figure 7 A; $p<0.01$) or T-independent (Figure 7 B; $p<0.001$) manner compared to the vehicle treated cells.

Figure 7. Piperine decreases T-dependent and T-independent proliferation of pre-activated B lymphocytes. Proliferation of B cells was induced by T-dependent activation with α-CD40 and α-BCR Abs (A), or by T-independent activation with LPS (B). Piperine (25, 50 and 100 μM) or vehicle control was added after 48 h, and cells were cultured for an additional 24 h. B cells were pulsed with [^{3}H]-TdR 18 h prior to completion of the assay to quantify proliferation. Significance is indicated in comparison to the vehicle treated cells. NA = Non-activated; Veh = Vehicle; (n=3, SEM ; ns $p>0.05$, $^{**}p<0.01$, $^{***}p<0.001$).


A
Normalized percent proliferation by [3H]-TdR incorporation
120
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NA
α-CD40 & α-BCR
Veh
[25]
[50]
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piperine [μM]
B
Normalized percent proliferation by [3H]-TdR incorporation
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[100]
piperine [μM]

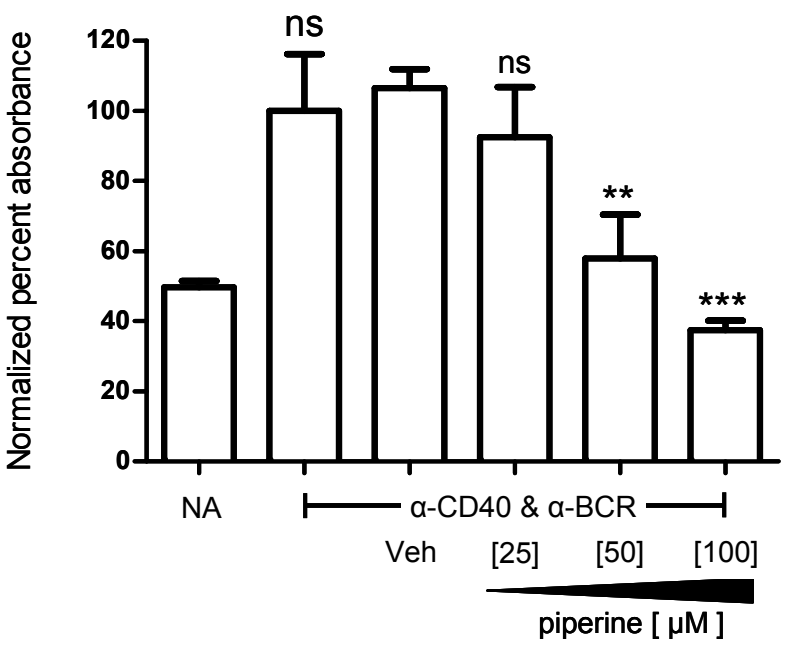
Figure 7 A-B

3.1.2 PIPERINE INHIBITS B LYMPHOCYTE METABOLIC ACTIVITY.

Cell proliferation is dependent on cell growth kinetics and viability. A MTT assay was used to evaluate the effects of piperine on B cell metabolic activity. The MTT assay measures the conversion of the MTT salt to an intensely coloured formazan product by mitochondrial enzymes (Gieni et al., 1995). The metabolic activity of B cells activated in a T-dependent (Figure 8 A) and T-independent (Figure 8 B) fashion was found to be significantly reduced by piperine in a dose dependent manner. Piperine at 50 μM ($p<0.01$) and 100 μM ($p<0.001$) significantly inhibited metabolic activity in T-dependent activated B cells (Figure 8 A) compared to vehicle treated cells. Furthermore, piperine at 50 μM ($p<0.001$) and 100 μM ($p<0.001$) significantly inhibited metabolic activity in T-independent activated B cells (Figure 8 B) compared to vehicle treated cells.

Figure 8. Piperine inhibits B lymphocyte metabolic activity following T- dependent and T-independent activation. Metabolic activity of B cells was examined after T-dependent activation with α-CD40 and α-BCR Abs (A) or after T-independent activation with LPS (B) in the presence of piperine (25, 50 and 100 μM) or vehicle for 72 h; Results were quantified by MTT-colorimetric assay. Significance is indicated in comparison to the vehicle treated cells. NA = Non-activated; Veh = Vehicle; (n=3, SEM; ns $p>0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

A



B

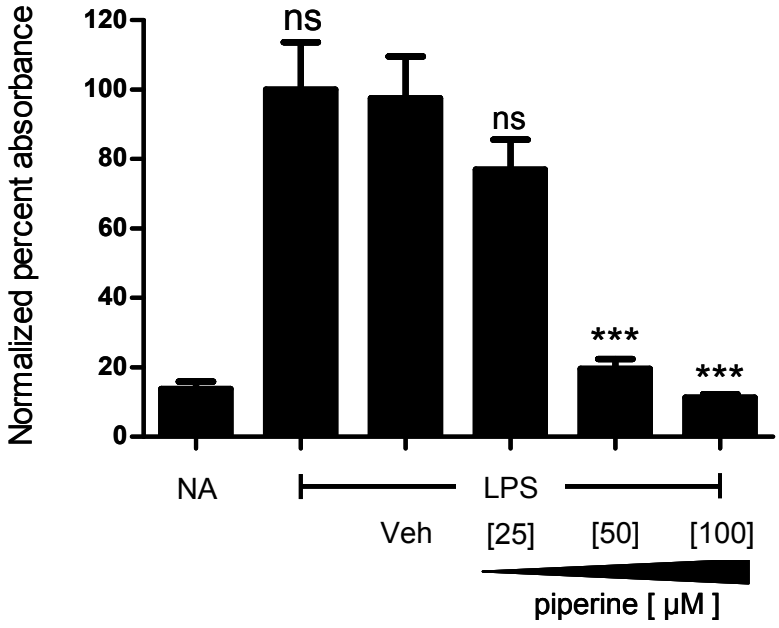


Figure 8 A-B

3.1.3 THE IMPACT OF PIPERINE ON B LYMPHOCYTE SURVIVAL.

To ensure that piperine treatment of activated B cells did not cause cell death, cell survival over a 72 h period of activation in the presence or absence of piperine was assessed by evaluating 7-AAD staining using flow cytometry. A representative dot-plot is shown in (Figure 9). A decrease in cell survival was observed for both T-dependent and T-independent activation (Figure 10 A and B); however, only the decreases in survival seen for the T-independent activated B cells were statistically significant. Both 50 μM ($p<0.001$) and 100 μM ($p<0.001$) piperine caused a significant decrease in the viability of T-independent activated B cells (Figure 10 B) compared to untreated controls.

B cell viability is notably higher in untreated activation groups compared to the non-activated cells (Figure 10A and 10B), suggesting that activation prevents B cell death. Furthermore, there was no significant difference between the amount of cell death in non-activated cells compared to those treated with piperine except at the highest concentration of piperine (100 μM) in the T-independent activation group. This suggests that cell death resulting from piperine is predominantly a consequence of its ability to prevent cell activation rather than a direct toxic effect.

Figure 9. T-independent activated B lymphocyte survival is decreased by piperine treatment. Representative data of T-independent activated B cells (by LPS) were treated with piperine (25, 50 and 100 μM) or vehicle for 72 h. Cell survival was assessed by flow cytometry using 7-AAD staining. All histogram cell counts were normalized and represented on the same ordinate scale. NA = Non-activated; Veh = Vehicle.

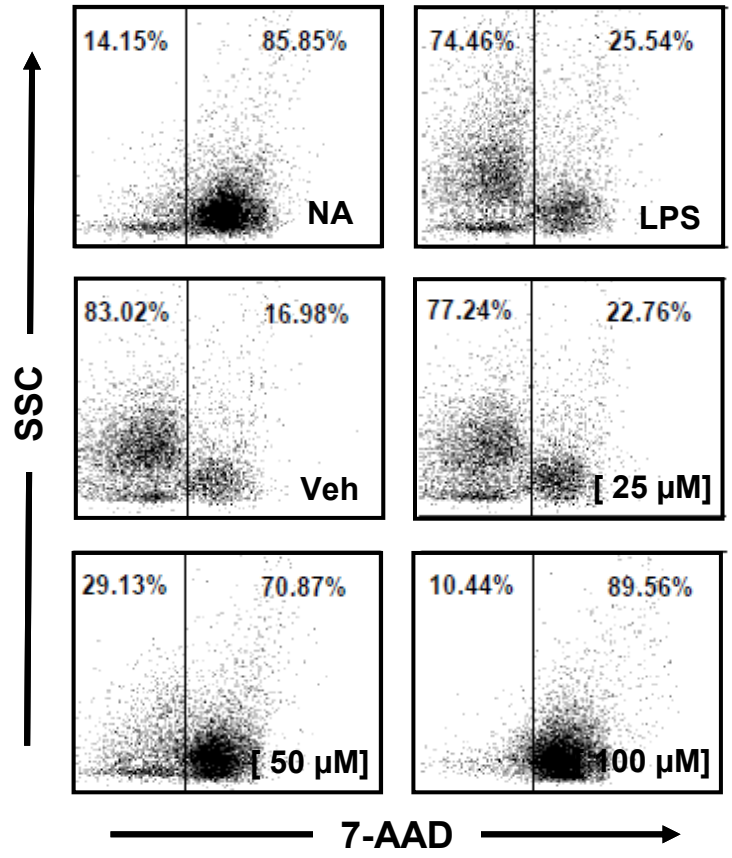

14.15%
85.85%
NA
74.46%
25.54%
LPS
83.02%
16.98%
Veh
77.24%
22.76%
[25 µM]
29.13%
70.87%
[50 µM]
10.44%
89.56%
100 µM]
SSC
7-AAD

Figure 9

Figure 10. T-dependent and T-independent activated B lymphocytes have decreased cell survival with piperine treatment over 72 h. B cells were activated in a T-dependent manner with α-CD40 and α-BCR Abs (A), or in a T-independent manner with LPS (B), in the presence of piperine (25, 50 and 100 μM) or vehicle for 72 h. Cell survival was assessed by flow cytometry using 7-AAD staining. Significance is indicated in comparison to vehicle treated cells. NA = Non-activated; Veh = Vehicle; (n=3, SD; ns $p>0.05$, and ***$p<0.001$).

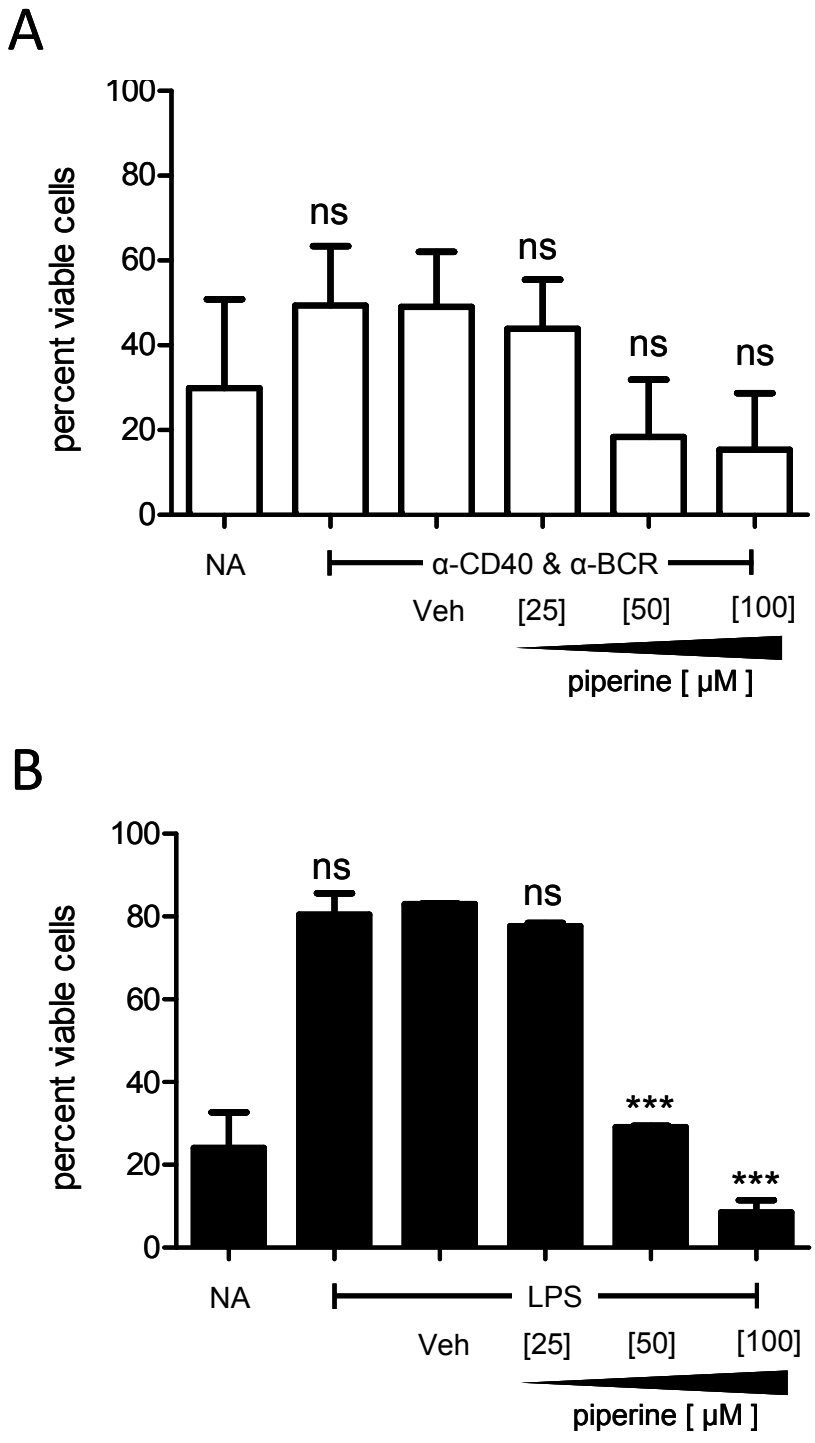

A
100
80
60
40
20
0
percent viable cells
ns
ns
ns
ns
NA
α-CD40 & α-BCR
Veh
[25]
[50]
[100]
piperine [μM]
B
100
80
60
40
20
0
percent viable cells
ns
ns
***
***
NA
LPS
Veh
[25]
[50]
[100]
piperine [μM]

Figure 10 A-B

To further clarify whether piperine has significant toxic effects on B cells we evaluated piperine pre-treatment of B cells prior to their activation. B cells were cultured in the presence or absence of piperine for 6 h prior to T-dependent or T-independent activation. If piperine treatment was toxic, B cells would be expected to have a reduced capacity to proliferate following activation, when compared to activated untreated B cells. However, both T-dependent (Figure 11 A) and T-independent (Figure 11 B) activated B cells demonstrated no significant decrease in proliferation when compared to the activated group that was pre-treated with the vehicle. This provides evidence for little immediate toxicity by piperine on B cells. A significant increase in proliferation was observed in 100 μM piperine pre-treated T-dependent activated B cells (Figure 11 A; $p<0.001$) compared to untreated controls. Similarly, a significant increase in proliferation was observed in 50 μM ($p<0.05$) and 100 μM ($p<0.001$) piperine pre-treated T-independent activated B cells compared to vehicle treated cells (Figure 11 B). This is attributed to a delayed proliferative response in piperine treated groups compared to the non-piperine treated groups; meaning, the non-piperine treated groups at 72 h may have reached a plateau, whereas, the piperine treated groups may still be in the exponential growth phase.

Figure 11. T-dependent or T-independent activated B lymphocyte proliferation was unaffected after 6 h piperine pre-treatment. B cells were cultured in the presence of piperine (25, 50 and 100 μM) or vehicle for 6 h. Subsequently, B cells were washed, and T-dependently activated with α-CD40 and α-BCR Abs (A) or T-independently activated with LPS (B) for 72 h. B cells were pulsed with [^{3}H]-TdR 18 h prior to completion of the assay completion to quantify proliferation Significance is indicated in comparison to the vehicle control (n=3, SEM; ns $p>0.05$, *$p<0.05$, **$p<0.01$, and ***$p<0.001$).

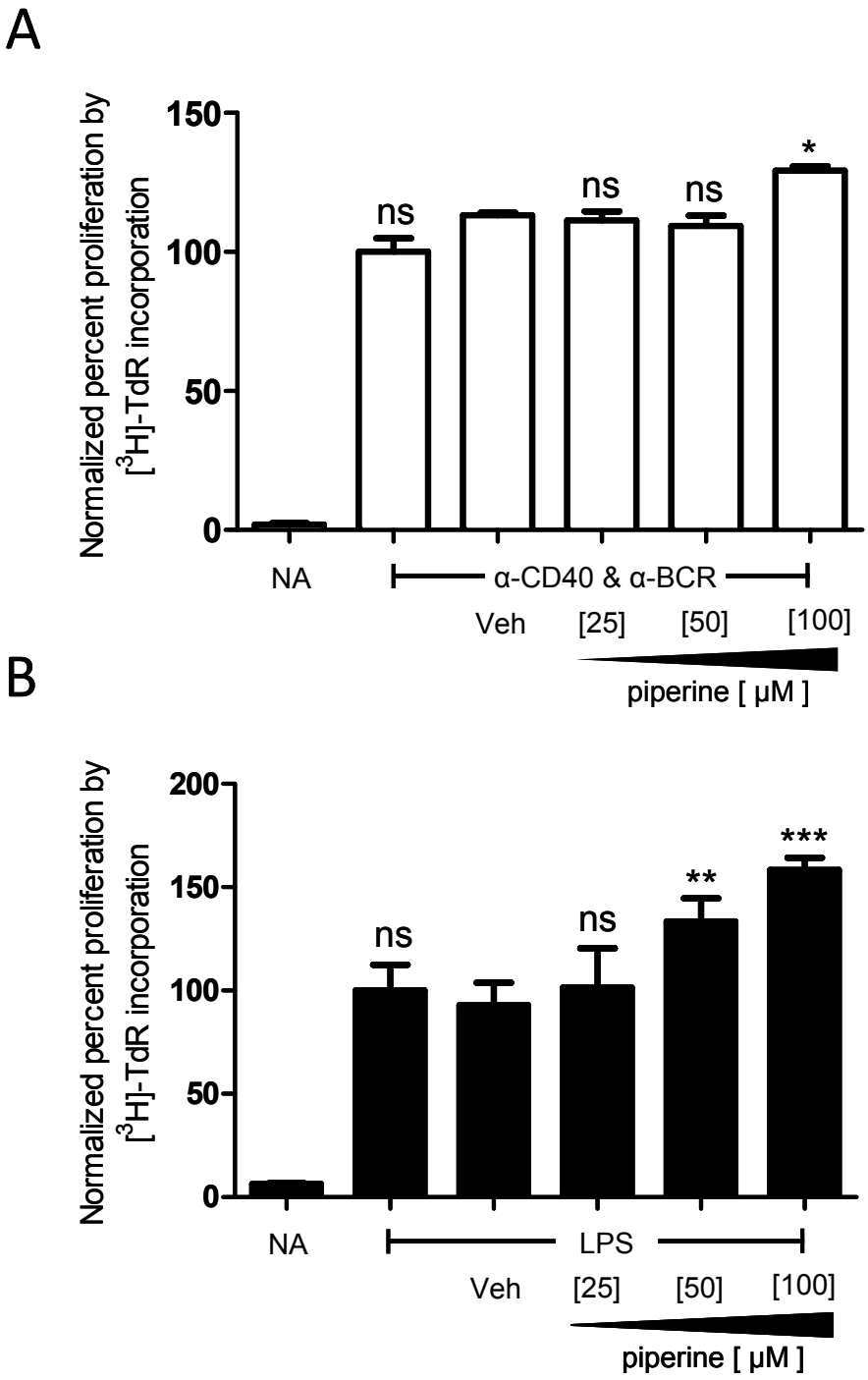

A
Normalized percent proliferation by [3H]-TdR incorporation
150
100
50
0
ns
ns
ns
*
NA
α-CD40 & α-BCR
Veh
[25]
[50]
[100]
piperine [μM]
B
Normalized percent proliferation by [3H]-TdR incorporation
200
150
100
50
0
ns
ns
**
***
NA
LPS
Veh
[25]
[50]
[100]
piperine [μM]

Figure 11 A-B

To investigate the effect of piperine on non-activated B cells we cultured naive B cells in the presence of high dose piperine (100 μM) or vehicle for 24 h and assessed cell viability at 3, 6, 12, 18 and 24 h time points using 7-AAD exclusion assay, (Figure 12). There were no significant differences in cell viability between the piperine treated and vehicle-treated B cells at the 3, 6 and 12 h time points. However, a significant decrease in B cell viability was observed at 18 h ($p<0.01$) and 24 h ($p<0.05$) when non-activated B cells were compared with 100 μM piperine treated B cells. Piperine may therefore have toxic effects on non-activated B cells at or beyond 18 h exposure. A significant decrease in survival for all B cell groups was observed beyond the 6 h time point ($p<0.001$). These results imply piperine has no immediate toxicity to activated or non-activated B cells, although toxicity becomes an issue following longer incubation periods.

Figure 12. Non-activated B lymphocyte survival was unaffected in the presence of piperine over 12 hour period. Non-activated B cells were cultured in the presence or absence of 100 μM piperine for 24 h. B cell viability was assessed by flow cytometry using 7-AAD staining at 3, 6, 12, 18 and 24 h. Significance is indicated in comparison to the vehicle control or previous time point (n=3, SD; ns $p>0.05$, ***$p<0.001$).

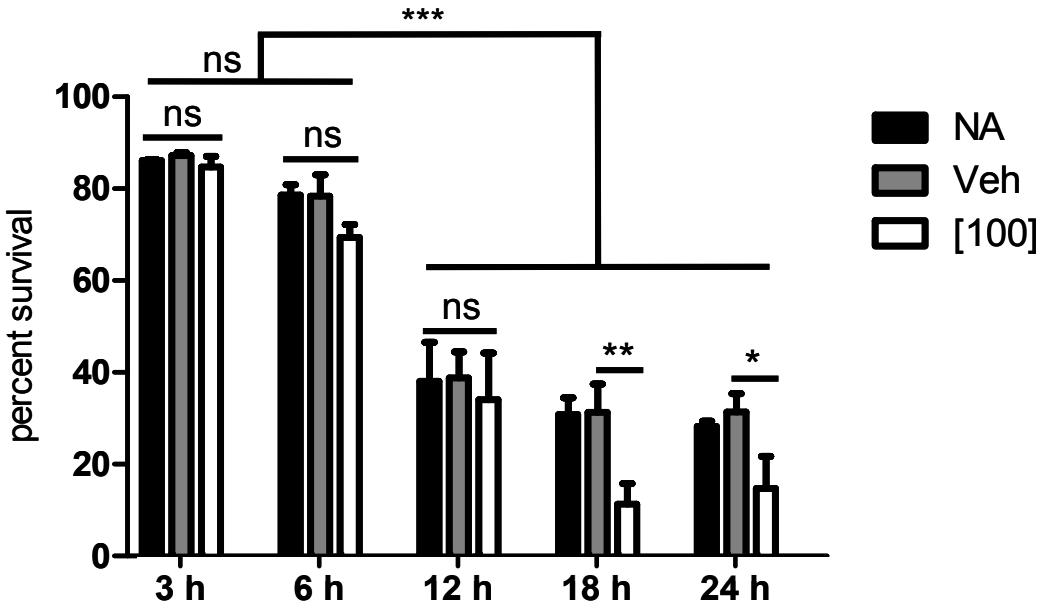

***
ns
ns
ns
ns
**
*
100
80
60
40
20
0
percent survival
3 h
6 h
12 h
18 h
24 h
NA
Veh
[100]

Figure 12

3.1.4 PIPERINE INHIBITS B LYMPHOCYTE CELL CYCLE PROGRESSION.

Having shown that piperine inhibits B cell proliferation, we next wanted to investigate the effects of piperine on cell cycle progression. B cells were activated in a T-dependent or T-independent manner in the presence or absence of piperine and cell cycle analysis was performed by flow cytometry using propidium iodide staining. Cell aggregates were gated out (Figure 13 B) and cell cycle phase peaks were quantified (Figure 13 A). A dose-dependent response to piperine was demonstrated on representative histograms for T-dependent (Figure 13 C), and T-independent (Figure 13 D) activated B cells. These results demonstrated that treatment of B cells with piperine at concentrations of 50 μM or higher significantly reduced the number of cells in S- and G2/M-phase (Figure 13 and 14). There were significantly more B cells in G0/1-phase following T-dependent (Figure 14 A: $p<0.001$) and T-independent (Figure 14 B: $p<0.001$) activation in piperine treated groups as compared to the control group. These findings suggest piperine induces a G0/1-phase arrest in B cells.

Figure 13. Piperine inhibits B lymphocyte cell cycle progression in response to T-dependent and T-independent activation. B cells were activated in a T-dependent (α-CD40 and α-BCR Ab; C), or T-independent manner (LPS; D), in the presence of piperine (25, 50 and 100 μM) or vehicle for 72 h. The B cells were then stained with propidium iodide and total nucleic acid was quantified by flow cytometry. B cells were gated on forward and side scatter plot to analyze the blast/proliferating cells; this region was determined examining the activated non-piperine treated group (data not shown). Panel A shows the cell cycle phase histogram with corresponding gates. Panel B shows the appropriate gate utilized to isolate single cells from doublets, excluding sub-G0-phase cells. Histograms shown are representative of 3 independent experiments. Numbers shown in the histograms for each treatment group represent the raw percentage of gated events collected for analysis.

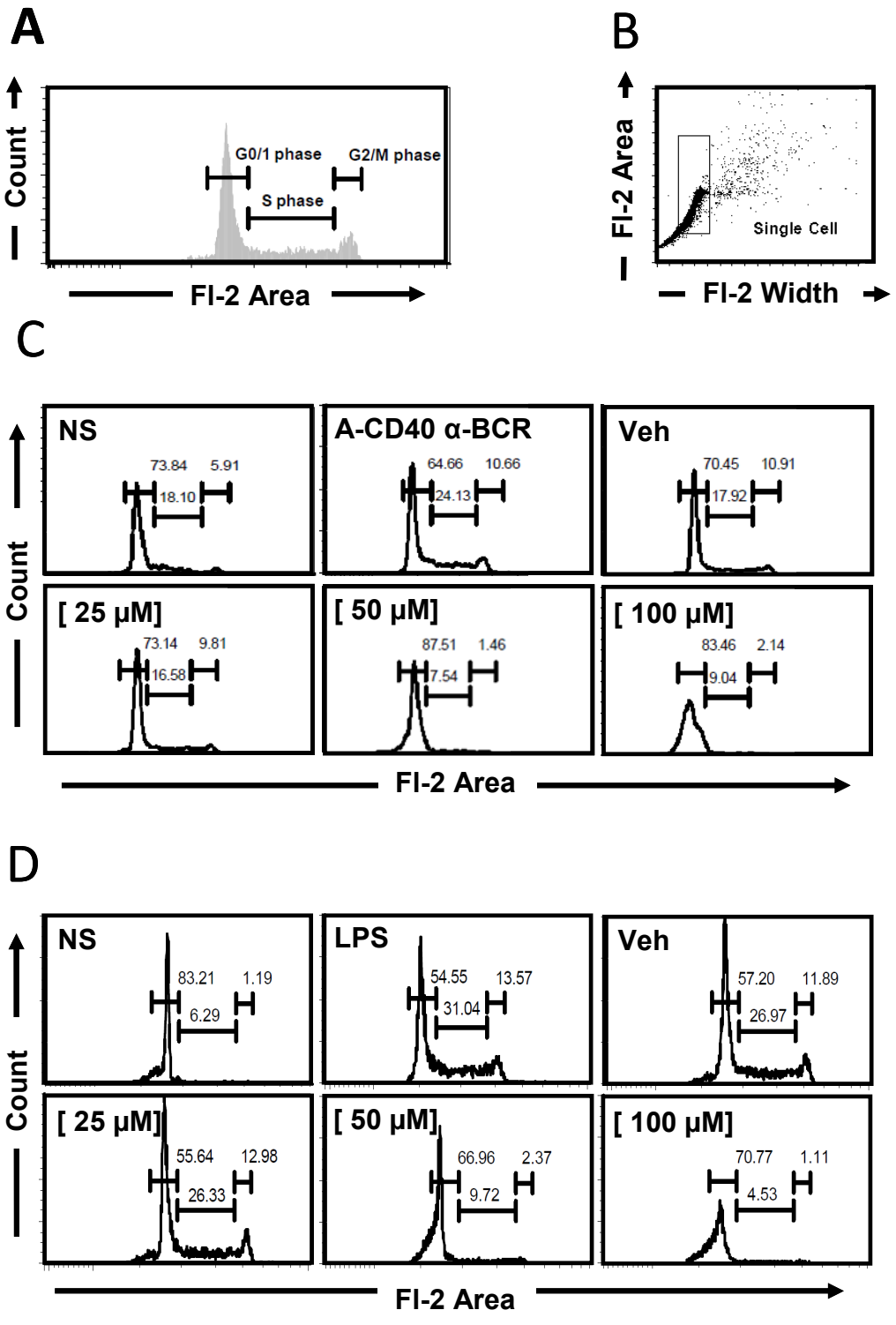

A
Count
G0/1 phase
G2/M phase
S phase
Fl-2 Area
B
Fl-2 Area
Single Cell
Fl-2 Width
C
NS
73.84
5.91
18.10
A-CD40 α-BCR
64.66
10.66
24.13
Veh
70.45
10.91
17.92
[25 μM]
73.14
9.81
16.58
[50 μM]
87.51
1.46
7.54
[100 μM]
83.46
2.14
9.04
Count
Fl-2 Area
D
NS
83.21
1.19
6.29
LPS
54.55
13.57
31.04
Veh
57.20
11.89
26.97
[25 μM]
55.64
12.98
26.33
[50 μM]
66.96
2.37
9.72
[100 μM]
70.77
1.11
4.53
Count
Fl-2 Area

Figure 13 A-D

Figure 14. Piperine treatment of T-dependent and T-independent activated B lymphocytes arrests cells in G0/1 phase. B cells were activated in a T-dependent (α-CD40 and α-BCR Abs; A), or T-independent manner (LPS; B), in the presence of piperine (25, 50 and 100 μM) for 72 h. The B cells were stained with propidium iodide and total nucleic acid was quantified by flow cytometry. Significance is indicated in comparison to vehicle treated cells, for each cell cycle phase (n=3, SD; ns $p>0.05$, # or Θ $p<0.05$, and ***, ### or ΘΘΘ $p<0.001$).

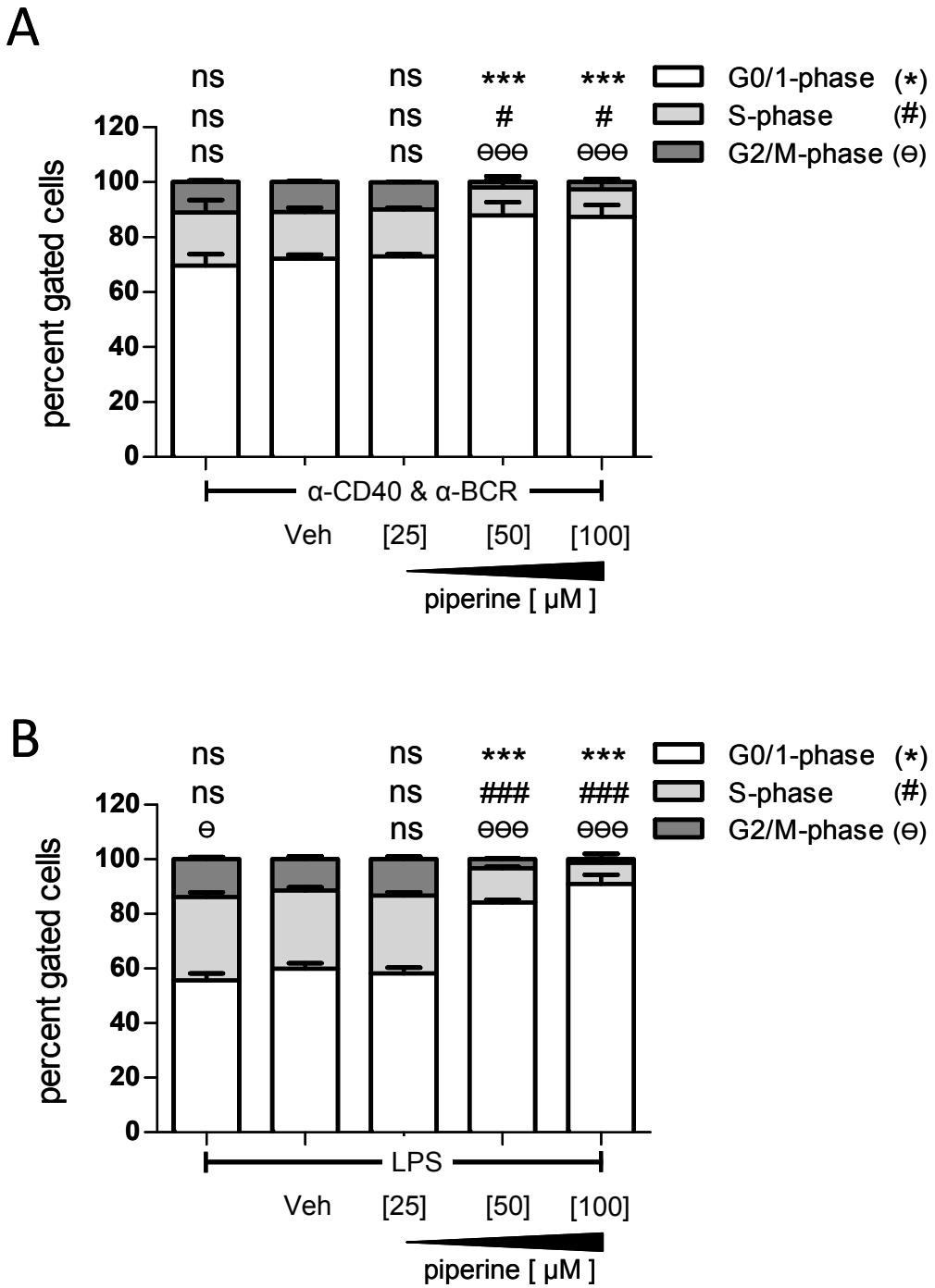

A
ns
ns
ns
ns
ns
ns
***
#
ϴϴϴ
***
#
ϴϴϴ
G0/1-phase (*)
S-phase (#)
G2/M-phase (ϴ)
percent gated cells
120
100
80
60
40
20
0
α-CD40 & α-BCR
Veh
[25]
[50]
[100]
piperine [µM]
B
ns
ns
ϴ
ns
ns
ns
***
###
ϴϴϴ
***
###
ϴϴϴ
G0/1-phase (*)
S-phase (#)
G2/M-phase (ϴ)
percent gated cells
120
100
80
60
40
20
0
LPS
Veh
[25]
[50]
[100]
piperine [µM]

Figure 14 A-B

Given that piperine appears to inhibit cell cycle progression at the G0/1-phase, and cyclin D1, D2, and D3 expression is important for transition into S-phase (Banerji et al., 2001; Harbour and Dean, 2000; Solvason et al., 2000), we examined the effects of piperine on the expression of cyclin D1, D2, and D3 in B cells by immunoblotting (Figure 15 A-C). Representative immunoblots of lysates from B cells activated in a T-dependent fashion, showed an increase in cyclin D1, D2, and D3 expression at 24 h compared to 0 h for all treatment groups. Our results indicate that piperine inhibits, in a dose-dependent manner, activation-induced cyclin D1 (Figure 15 A) cyclin D2 (Figure 15 B) and cyclin D3 (Figure 15 C) protein expression. Preliminary results also indicate that piperine inhibits T-independent activated B cell expression of cyclin D1, D2 and D3 (data not shown).

3.1.5 PIPERINE INHIBITS B LYMPHOCYTE ERK AND AKT PHOSPHORYLATION

ERK1/2 are MAPKs, which are a class of intracellular serine/threonine protein kinases that are involved in cyclin D1, D2 and D3 regulation (Roux and Blenis, 2004). Similarly, Akt has been implicated in cell survival and growth (Vanhaesebroeck and Alessi, 2000). Therefore, phosphorylation status of ERK1/2 (Figure 16) and Akt (Figure 17) were examined following T-dependent activation of B cells treated with increasing concentrations of piperine (25, 50 and 100 μM). As observed on representative immunoblots of lysates from B cells activated in a T-dependent fashion showed an increased the phosphorylation of both ERK1/2 (Figure 16) and Akt (Figure 17), which was inhibited by piperine in a dose dependent manner.

Figure 15. Piperine treatment decreases cyclin D1, D2 and D3 protein expression in T-dependent activated B lymphocytes. B cells were analyzed for cyclin D1 (Panel A), D2 (Panel B), and D3 (Panel C) expression 24 h post T-dependent activation with α-CD40 and α-BCR Abs in the presence of 25, 50 or 100 μM piperine or vehicle. B cells were harvested, washed and cell lysates were prepared, resolved by SDS-PAGE, and immunoblotted for cyclin D1, D2 and D3, independently. Equal protein loading was confirmed by probing for the presence of actin. Results are representative of 3 independent experiments.

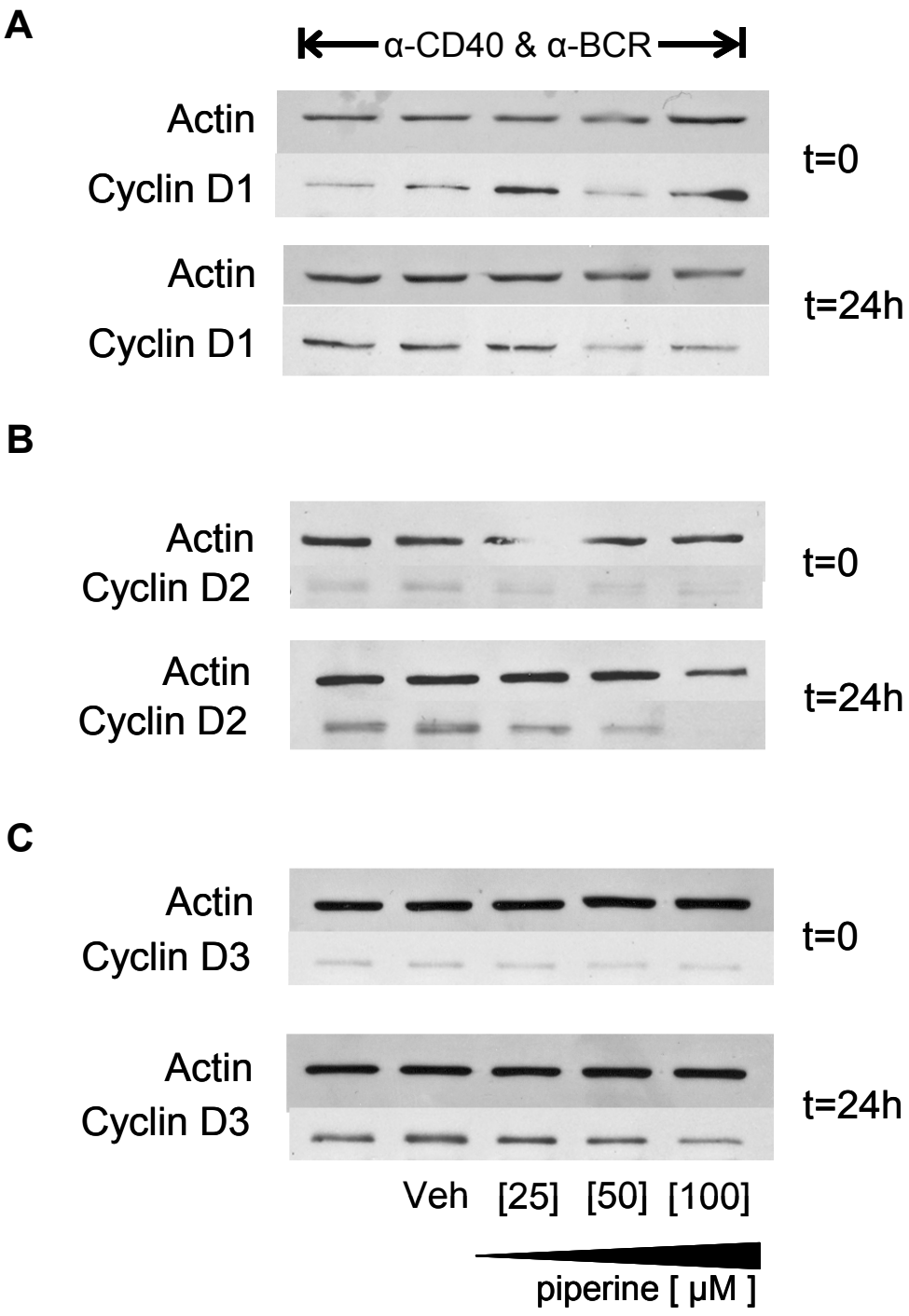

A
α-CD40 & α-BCR
Actin
Cyclin D1
t=0
Actin
Cyclin D1
t=24h
B
Actin
Cyclin D2
t=0
Actin
Cyclin D2
t=24h
C
Actin
Cyclin D3
t=0
Actin
Cyclin D3
t=24h
Veh [25] [50] [100]
piperine [μM]

Figure 15 A-B

Figure 16. Piperine decreases ERK1/2 phosphorylation in T-dependent activated B lymphocytes in a dose-dependent manner. B cells were analyzed for phospho-ERK1/2 expression at 20 and 60 min post T-dependent activation with α-CD40 and α-BCR Abs in the presence of 25, 50 or 100 μM piperine or vehicle. B cells were harvested, washed and cell lysates were prepared, resolved by SDS-PAGE, and immunoblotted for phospho-ERK1/2. Equal protein loading was confirmed by probing for total-ERK. Results are representative of 3 independent experiments.

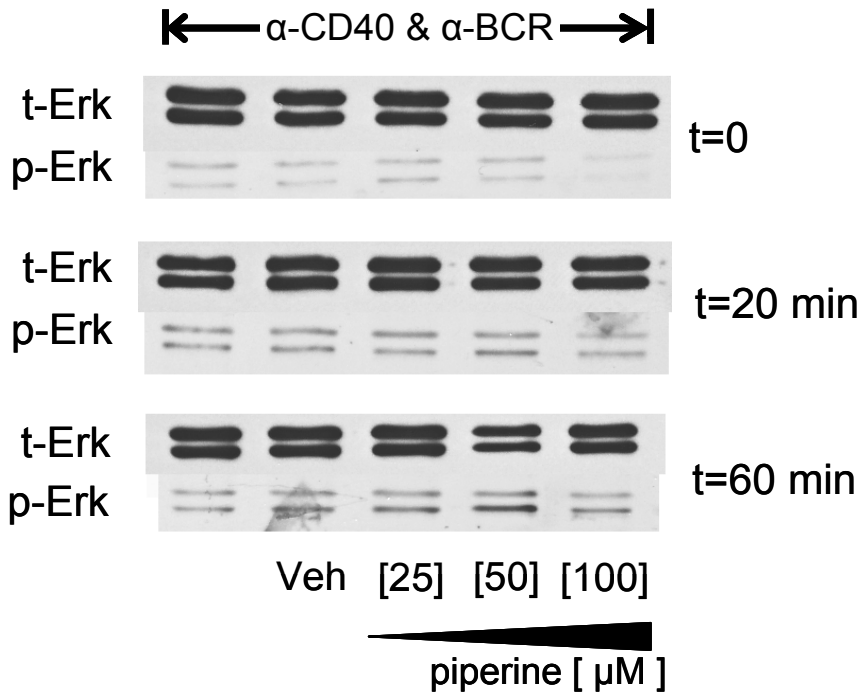

α-CD40 & α-BCR
t-Erk
p-Erk
t=0
t-Erk
p-Erk
t=20 min
t-Erk
p-Erk
t=60 min
Veh
[25]
[50]
[100]
piperine [μM]

Figure 16

Figure 17. Piperine decreases Akt phosphorylation in T-dependent activated B lymphocytes in a dose-dependent manner. B cells were analyzed for phospho-Akt expression at 20 and 60 min post T-dependent activation with α-CD40 and α-BCR Abs in the presence of 25, 50 or 100 μM piperine. B cells were harvested, washed and cell lysates were prepared, resolved by SDS-PAGE, and immunoblotted. Equal protein loading was confirmed by probing for total Akt. Results are representative of 3 independent experiments.

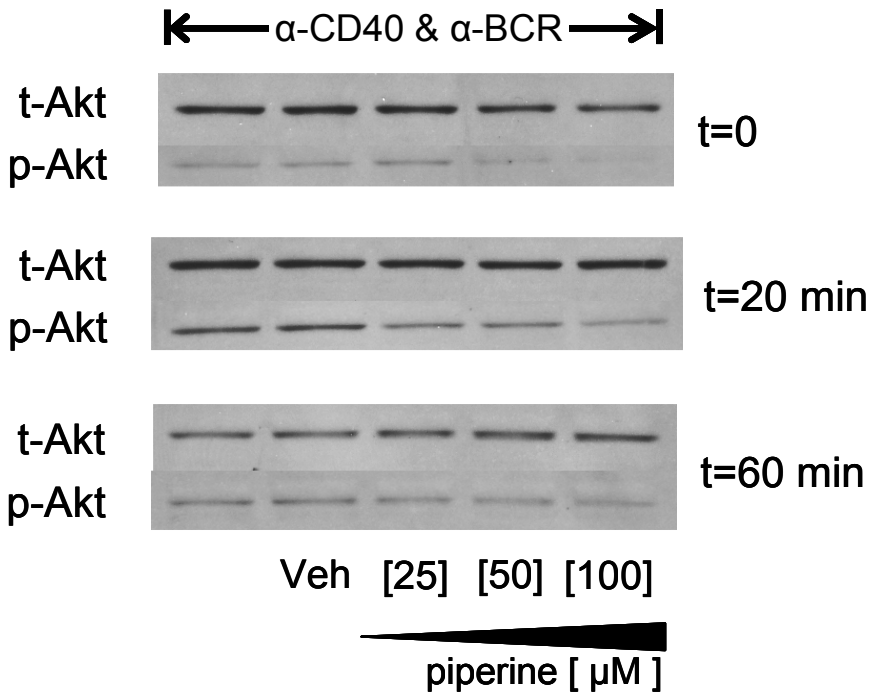

α-CD40 & α-BCR
t-Akt
p-Akt
t=0
t-Akt
p-Akt
t=20 min
t-Akt
p-Akt
t=60 min
Veh [25] [50] [100]
piperine [μM]

Figure 17

3.1.6 INHIBITORY EFFECTS OF PIPERINE ON B CELL ACTIVATION ARE INDEPENDENT OF TRPV1 RECEPTOR.

As TRPV1 is the only known receptor for piperine (McNamara et al., 2005), we examined the effects of piperine on TRPV1 knockout B cells. TRPV1 knockout B cells were activated in a T-dependent or T-independent manner in the presence or absence of piperine. As previously seen with wild-type B cells, proliferation of TRPV1 knockout B cells was significantly inhibited with 50 μM piperine following T-dependent (Figure 18 A: $p<0.001$) and T-independent (Figure 18 B: $p<0.001$) activation. This demonstrates that TRPV1 signalling is not required for the inhibitory effects of piperine on B cell activation.

Figure 18. T-dependent and T-independent activated TRPV1$^{-/-}$ B lymphocyte proliferation is inhibited by piperine treatment. Proliferation of TRPV1$^{-/-}$ B cells was induced by T-dependent activation with α-CD40 and α-BCR Abs (A), or by T-independent activation with LPS (B), in the presence of piperine (25, 50 and 100 μM) or vehicle for 72 h. B cells were pulsed with [^{3}H]-TdR 18 h prior to completion of the assay to determine percent proliferation, which was normalized to the activated group. Significance is indicated in comparison to the vehicle treated cells. NA = Non-activated, Veh = Vehicle (n=3, SEM, ns $p>0.05$, *** $p<0.001$).

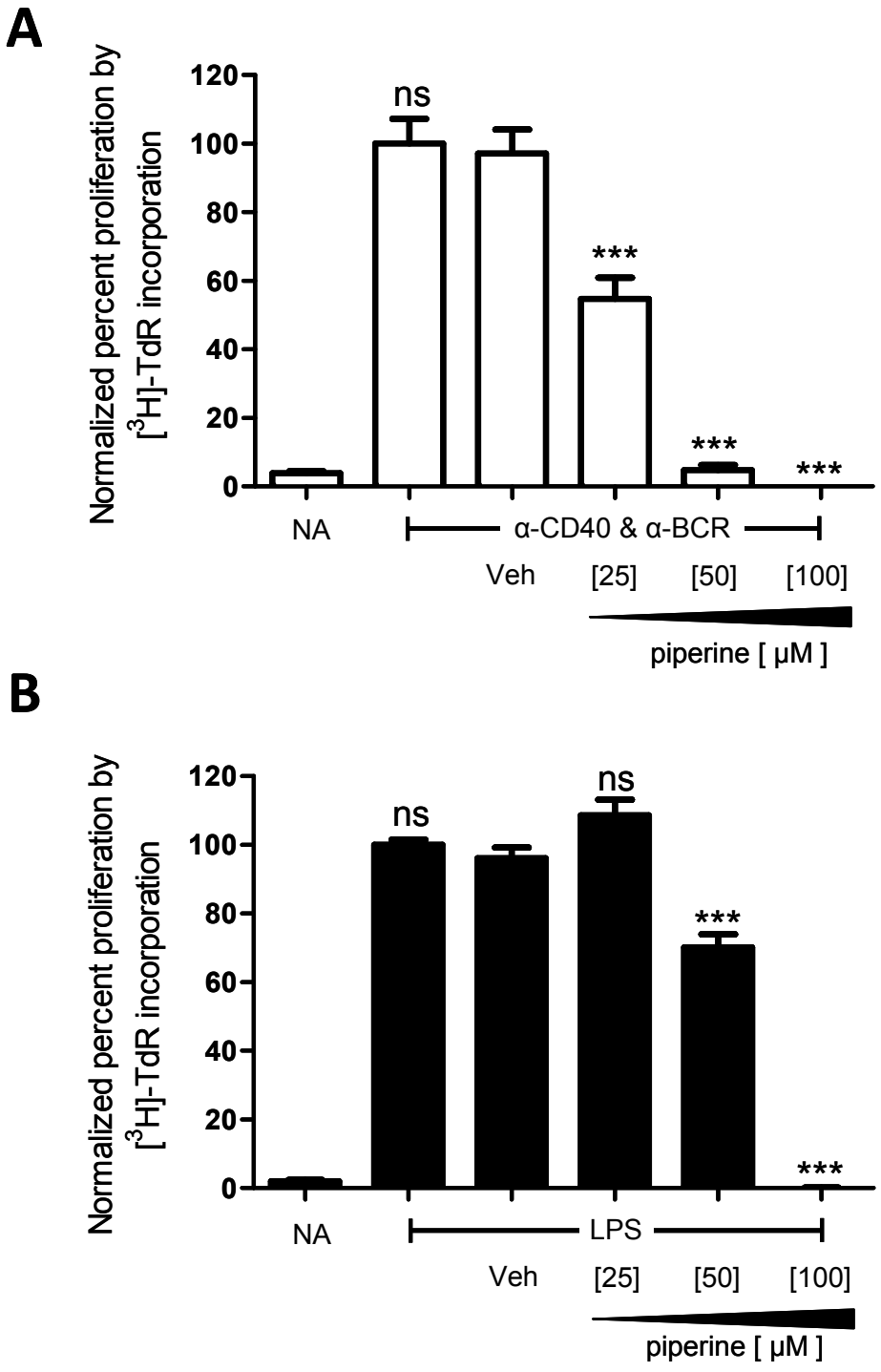

A
Normalized percent proliferation by [3H]-TdR incorporation
120
100
80
60
40
20
0
ns
***
***
***
NA
α-CD40 & α-BCR
Veh
[25]
[50]
[100]
piperine [μM]
B
Normalized percent proliferation by [3H]-TdR incorporation
120
100
80
60
40
20
0
ns
ns
***
***
NA
LPS
Veh
[25]
[50]
[100]
piperine [μM]

Figure 18 A-B

3.2 THE EFFECTS OF PIPERINE ON B LYMPHOCYTE EFFECTOR FUNCTION.

The effector function of B cells is multifaceted. Current literature has implicated activated B cells as important APCs which stimulate a strong T cell response, secrete cytokines that direct immune system responses, as well as differentiate into antibody-secreting plasma cells (Harris et al., 2000; Lund, 2008; Mizoguchi and Bhan, 2006; Rivera et al., 2001). Knowing the importance of B cell effector function, we examined the effect of piperine on activated B cell cytokine and antibody production by B cells that were activated with α-CD40 and α-BCR Abs. However, previous work has demonstrated that even in the presence of T_H cell cytokine secretion and CD40 binding, T-dependent antigen-specific antibody responses are diminished in the absence of TLR ligands *in vivo* (Pasare and Medzhitov, 2005). This suggests that although mature B cells may become activated in a T-dependent manner, TLR ligation is also needed for a robust B effector plasma cell response. I therefore cultured B cells with an LPS concentration that showed not cause B cell proliferation (100 ng/ml; data not shown). I subsequently refer to this as "sub-proliferative concentrations of LPS" hereon out. During this time, I co-cultured the activated B cells in the presence or absence of α-CD3 and α-CD28 bead-stimulated T cells separated by a transwell system for 48 h. The B cells were then separated, washed, re-plated and reactivated with α-CD40, α-BCR antibody and sub-proliferative levels of LPS in the presence or absence of 100 μM piperine. I then collected sample supernatants 1 h after washing the B cells to check for residual cytokines remaining from earlier T cell help. The remaining re-activated B cells were cultured for and additional 48 h before supernatants were collected for analysis by ELISA to assess cytokine and antibody

production. The results of these experiments are described in sections 3.2.1 and 3.2.2 below.

3.2.1 PIPERINE INHIBITS B LYMPHOCYTE CYTOKINE PRODUCTION BY B LYMPHOCYTES.

Although B cells have not historically been considered an important source of cytokines in immune responses, current literature demonstrates that B cells produce a wide variety of cytokines which that influence the Th1/Th2 balance (Harris et al., 2000), and help regulate inflammatory reactions (Mizoguchi and Bhan, 2006). Therefore, it was pertinent to investigate the effects of piperine on activated B cell cytokine production. B cells activated in the presence of stimulated T cells, and subsequently cultured in the presence of piperine showed a significant decrease in IL-6 (Figure 19 A; $p<0.001$) and IL-10 (Figure 19 B; $p<0.001$) production, when compared to activated cells alone. Negligible quantities of IL-4, IL-12 and IFN-γ were detected (data not shown). The 1 h supernatants did not constrain significant levels of the cytokines tested (data not shown). This indicates that piperine treated B cells have a reduced capacity for cytokine production following activation.

3.2.2 PIPERINE INHIBITS B LYMPHOCYTE IMMUNOGLOBULIN PRODUCTION.

B cell activation results in the production of antibodies, which are integral to the adaptive humoral immune response and aid in the clearance of pathogens (Nimmerjahn and Ravetch, 2010). Thus, it was necessary for us to investigate the effect of piperine on

B cell antibody production. B cells activated in the presence of stimulated T cells, and subsequently cultured in the presence of piperine had a significant decrease in IgG2b ($p<0.001$), IgG3 ($p<0.01$), and IgM ($p<0.01$) secretion when compared to cells that didn't receive piperine (Figure 20 A). Only low levels of IgG1, IgG2a and IgGA were produced by activated B cells and piperine did not have a significant effect on production of these immunoglobulin subclasses. Taken together these data indicate that piperine inhibits the production of some immunoglobulin classes by activated B cell.

Figure 19. Piperine decreases IL-6 and IL-10 cytokine production by activated B lymphocytes. IL-6 (A) and IL-10 (B) secretion by B cells was measured by ELISA. B cells were activated by α-BCR, α-CD40 and a sub-proliferative concentration of LPS. B cells were activated in the presence or absence of activated T cells in a transwell system for 48 h. B cells were then separated, washed, replated without T cells and reactivated with α-BCR, α-CD40 and a sub-proliferative concentration of LPS in the presence or absence of 100 μM piperine for an additional 48 h. Supernatants were subsequently collected and assayed for cytokine production by ELISA. Box-and-whisker plots indicate significance in comparison to activated B cells co-cultured with T cells (n=3, ***$p<0.001$); with a horizontal line at the median (50th percentile), the ends of the box are at the upper and lower quartiles (spread), and the whiskers indicate the smallest to the largest value (range).

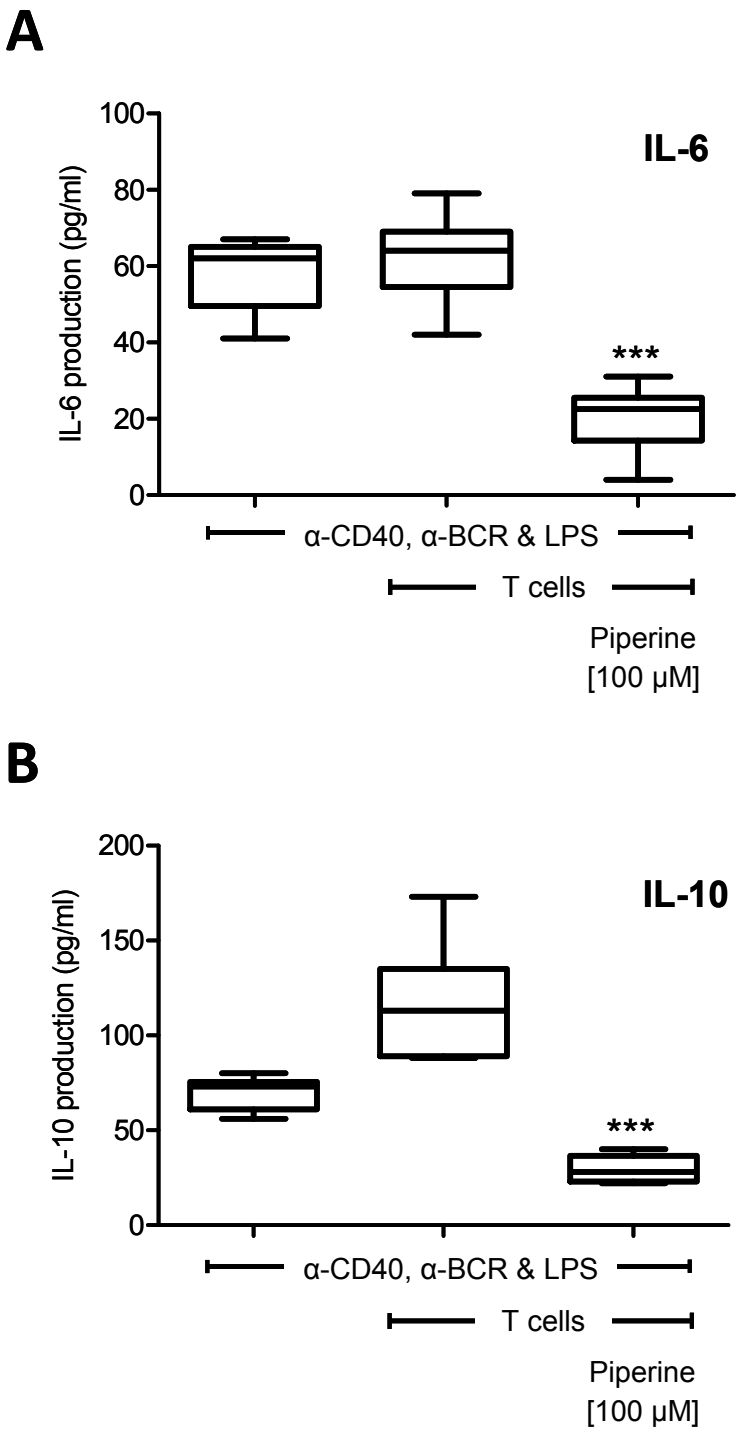

A
IL-6
IL-6 production (pg/ml)
100
80
60
40
20
0
***
α-CD40, α-BCR & LPS
T cells
Piperine
[100 μM]
B
IL-10
IL-10 production (pg/ml)
200
150
100
50
0
***
α-CD40, α-BCR & LPS
T cells
Piperine
[100 μM]

Figure 19 A-B

Figure 20. Piperine treatment of activated B lymphocytes decreases immunoglobulin production. IgG1, IgG2a, IgG2b, IgG3, IgA and IgM secretion by B cells was measured by ELISA. B cells were activated by α-BCR, α-CD40 and a sub-proliferative concentration of LPS. B cells were activated in the presence or absence of T cells, separated by a 0.4 μm pore transwell for 48 h. B cells were then separated, washed, replated and reactivated with α-BCR, α-CD40 and a sub-proliferative concentration of LPS in the presence or absence of 100 μM piperine for an additional 48 h. Supernatants were then collected and assayed for antibody production by ELISA. Significance is indicated when compared to activated B cells co-cultured with T cells (n=3, SEM; ns $p>0.05$, *$p<0.05$, ***$p<0.001$).

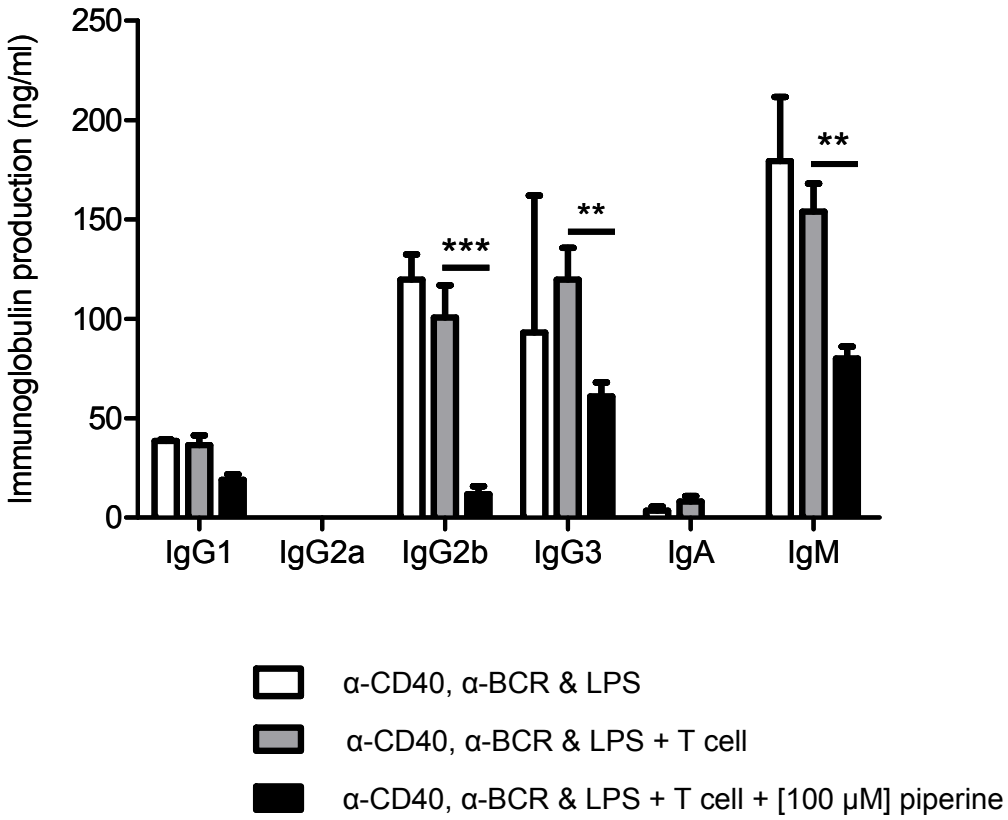

Immunoglobulin production (ng/ml)
250
200
150
100
50
0
***
**
**
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
α-CD40, α-BCR & LPS
α-CD40, α-BCR & LPS + T cell
α-CD40, α-BCR & LPS + T cell + [100 μM] piperine

Figure 20

3.2.3 PIPERINE INHIBITS B LYMPHOCYTE MHC II AND CO-STIMULATORY MOLECULE EXPRESSION.

During B cell activation, antigen is processed and presented in the context of MHC class II to attract cognate antigen-specific T cell help. Critical for full activation, the co-stimulatory molecule CD40 binds CD40L on T cells, which provides survival and class switch signals to the B cell. Furthermore, co-stimulatory molecule CD86 binding to CD28 on T cells is important for T cell stimulation, enhanced B cell proliferation and antibody production (Clark et al., 2003; Clatza et al., 2003; Hathcock et al., 1994; Lenschow et al., 1996; Ren et al., 1994; Rivera et al., 2001). Therefore, we investigated the expression of these important molecules during B cells activation in the presence or absence of piperine. During T-dependent activation of B cell, a significant dose dependent reduction in the expression of MHC II (Figure 21 A; Figure 22 A) and co-stimulatory molecules CD40 (Figure 21 B; Figure 22 B) and CD86 (Figure 21 C; Figure 22 C) was observed in piperine treated groups when compared to untreated controls. Similarly, a significant dose dependent reduction in MHC II (Figure 23 A; Figure 24 A) and co-stimulatory molecules CD40 (Figure 23 B; Figure 24 B) and CD86 (Figure 23 C; Figure 24 C) expression was observed for T-dependent and T-independent activated B cells in the presence of piperine when compared to untreated controls. B cell expression of MHC II, CD40 and CD86 following 100 μM piperine treatment approached the expression levels of non-activated B cells (Figures 21-24). These results indicate that piperine negatively affects expression levels of molecules are important for B cell antigen presentation.

Figure 21. The effect of piperine on MHC II, CD40 and CD86 expression following T-dependent activation of B lymphocytes. B cells were activated with α-CD40 and α-BCR Ab for 24 h in the presence of piperine (25, 50 and 100 μM) or vehicle. B cell expression of MHC II (A), CD40 (B) and CD86 (C) was assessed by flow cytometry, and gated on live cells. Representative histograms of 3 independent experiments are shown. NA = Non-activated, A = Activated, Veh = Vehicle, (n=3).

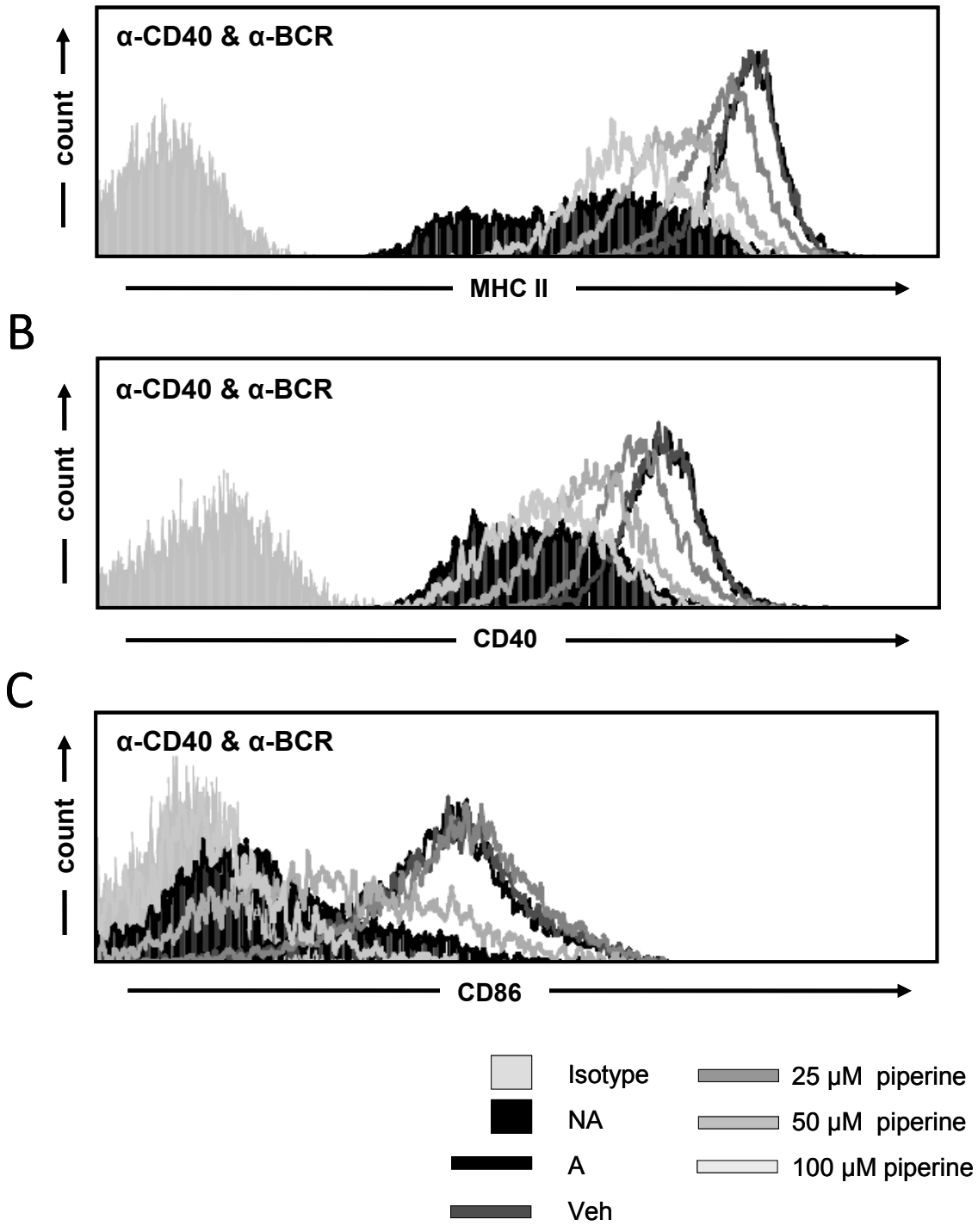

α-CD40 & α-BCR
count
MHC II
B
α-CD40 & α-BCR
count
CD40
C
α-CD40 & α-BCR
count
CD86
Isotype
NA
A
Veh
25 μM piperine
50 μM piperine
100 μM piperine

Figure 21 A-C

Figure 22. Piperine treatment inhibits MHC II, CD40 and CD86 expression following T-dependent activation of B lymphocytes. B cells were activated with α-CD40 and α-BCR Ab for 24 h in the presence of piperine (25, 50 and 100 μM) or vehicle. B cell expression of MHC II (A), CD40 (B) and CD86 (C) was assessed by flow cytometry and gated on live cells. Mean channel fluorescence backshift was used for statistical comparison to vehicle group. Significance is indicated in comparison to the vehicle group; NA = Non-activated, Veh = Vehicle, (n=3, ***$p<0.001$, *$p<0.05$, ns=not significant, SD).

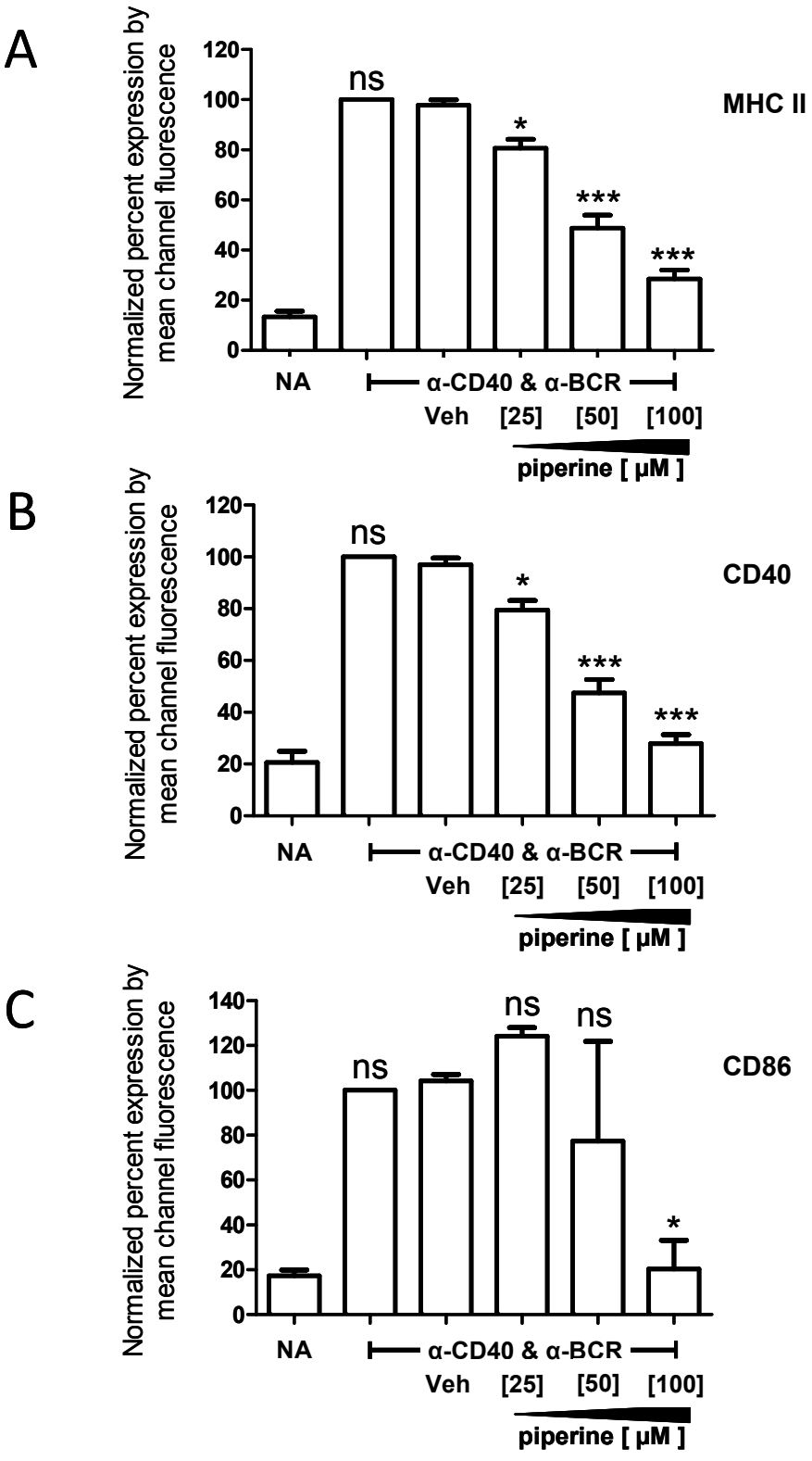

A
Normalized percent expression by mean channel fluorescence
120
100
80
60
40
20
0
ns
*
***
***
MHC II
NA
α-CD40 & α-BCR
Veh
[25]
[50]
[100]
piperine [μM]
B
Normalized percent expression by mean channel fluorescence
120
100
80
60
40
20
0
ns
*
***
***
CD40
NA
α-CD40 & α-BCR
Veh
[25]
[50]
[100]
piperine [μM]
C
Normalized percent expression by mean channel fluorescence
140
120
100
80
60
40
20
0
ns
ns
ns
*
CD86
NA
α-CD40 & α-BCR
Veh
[25]
[50]
[100]
piperine [μM]

Figure 22 A-C

Figure 23. The effect of piperine on MHC II, CD40 and CD86 expression following T-independent activation of B lymphocytes. B cells were activated with LPS for 24 h in the presence of piperine (25, 50 and 100 μM) or vehicle. B cell expression of MHC II (A), CD40 (B) and CD86 (C) was assessed by flow cytometry and gated on live cells. Representative histograms of 3 independent experiments are shown. NA = Non-activated, A = Activated, Veh = Vehicle, (n=3).

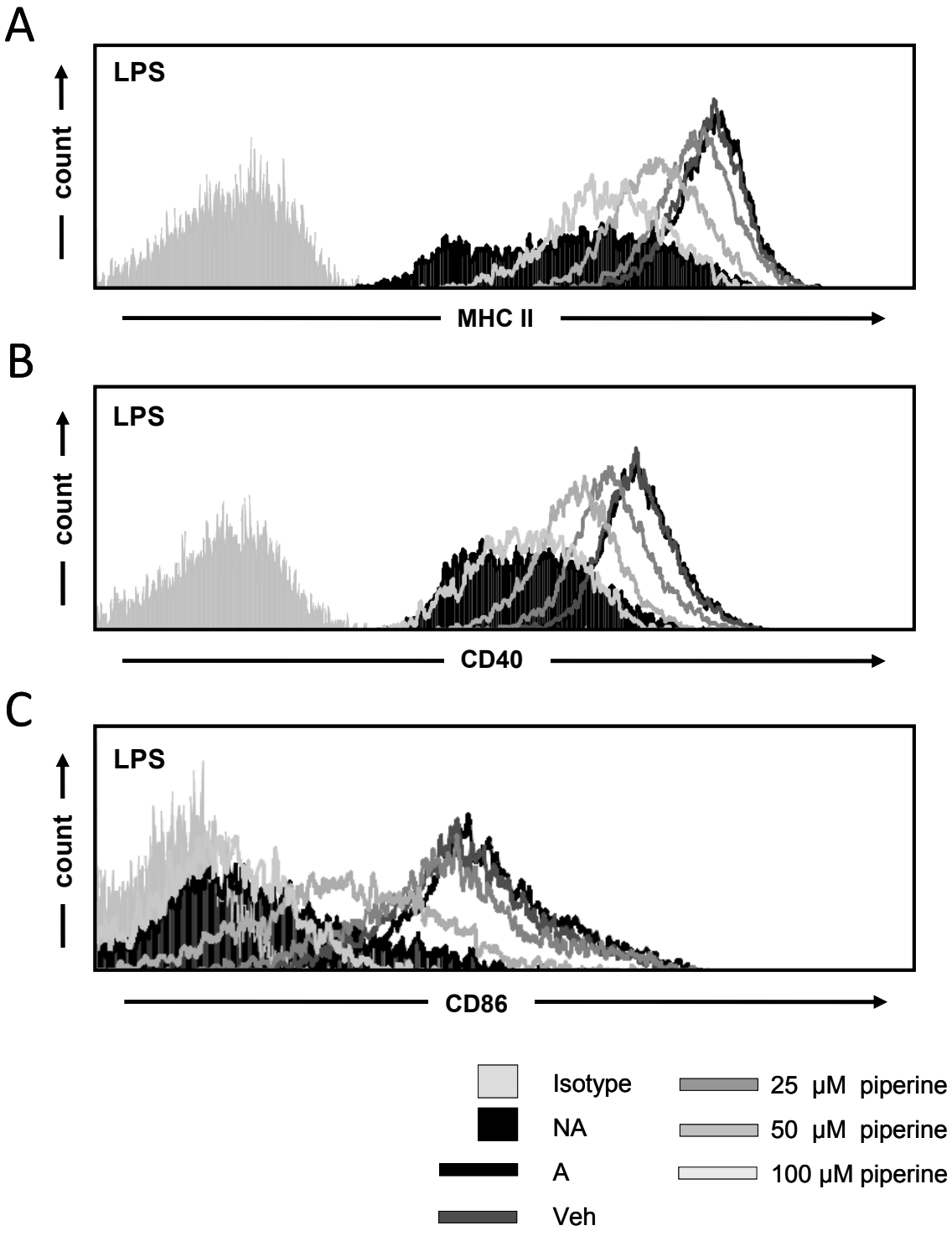

A
LPS
count
MHC II
B
LPS
count
CD40
C
LPS
count
CD86
Isotype
NA
A
Veh
25 μM piperine
50 μM piperine
100 μM piperine

Figure 23 A-C

Figure 24. Piperine treatment inhibits MHC II, CD40 and CD86 expression following T-independent activation of B lymphocytes. B cells were activated with LPS for 24 h in the presence of piperine (25, 50 and 100 μM) or vehicle. B cell expression of MHC II (A), CD40 (B) and CD86 (C) was assessed by flow cytometry and gated on live cells. Mean channel fluorescence backshift was used for statistical comparison to vehicle group. Significance is indicated in comparison to the vehicle group. NA = Non-activated, Veh = Vehicle, (n=3, ***$p<0.001$, **$p<0.01$, ns=not significant, SD).

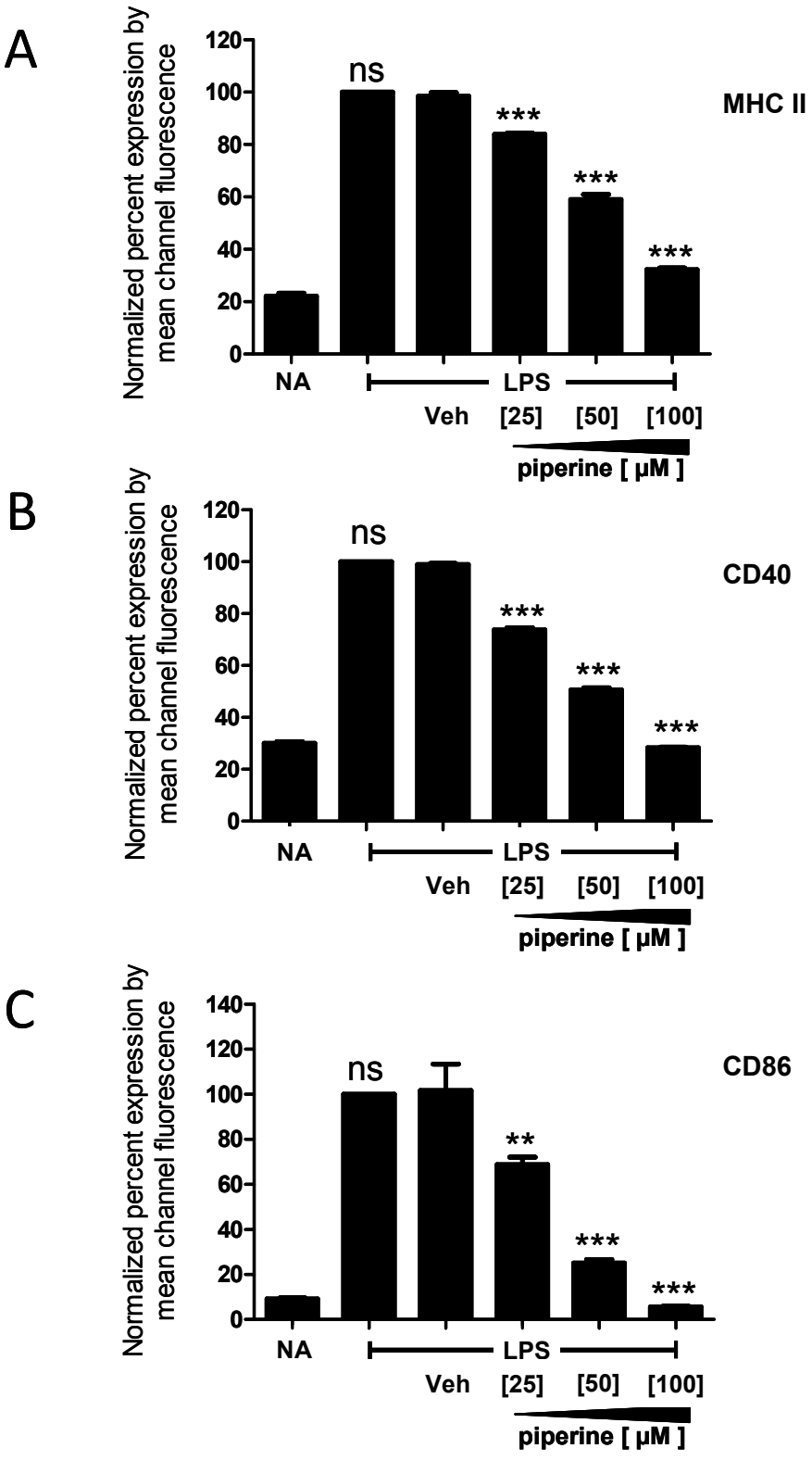

A
Normalized percent expression by mean channel fluorescence
120
100
80
60
40
20
0
ns
***
***
***
MHC II
NA
LPS
Veh
[25]
[50]
[100]
piperine [µM]
B
Normalized percent expression by mean channel fluorescence
120
100
80
60
40
20
0
ns
***
***
***
CD40
NA
LPS
Veh
[25]
[50]
[100]
piperine [µM]
C
Normalized percent expression by mean channel fluorescence
140
120
100
80
60
40
20
0
ns
**
***
***
CD86
NA
LPS
Veh
[25]
[50]
[100]
piperine [µM]

Figure 24 A-C

3.2.4 PIPERINE INHIBITS B LYMPHOCYTE INDUCED T CELL PROLIFERATION.

At the start of a humoral response the BCR binds to antigen, which activates the B cell. The antigen is subsequently internalized, processed and presented in the context of MHC II, which stimulates an antigen-specific helper T cell. This in turn co-activates both the T and B cell, allowing for a robust T cell response and development of effector B cell response (Rivera et al., 2001; Vascotto et al., 2007). Therefore, the effect of piperine on B cell presentation of antigen to T cells was assessed. B cells were activated by an ovalbumin labelled α-BCR Ab complex, α-CD40 Ab, and sub-proliferative levels of LPS for 6 h in the presence or absence of 100 μM piperine. B cells were then washed and incubated with OVA-specific TCR transgenic OT-II T cells for 72 h. T cell proliferation was then assessed by flow cytometry using an Oregon Green dye dilution assay. Representative data illustrates a marked dose-dependent inhibition by piperine of B cell-mediated T cell activation (Figure 25 B). Non-stimulated T cells (Figure 25 A) were used to control for spontaneous proliferation. These results indicate that piperine may inhibit B cell antigen-presenting function of B cells.

Figure 25. Piperine inhibits B lymphocyte-mediated T cell activation in a dose-dependent manner. B cells were activated by α-CD40, and biotin-labelled α-BCR Ab, secondary OVA-labelled α-biotin Ab, and a sub-proliferative concentration of LPS in the presence of piperine (25, 50, and 100 μM) or vehicle for 6 h. B cells were subsequently washed and co-cultured with Oregon Green stained transgenic OT-II CD4+ T cells for 72 h. T cells stimulated by activated B cells (B) were measured by flow cytometry using an Oregon Green dye dilution assay. Cell counts were normalized and represented on the same ordinate scale within each specific method of activation. Non-stimulated T cells (A) were used to control for spontaneous proliferation. Representative histograms; A = Activated, Veh = Vehicle, (n=2).

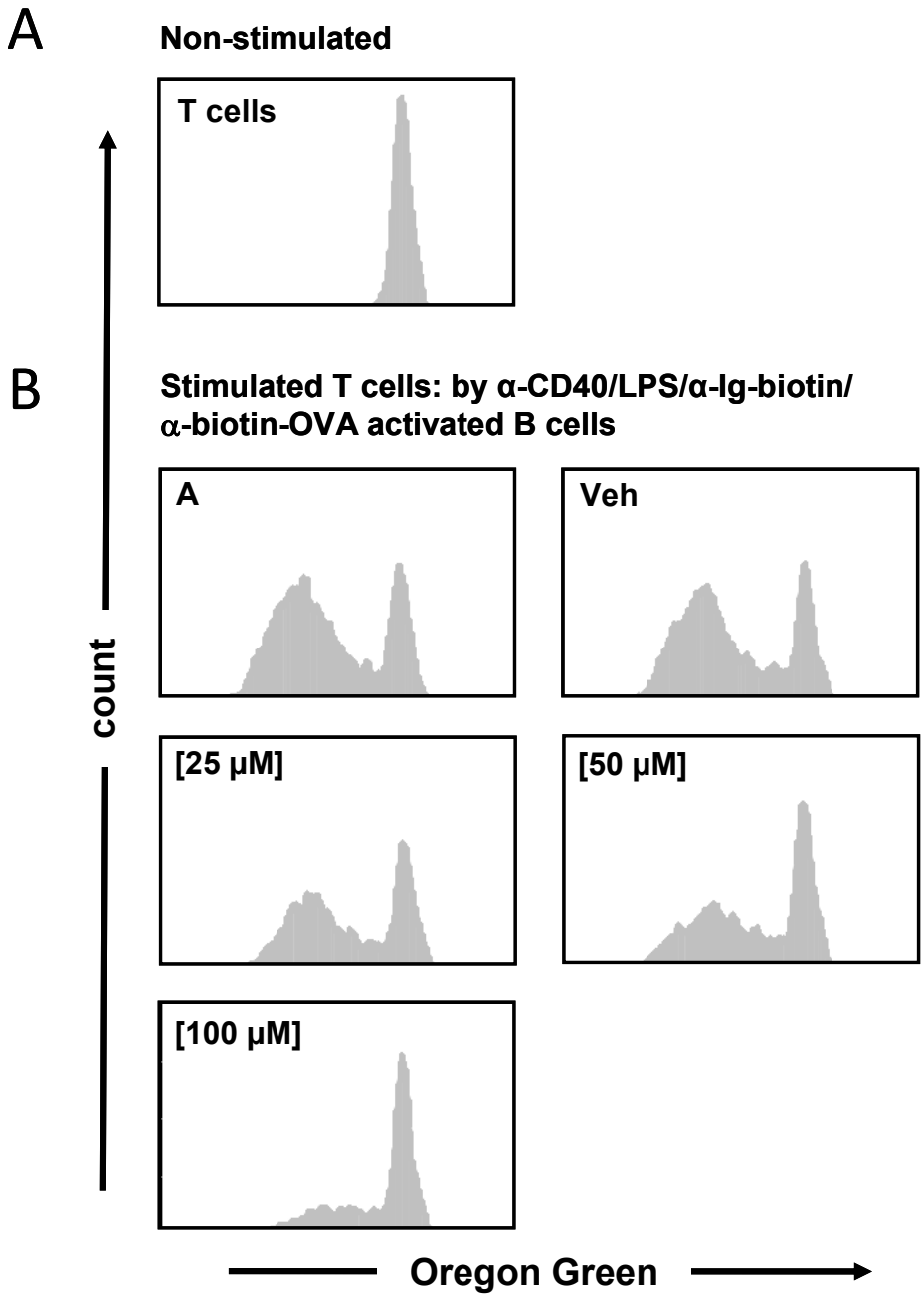

A
Non-stimulated
T cells
B
Stimulated T cells: by α-CD40/LPS/α-Ig-biotin/
α-biotin-OVA activated B cells
A
Veh
[25 μM]
[50 μM]
[100 μM]
count
Oregon Green

Figure 25 A-B

CHAPTER 4. DISCUSSION

4.1 DISCUSSION

The results presented in our study demonstrate that piperine inhibits B cell activation and effector function. Results obtained by examining proliferation, survival, and signal transduction, indicate that piperine inhibits multiple aspects of B cell activation. Furthermore, this study has demonstrated that piperine inhibits MHC II and co-stimulatory molecule up-regulation, cytokine and antibody production by B cells, as well as preventing effective T cell stimulation B cells.

Piperine inhibited B cell proliferation in a dose-dependent manner (Figure 6) by preventing B cells from transitioning from G0/1 to S-phase of the cell cycle (Figure 14). The cyclin Ds play an important role in this transition (Banerji et al., 2001; Harbour and Dean, 2000; Solvason et al., 2000), and thus provide an important focal point of this study. Furthermore, the cyclin Ds may be particularly important in the haematopoietic system, as mice with defective cyclin Ds die due to severe anaemia, yet have several normally development of several other tissues and organs, suggesting that haematopoietic cells may be particularly dependent on cyclin-D compared to other cell types (Massague, 2004).

Cyclins D2, D3, E and A, but not cyclin D1, are up-regulated during BCR engagement and proliferation (Hsia et al., 2002; Solvason et al., 2000). My results are in agreement with these findings in that I demonstrate cyclin D2 and D3 up-regulation during T-dependent (Figure 15) and T-independent (data not shown) activation of B cells. However, I also demonstrated cyclin D1 up-regulation during B cell activation. Furthermore, the expression of these cyclins was inhibited by piperine in a dose-

dependent manner. Previous studies using immature WEHI 231 B cells demonstrated that cyclin D2 expression is critically important in BCR induced G0/1 to S-phase transition in immature B cells, as well as for B cell survival (Banerji et al., 2001; Solvason et al., 2000). Thus, the G0/1-phase cell cycle arrest observed in my study (Figure 14) may be a direct result of diminished cyclin D2 expression due to piperine treatment. Although the decreased level of cyclin D expression during piperine treatment correlates with decreased B cell proliferation, it remains to be determined if this is the primary mechanism.

To determine if diminished cyclin D expression is the direct cause of inhibited B cell proliferation in the presence of piperine, we could use a protein transduction method as previously described (Hsia et al., 2002). This technique uses purified protein preparations of cyclin D linked to the HIV1 *trans*-activator gene product TAT, which allows the construct to penetrate the cell membrane while the fused cyclin D protein restores or elevates endogenous cyclin D levels (Ford et al., 2001; Hsia et al., 2002). This approach could be used in primary B cell cultures treated with piperine and would allow elucidation of whether cyclin D inhibition is involved in the anti-proliferative effect of piperine on B cells. Alternatively, to determine if the inhibitory effects of piperine are downstream of the cyclin Ds, one could utilize mitogenically activated pRb knockout B cells. Cyclins D1, D2, and D3 function as key regulators for B cell cycle progression by activating CDK4/6, driving the cell towards a G0/1- to S-phase transition. Activated CDK4/6 phosphorylates the retinoblastoma protein (pRb), which releases pRb from the pro-cell cycle E2F family of DNA-binding transcription factors. The now free E2F transcription factors promote cell cycle progression and proliferation (Harbour and Dean,

2000; Piatelli et al., 2004). Cells that lack pRb may no longer depend on the D-type cyclins to proliferate (Sherr, 2004). Thus, if activated pRb knockout B cells are able to proliferate in the presence of piperine, it would suggest that the inhibitory effects of piperine are specific to cyclin D activity, or lie upstream of cyclin D expression. However, pRb protein has also been implicated in playing an important role in regulating cell differentiation, and pRb knockout mice die within 14 to 15 days of gestation (Corn and El-Deiry, 2002), indicating a need to utilize a conditional or inducible pRb knockout. One possible method is an *in vivo* Cre-lox system, which can mediate the deletion of the pRb gene upon treatment of mice with an inducing agent such as tamoxifen (Jaisser, 2000). This would allow temporal control over knocking out B cell specific pRb.

Many signalling pathways have been implicated in activating cyclin D1, D2 and D3. Among these, Akt and ERK1/2 phosphorylation, as well as translocation of NF-κB transcription factors are relevant to my study (Glassford et al., 2001; Piatelli et al., 2004). Activation of the PI3K-Akt pathway and phosphorylation of ERK1/2 are known to be involved in cell growth, survival and metabolism (Banerji et al., 2001; Vanhaesebroeck and Alessi, 2000), and result from BCR engagement (Okkenhaug and Vanhaesebroeck, 2003; Gold et al., 2000), as well as CD40 (Arron et al., 2001) and TLR4 ligation (Vanhaesebroeck and Alessi, 2000).

Akt is important for cell survival, growth and proliferation. My study is the first documentation of diminished B cell phosphorylation of Akt in the presence of piperine, which correlates with the inhibitory effect of piperine on B cell proliferation. Additionally, functions downstream of Akt, such as cell survival and cell cycle

progression, were also inhibited by piperine treatment (Vivarelli et al., 2004; Laird et al., 2009; Vanhaesebroeck and Alessi, 2000).

Signalling pathways emanating from BCR, CD40 and TLR4 all have the capacity to activate Akt. In B cells, both TLR4 and CD40 ligation activate the PI3K/Akt pathway by TRAF6. BCR-induced signalling transiently increases PI3K/Akt activity (Banerji et al., 2001). However, without simultaneous CD40 ligation, this activation of PI3K/Akt is not sustained. Without continued Akt activity during BCR engagement, B cell death may occur (Banerji et al., 2001), indicating the important role that Akt plays in cell survival. For example, Akt phosphorylates IKK, which results in NF-κB activity, and induction of the anti-apoptotic protein, B cell lymphoma-extra large (Bcl-xL) (Kane et al., 1999). Dephosphorylated Bcl-2-associated death promoter (BAD) protein normally binds and inactivates Bcl-xL, leading to apoptosis. Akt is known to phosphorylate BAD, causing it to disassociate from the anti-apoptotic Bcl-xL protein, thus inhibiting apoptosis (Gold et al., 2000). Akt is also known to inhibit FOXO-dependent expression of pro-apoptotic Bim (Massague, 2004). Thus, the long-term diminishment of phosphorylated Akt over time may weaken B cell survival. In my study, piperine was not shown to be immediately toxic (Figure 11), yet over a prolonged period of time piperine increased cell death in activated (Figure 10 B) and non-activated B cells (Figure 12). This may be a result of diminished Akt phosphorylation during prolonged exposure to piperine and a loss of the pro-survival signals that lie downstream of Akt activation.

In addition to the important role of Akt in cell survival, Akt also regulates cell cycle progression by increasing cyclin D expression and inhibiting $p27^{KIP1}$ expression (Liang and Slingerland, 2003). As cyclin Ds and $p27^{KIP1}$ regulate the activity of CDK4/6

and the resulting phosphorylation of Rb, as mentioned previously, Akt activation promotes the transition of cells from G0/G1 to the S-phase of the cell cycle. My results demonstrated a very modest increase in Akt phosphorylation following B cell stimulation in the presence of piperine; however, activation of Akt was not sustained, as a dose-dependent reduction in Akt phosphorylation was observed at 20 min (Figure 17). As my study demonstrated a dose-dependent reduction in Akt phosphorylation and cyclin D expression, as well as a block in cell cycle progression in the presence of piperine, an increase in $p27^{Kip1}$ may result with a corresponding inactivation of E2F. Future studies should investigate the effects of piperine on the expression of these factors during B cell activation.

Just as Akt phosphorylation is important for cell growth and survival (Banerji et al., 2001; Vanhaesebroeck and Alessi, 2000), ERK1/2 phosphorylation is also important in mitogenic activation, cell growth and cell cycle progression (Roux and Blenis, 2004). Likewise, signal transduction by BCR, CD40 and TLR4 can also induce ERK1/2 phosphorylation (Barton and Medzhitov, 2003; Gerondakis et al., 2007; Purkerson and Parker, 1998). The current study has demonstrated a dose-dependent reduction in ERK1/2 phosphorylation on activated B cells in the presence of piperine.

ERK1/2 phosphorylation promotes CDK activation through the stabilization of c-myc, a transcription factor that mediates direct transcriptional activation of cyclin D expression (Massague, 2004; Sutherland et al., 1996). Additionally, c-myc indirectly removes negative regulatory phosphates from CDKs, as well as induces ubiquitination of CDK inhibitors, such as $p21^{WAF1}$ and $p27^{KIP1}$ (Sears and Nevins, 2002; Harnett, 2004). This reiterates the importance of ERK1/2 signalling in cell cycle regulation, as a

reduction in ERK1/2 phosphorylation as I observed, would inhibit c-myc stabilization and reduce cyclin D activity (Figure 15). Future investigation of $p27^{KIP1}$ status in piperine-treated activated B cells would be pertinent, as a reduction in both ERK1/2 and Akt phosphorylation can also lead to increased $p27^{KIP1}$ levels, as previously mentioned.

The results of my study are consistent with studies using other cell types in which piperine was shown to inhibit ERK1/2 phosphorylation. For example, piperine inhibited ERK1/2 phosphorylation in interleukin-1β-stimulated synoviocytes (Bang et al., 2009). Furthermore, Hwang et al., (2011) examined a fibrosarcoma cell line and, demonstrated that piperine inhibited PMA-induced phosphorylation of PKC-α and ERK1/2. Since my study has demonstrated a modest reduction in ERK1/2 phosphorylation due to piperine treatment of B cells (Figure 16), the effect of piperine on ERK1/2 activation may be uniform across multiple cells types, and could be a major mechanism by which piperine decreases cell proliferation, as observed in my study and others (Harris et al., 2005; Kakarala et al., 2010).

In response to TLR4 activation, NF-κB and ERK signalling cascades are coordinated through IκB-Kinase (IKK). IKK is therefore a probable candidate for investigation as a site of inhibition by piperine. It is suggested that TLR4 signalling triggers the activation of IKK, which frees Tpl2 to activate ERK1/2, resulting in c-myc stabilization and cyclin D expression. Furthermore, IKK phosphorylation results in the nuclear translocation of NF-κB c-Rel, and subsequent induction of transcription of the pro-survival factor Bcl-xL. C-Rel also regulates G1-to S-phase progression and Rb hyper-phosphorylation (Gerondakis et al., 2007; Massague, 2004; Sutherland et al., 1996; Sears and Nevins, 2002; Harnett, 2004). Hsia et al., (2002) also demonstrated impaired

of G1-phase cyclins using c-Rel knockout mice, which was independent of $p27^{KIP1}$ turnover. Furthermore, B cells deficient in c-Rel expression have a reduced capacity to proliferate in response to CD40 and LPS signalling, as well as having defects in survival (Schmidt et al., 2003).

Although the NF-κB and ERK1/2 pathways are not fully understood, they remain critically important in B cell signalling. Therefore, considering the effects of piperine on inhibiting B cell proliferation (Figure 5), inducing G0/1-phase cell cycle arrest (Figure 14), and increased B cell death during prolonged piperine treatment (Figure 10 &12), it is likely that piperine may be mediating its effects within this coordinated NF-κB/ERK signalling cascade. As TRAF6 lies upstream of both IKK−NF-κB and Tpl2−ERK1/2 pathways, and IKK is necessary for Tpl2 release and ERK 1/2 activation, both TRAF6 and IKK remain probable sites for future investigation.

There is supporting evidence concerning the effect of piperine on NF-κB signalling pathways. For example, Pradeep and Kuttan (2004) investigated immune regulation and inflammatory response with respect to B16F-10 melanoma cells, and showed that piperine inhibits NF-κB subunits p65, p50 and c-Rel. Kumar et al., (2007) examining endothelial cells in an inflammatory setting, demonstrated that piperine inhibited NF-κB by attenuating IKK activity. Furthermore, reiterating the link between ERK and NF-κB signalling pathways to the effect of piperine, a recent study by Hwang et al., (2011) has demonstrated that piperine inhibits PMA-induced tumour cell invasion in a dose-dependent manner by blocking PKC/ERK, and NF-κB signalling pathways.

The major signalling cascades delineated above are not wholly separate, but are rather intrinsically involved with each other. To ensure robust Akt and ERK1/2

activation, as well as an antibody response, CD40 ligation must occur in conjunction with BCR engagement (Parry et al., 1994; Hanissian and Geha, 1997; Pullen et al., 1999). TLR4 ligation promotes antigen-specific antibody responses (Pasare and Medzhitov, 2005) as well as inflammatory activities of NF-κB (Gerondakis et al., 2007). Furthermore, evidence is accumulating that TLR agonism is needed during a T-dependent response. For example, Ruprecht and Lanzavecchia (2006) have demonstrated that T-dependent activated human B cells will undergo abortive proliferation and differentiation, unless co-stimulated with a TLR agonist such as LPS. Likewise, Pasare and Medzhitov (2005) demonstrated, using MyD88 knockout mice, that generation of T-dependent antigen-specific antibody responses require co-activation of TLR on B cells. In this respect, it is difficult to delineate the site of piperine's actions, as BCR, CD40 and TLR4 signalling cascades are dependent on each other for B cell activation, survival, and proliferation.

As TRPV1 is the only known receptor for piperine (McNamara et al., 2005), it was important to determine whether TRPV1 is required for the inhibitory effects of piperine on activated B cells. My study demonstrated no significant difference in proliferation when comparing activation of TRPV1 knockout B cells to wild type B cells in the presence of piperine (Figure 18). Additionally, previous work done by Inada et al., (2006) demonstrated that TRPV1 was not detected on splenocytes, and was indeterminable on avian DT40 B cells (Inada et al., 2006). Taken together, these results suggest that TRPV1 signalling is not involved in the inhibitory effects of piperine on B cell function.

Since ERK1/2 phosphorylation was inhibited by piperine treatment of B cells following BCR engagement, as well as CD40 and TLR4 ligation, I thought it important to investigate cytokine production by B cells. ERK1/2 and NF-κB transcriptional co-regulation have shown to play an important role in IL-10 production by macrophages (Banerjee et al., 2006). Correspondingly, I found that B cells activated in the presence of piperine had diminished IL-10 production (Figure 19).

IL-10 production by B cells is implicated in immune regulation by suppressing exacerbated and non-specific inflammation (Lund, 2008; Mizoguchi and Bhan, 2006; Duddy et al., 2004), which may aid in antigen-directed germinal centre affinity maturation and minimize bystander cell activation. Moreover, IL-10-producing B cells have been demonstrated experimentally to inhibit the progression of experimental autoimmune encephalomyelitis (EAE); similar findings correlate to clinical detection of IL-10-producing B cells in systemic lupus erythematosus (SLE) (Mizoguchi and Bhan, 2006). Thus, the reduction in IL-10 production by piperine-treated activated B cells suggests that piperine may interfere with regulatory B effector cell functions. Furthermore, through inhibiting IL-10 production, piperine may promote susceptibility to autoimmunity, as was observed when IL-10-deficient B cells were unable to confer protection from arthritis experimentally (Evans et al., 2007).

Interleukin-6 can augment B cell differentiation, increase immunoglobulin production, and promote cellular growth (Hobbs et al., 1991). IL-6 is produced in a variety of clinical inflammatory settings, including SLE and rheumatoid arthritis; furthermore, over-expression of IL-6 can produce arthritic-like autoimmunity in mice. B cells are known to produce IL-6, which contributes to inflammation (Maeda et al., 2010).

As observed with IL-10, IL-6 production by B cells was inhibited in the presence of piperine (Figure 19), potentially as a result of diminished NF-κB activity, although this remains to be elucidated. However, as many other studies that examined the effects of piperine on other cell types have found a reduction in NF-κB activity (Hwang et al., 2011; Kumar et al., 2007; Pradeep and Kuttan, 2004), this is a likely target of piperine.

Although the NF-κB pathway regulates both IL-6 and IL-12 production (Takeda and Akira, 2004; Bae et al., 2010), I was unable to demonstrate the presence of IL-12 in B cell cultures (data not shown). Previous work has demonstrated that after a 4 day activation period, B cells were able to produce significant quantities of IL-12 (Harris et al., 2000). As my study examined B cells activated in culture for only 2 days, perhaps a longer culture period would have produced measurable concentrations of IL-12. Although diminished IL-6 production by B cells in the presence of piperine alludes to a possible anti-inflammatory role for piperine, decreased IL-10 production observed in our study may suggest pro-inflammatory activity. Studying IL-12 production would provide further information to elucidate the exact effect of piperine on B cell-mediated inflammation.

Cytokine production is also known to drive antibody production, such as IL-6 mediated enhancement of IgG production (Maeda et al., 2010). However, previous work has demonstrated that even in the presence of T_H cell cytokine secretion and CD40 binding, T-dependent antigen-specific antibody responses are diminished in the absence of TLR ligands *in vivo* (Pasare and Medzhitov, 2005). This suggests that although mature B cells may become activated in a T-dependent manner, TLR ligation is also needed for a robust B effector plasma cell response. I therefore co-cultured B and T cells

with sub-proliferative concentrations of LPS to determine B effector antibody production in the presence of piperine. In the presence of vehicle, significant quantities of IgG2b, IgG3 and IgM were produced upon B cell activation. Importantly, I observed that production of these antibody isotypes by activated B cells was significantly reduced in the presence of piperine (Figure 20), indicating that piperine inhibits B cell antibody production. Interestingly, steady state and antigen-specific IgM and IgG production are considerably reduced and in some instances completely abolished in MyD88 knockout mice (Pasare and Medzhitov, 2005). Previously, piperine has been found to inhibit NF-κB and ERK1/2 (Bae et al., 2010; Kumar et al., 2007; Pradeep and Kuttan, 2004), which lie downstream of the TLR4-MyD88 pathway, providing a possible mechanism behind the piperine-mediated reduction in antibody production observed in this study.

Although TLR4 co-activation with BCR and CD40 may be a requirement for certain immunoglobulin class switches, TLR4 signalling alone can activate and induce class switch to IgG3, as well as IgG1 and IgE when co-stimulated with IL-4 (Li et al., 1997; Dil and Marshall, 2009). A recent study demonstrated that LPS-TLR4 signalling requires PI3K to induce B cell proliferation, yet PI3K is not required for differentiation into plasma cells or antibody class switch (Dil and Marshall, 2009). Indeed, this current study demonstrated that LPS-stimulated B cell proliferation was inhibited by piperine, and the effect of piperine extended to the inhibition of antibody production; however, the effect of piperine on class switching was not examined. As this study has also demonstrated that piperine inhibits activation of Akt, which lies downstream of PI3K, it would be interesting to investigate the effects of piperine on antibody class switch, to

determine whether piperine has additional effects that are independent of the PI3K-Akt axis.

Although cytokine and antibody production are important aspects of B cell effector function, antigen presentation by B cells is also required to stimulate a strong T cell response. Following antigen binding to the BCR, B cells become activated and subsequently internalize and deliver antigen to sub-cellular compartments for processing into peptides that are presented to T cells in the context of MHC II (Song et al., 1995; Clark et al., 2003). During B cell activation and antigen presentation, co-stimulatory molecules CD80, CD86 and CD40 are known to increase in expression; however, CD86 was examined instead of CD80 in my study, as BCR cross-linking alone has previously been shown to up-regulate CD86 and not CD80 (Lenschow et al., 1996).

The interaction between B cell CD86 and T cell CD28 is vital in T cell stimulation; moreover, this interaction also contributes to the production of IgG1 and IgE and enhances anti-apoptotic Bcl-xL protein expression during T-dependent activation of B cells (Schmidt et al., 2003). As piperine inhibits T-dependent (Figure 22) and T-independent (Figure 24) expression of CD86 on activated B cells, it is apparent that piperine-treated B cells may have a reduced capacity to stimulate T cells, and which reduces the capacity of T cells to offer help to B cells, potentially resulting in a negative impact on B cell survival and antibody production, as my study has demonstrated (Figure 20).

Although LPS and CD40 ligation result in up-regulation of CD86 on B cells, previous work has also demonstrated an up-regulation of CD54 (ICAM-1) (Vivarelli et al., 2004). Non-circulating mature B cells are thought to be retained within the marginal

zone of the spleen, in part, due to stromal cell ICAM-1 and VCAM-1 binding to B cell LFA-1 and VLA-4 molecules, respectively. As piperine has been previously shown to inhibit ICAM-1 expression on endothelial cells, and BCR-mediated activation induces LFA-1 expression (Boscacci et al., 2010; Carrasco et al., 2004; Lu and Cyster, 2002), perhaps piperine may inhibit ICAM-1 expression on splenic stromal cells, and therefore inhibit B cell retention and activation *in vivo* within the spleen. Thus, future investigation of the effect of piperine treatment on CD54 expression by activated B cells is warranted.

Also important to B cell-induced T cell proliferation is CD40. CD40 is implicated in B cell survival and differentiation, and binding of CD40 to CD40-Ligand on T cells up-regulates co-stimulatory molecules, such as CD86, as well as inducing strong NF-κB directed survival signals (Berberich et al., 1994; von Bergwelt-Baildon et al., 2004). Interestingly, RelB, a NF-κB subunit, up-regulates CD40 expression on B cells (O'Sullivan et al., 2000). As previously mentioned, piperine inhibits NF-κB in other cell types (Kumar et al., 2007; Pradeep and Kuttan, 2004); therefore, inhibition of NF-κB may be a possible mechanism by which piperine mediates inhibition of CD40 expression, resulting in reduced T cell proliferation.

Antigen cross-linking of the BCR results in internalization of the antigen and its passage through the endocytic pathway to endosomal compartments rich in MHC II (Vascotto et al., 2007). The antigen-BCR complex in the endosomal compartment signals similarly to cell surface BCR engagement, resulting in ITAM recruitment of Syk, phosphorylation of BLNK, Grb2, PLC-γ2, Vav and other associated molecules (DeFranco, 1997; Ishiai et al., 1999). ERK1/2, as described above in reference to surface BCR-mediated signalling, is activated as a result (DeFranco, 1997; Ishiai et al.,

1999). More recently, Al-Alwan et al. (2007) have demonstrated that PI3K play an important role in antigen presentation by using primary wild-type and PI3K knockouts (Al-Alwan et al., 2007). As both ERK1/2 and PI3K activity are required for antigen presentation, and PI3K signal transduction can activate Akt, it is reasonable to correlate these intracellular mediators to the diminished capacity of B cells to stimulate T cells in the presence of piperine. However, as ERK1/2 and Akt phosphorylation occurs during B cell activation and antigen presentation, it is difficult to delineate whether the inhibitory effects of piperine are on B cell activation or antigen processing. To resolve this issue, a fluorescently labelled anti-BCR ovalbumin labelled complex could be used to determine whether piperine inhibits antigen internalization by confocal microscopy as previously done (Al-Alwan et al., 2007).

This study has demonstrated the inhibitory effect of piperine on B cell proliferation and effector functions. In this regard, piperine may represent a possible anti-inflammatory agent for future investigation. However, previous work by Pathak and Khandelwal (2007) has demonstrated that piperine alone on LPS-activated splenocytes had no influence on splenocyte proliferation (Pathak and Khandelwal, 2007). Furthermore, the above mentioned study used the same mitogenic concentration of LPS as in my study, yet Pathak and Khandelwal (2007) used 175 μM, or, 1.75× greater concentration of piperine than my study. I have shown in my study a near ablation in proliferation at a piperine concentration of 100 μM piperine (Figure 7), which indicates my results conflict directly with previous findings. The differing results between above mentioned study and my research may be a result of differing vehicles used with piperine. Unfortunately, the vehicle used by Pathak and Khandelwal (2007) is not

transparent, and thus conclusions cannot be drawn. Pathak and Khandelwal (2007) have also determined that cadmium chloride treated B and T cells exhibit normal proliferation and cytokine production by piperine treatment (Pathak and Khandelwal, 2007). Interestingly, in 1856, Galletly documented that cadmium chloride precipitates as a salt with piperine (Galletly, 1856), and more recently, treatment with piperine has been patented as method of preventing gastrointestinal absorption of cadmium by forming an insoluble salts (Majeed, 1996). Therefore, it is possible that piperine rescued lymphocytes not from cadmium poisoning, but rather prevented cadmium cellular absorption by cadmium-piperine salt formation and precipitation. Meaning, if the cadmium and piperine precipitate out, presumably neither would be affecting the B cells, and thus piperine would not inhibit proliferation and cytokine production by activated B cells.

Interestingly, oral administration of piperine at 4.5 mg/kg for 5 days leads to a decrease in thymus, spleen and mesenteric lymph node weight, as well as a reduction in antibody-forming cells and serum antibody titer (Dogra et al., 2004). Conversely, Sunila and Kuttan (2004) found that at treatment with a higher concentration of piperine, 60 mg/kg for the same time period, resulted in an increase in immune cell number, as well as antibody titer (Sunila and Kuttan, 2004). These results indicate that the effects of piperine treatment *in vivo* may differ with administration route and concentration.

4.2 FUTURE DIRECTIONS.

For many years, there has been considerable effort to identify new medicinal anti-inflammatory agents from natural sources. As this is the first report on the effects of

piperine on B cell activation and effector function, there are many exciting avenues that remain open for future investigation.

Cytokine production by effector B cells is important to both humoral and cellular immune responses. However, B cell cytokine production may amplify effector T cell activity, and propagate an inappropriate immune response, such as allergy and autoimmunity (Wojciechowski et al., 2009). As I have shown that piperine can inhibit a multiplicity of effector B cell functions, it may be possible to use piperine as an anti-inflammatory agent. Interestingly, piperine inhibits both the activation and proliferation of T cells (Carolyn Doucette, personal communication). Similarly, it has recently been shown that piperine can inhibit DC proliferation during maturation (Rodgers, 2010). As piperine inhibits the activation and proliferation of multiple immune cell types, there are many disease models in which piperine may prove to be useful as a therapeutic agent.

Although there is immunological rationale to use piperine *in vivo*, there are obstacles to overcome before progressing to an *in vivo* model. LD_{50} values for piperine when administered intravenously (i.v.), intraperitoneally (i.p.), subcutaneous (s.c.), intragastrically (i.g.), and intramuscularly (i.m.) in mice are 15.1, 43, 200, 330, and 400 mg/kg bodyweight, respectively. Morbidity was attributed to multi-organ dysfunction, including hemorrhagic necrosis, gastrointestinal edema, and respiratory failure (Piyachaturawat et al., 1982; Piyachaturawat et al., 1983). With this in mind, investigation of piperine in an *in vivo* model is problematic; however, recent research has used piperine successfully in *in vivo* anti-inflammatory models. For example, daily 30 mg/kg i.p injections of piperine reduced monosodium urate crystal-induced inflammation in murine gouty arthritis model, which decreased paw swelling and suggested a reduced

capacity for leukocyte migration to areas of inflammation (Sabina et al., 2011). Oral administration of piperine has also seen recent success, as daily administration of 100 mg/kg of piperine reduced nociceptive and arthritic effects of carrageenan-induced acute paw pain and arthritis (Bang et al., 2009). Another obstacle is apparent when concerning piperine delivery, as piperine is not easily solubilized. In particular, piperine is essentially insoluble in water making *in vivo* delivery difficult (Raman and Gaikar, 2002); however, uses of polar aprotic solvents such as dimethylsulphoxide (McNamara et al., 2005) have been established. Current research into viscosity modifiers or emulsions to stabilize piperine such as carboxymethylcellulose (Sabina et al., 2011), or by using nanoparticle encapsulation, as done recently with curcumin (Shaikh et al., 2009), presents optimistic future *in vivo* use.

Since TRPV1 is a receptor for piperine and is known to be present on sensory neurons and involved in neuropathic pain (McNamara et al., 2005; Mori et al., 2002), there are additional considerations for future *in vivo* use of piperine. Namely, to avoid the associated morbidity and mortality due to the effect of piperine on TRPV1 agonism (Piyachaturawat et al., 1983), we could reconstitute TRPV1 knockout mice with wild-type bone marrow. In this manner, the effect of piperine could be examined on wild-type B cells *in vivo*, without affecting the TRPV1 knockout mouse. Another way to determine the effect of piperine *in vivo* on immune function would be to inoculate a TRPV1 knockout mouse with allogeneic lymphocytes to assess *ex vivo* B cell function following piperine treatment *in vivo*, as previously described (Dogra et al., 2004). Briefly, syngeneic whole blood from the donor mouse is re-suspended in gel, and the activated recipient allogeneic B cells are cultured on top in the presence of complement. Plaque

formation would indicate secretion of directed IgM at early time points, and IgG at late points by B cells.

As the PI3K/Akt axis and EKR1/2 signalling proteins are major effectors in cancer progression (Hwang et al., 2011), piperine may be beneficial in cancer therapies. For example, diffuse large B cell lymphoma (DLBCL) is the most common haematological malignancy in adults, with poor prognosis (Bhalla et al., 2011). DLBCL is understood to involve Akt activation, and has recently been demonstrated to involve ERK1/2 activation since agents that diminish ERK1/2 phosphorylation by inhibiting MAP2K result in increased death of malignant cells (Bhalla et al., 2011; Ranger et al., 2003). As both Akt and ERK1/2 phosphorylation are affected by piperine treatment of B cells, it is reasonable to extend this current study into B cell malignancies. Furthermore, using an *in vivo* adoptive transfer model, TRPV1 knockout mice could be inoculated with DLBCL cells and treatment with piperine, allowing for gross, histological, and immunological assays to be used to determine the effect of piperine in an *in vivo* B cell malignancy model.

Of interest, it has been noted that medicinal application of piperine in Thailand involves the administration of no more than 7.5 g of piperine. Assuming total body distribution and bioavailability, a person of 50 kg would presumably achieve a 9−13 mg/kg dose which is far below the LD_{50} of 330 mg/kg examined by mice (Piyachaturawat et al., 1983). Thus, in my opinion, reasonable dietary intake of pepper, thus piperine, should possess little to no threat to human health. However, as piperine is a potent inhibitor of drug metabolism (Atal et al., 1985) and may be contraindicated when used with certain pharmaceuticals such as carbamazepine, which require metabolism for

elimination (Pattanaik et al., 2009), caution is advised with repeated or prolonged intake of piperine.

4.3 CONCLUSION.

This study has demonstrated the inhibitory effect of piperine on B cell activation and effector functions. Results obtained by examining the proliferation of piperine-treated B cells demonstrated a G0/1-phase cell cycle arrest, which was associated with reduction in cyclin D1, D2 and D3 expression, as well as diminished Akt and ERK1/2 phosphorylation. This study demonstrated that piperine inhibits B cell effector functions, including production of the cytokines IL-6 and IL-10, and antibodies. Piperine significantly reduced expression of MHC II, and the co-stimulatory molecules CD40 and CD86 on activated B cells. In addition, piperine treatment diminished B cell-mediated antigen presentation to T cells. This *in vitro* study shows that piperine has potent immuno-suppressive effects on B cells.

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