DIFFERENTIAL ROLES OF ERK-MAPKinase IN WEHI-231 CELL APOPTOSIS AND GROWTH.

A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow

Stephen Baxter Gauld

12th October 2000

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Maggie Harnett, for all her help and guidance during experimental work and also for all her helpful comments during the writing of this thesis. I would also like to thank other workers in our research group including Sandra Seatter, Maureen Deehan, Elad Katz, Kirsty Brown and Claire Ackerman for all their advice and support during my Ph.D.

I would also like to thank other members of the Department of Immunology and Bacteriology, including my advisor of studies, Jim Brewer, for all their help.

Finally, I would also like to thank the Medical Research Council (UK) for my research studentship.

CONTENTS

FIGURES.

TABLES.

ABBREVIATIONS.

adenosine 5'triphosphate	ATP
antigen	Ag
antigen-presenting cell	APC
arachidonic acid	AA
B cell linker protein	BLNK
B-cell receptor	BCR
BCR-associated protein	BAP
brutons tyrosine kinase	Btk
carboxy-fluorescein diacetate succinimidyl ester	CFSE
conventional phospholipase D	PtdCho-PLD
cyclin dependent kinase	CDK
cyclooxygenase	COX
cytosolic phospholipase A_2	$cPLA_2$
cytotoxic T lymphocyte	T_C
diacylglycerol	DAG
extracellular signal-regulated protein kinase	ERK-MAPKinase
follicular dendritic cell	FDC
germinal centre	GC
GTP-binding protein	G-protein
guanine nucleotide exchange factor	GEF
haematopoietic stem cell	HSC
helper T lymphocyte	T_H
hen-egg lysosyme	HEL
immature-transitional B cell	T1
immunoreceptor tyrosine-based activation motif	ITAM
immunoreceptor tyrosine-based inhibitory motif	ITIM
immunoglobulin D	IgD
immunoglobulin M	IgM
inositol 1,4,5-trisphosphate	IP_3
lipopolysaccharide	LPS
Ly	Ly294002
MAPKinase kinase	MEK
MAPKinase kinase kinase	MEKK
membrane immunoglobulin M	mIgM
mitochondrial transmembrane potential	$\Delta\psi_m$
periarteriolar lymphoid sheath	PALS

phosphatidylinositol 4,5-bisphosphate	$PtdIns(4,5)P_2$
phosphatidylinositol 3,4,5-trisphosphate	$PtdIns(3,4,5)P_3$
phosphoinositide 3-kinase	PI(3)K
phospholipase C	PLC
protein kinase C	PKC
protein tyrosine kinase	PTK
recombination-activating gene	RAG
retinoblastoma protein	Rb
Src Homology 2 Domain containing 5' Inositol Phosphatase	SHIP
SH2 phosphatase 1	SHP-1
Son of sevenless	Sos
Src-homology domain	SH2
sphingosine-1-phosphate	SPP
stress-activated protein kinase	JNK
TNF receptor-associated protein	TRAF
tumour necrosis factor	TNF
Wm	wortmannin
phorbol-12-myristate acetate	PMA
propidium iodide	PI

SUMMARY.

BCR-ligation in B lymphocytes results in differential biological responses according to the maturational stage of the cell. Thus, while mature B lymphocytes become activated and proliferate in response to antigen receptor crosslinking, immature B lymphocytes become unresponsive or undergo apoptosis. Although these biological responses to antigen are well reported, the signalling pathways activated in each scenario are still poorly defined. In particular, the signalling cascades that result in the apoptosis of immature B lymphocytes which recognise self-antigen are still unclear.

Recent studies within our group have highlighted the phospholipase, $cPLA_2$, as a possible transducer of apoptotic signals following BCR-ligation (53) in WEHI-231 cells. Additionally, CD40 co-stimulation has been shown to rescue immature B lymphocyte from BCR-mediated apoptosis. We were therefore interested in investigating the signalling pathways linking the BCR to $cPLA_2$ activation and the mechanisms utilised by CD40 co-stimulation in the reversal of BCR-mediated apoptosis.

Initial studies examined the activation of the MAPKinase family of enzymes as these protein kinases, along with Ca^{2+}, have been implicated in the activation of $cPLA_2$. Our studies indicated that BCR-ligation induced the activation of a short, transient, ERK-MAPKinase signal, but not p38 MAPKinase or JNK. Additional studies have shown that inhibition of this ERK-MAPKinase signal by pharmacological means reduces BCR-induced apoptosis and $cPLA_2$ activation. Interestingly, CD40 co-stimulation was shown to uncouple the BCR from ERK-MAPKinase activation, suggesting the prevention of $cPLA_2$ activation may account for the anti-apoptotic properties of CD40 co-stimulation.

BCR-induced apoptosis of WEHI-231 cells is known to occur approximately 24-48 hours post receptor ligation. We therefore examined the activity of ERK-MAPKinase over these time periods. Interestingly, untreated WEHI-231 cells were shown to exhibit a basal cycling, ERK-MAPKinase activity, suggesting this protein was involved in the normal proliferation of these cells. BCR-ligation was shown to abolish this signal after approximately 2-4h suggesting that BCR-induced growth arrest involves the down-regulation of this cyclic ERK-MAPKinase activity. Additionally, CD40 co-stimulation was shown to restore this proliferative ERK-MAPKinase signal. Inhibitor studies corroborated this proposal that these late, cycling ERK-MAPKinase signals contribute to basal and CD40-rescued growth and proliferation of WEHI-231 cells. In summary, these results suggest differential roles for ERK-MAPKinase in regulating WEHI-231 cell fate. Firstly, an early, BCR-stimulated apoptotic signal, and secondly, a long-term proliferative signal. They also suggest that the induction of

growth arrest and apoptosis in WEHI-231 cells result from the activation of distinct signalling pathways.

We therefore investigated possible mechanisms involved in both the suppression, and re-establishment, of proliferative ERK-MAPKinase activity by BCR-ligation and CD40 co-stimulation respectively. Firstly, we examined the role of PI(3)K which is known to be involved in the induction of survival signals and ERK-MAPKinase activation in other cell systems. Our results suggested that PI(3)K mediated PtdIns(3,4,5)P_3 generation does not play a role in CD40-mediated rescue from BCR-induced growth arrest. These results were corroborated by the inability to detect activity of the anti-apoptotic protein, Akt, following CD40 co-stimulation. Inhibitor studies did however indicate that PI(3)K activity plays a role in the basal proliferation of WEHI-231 cells. However, PtdIns(3,4,5)P_3 generation, unlike basal ERK-MAPKinase, was not found to be cyclic in nature. These findings do not rule out a role for the involvement of other phosphatidylinositol species generated by PI(3)K activity, including PtdIns(3,4)P_2 which is known to activate ERK-MAPKinase. Additionally, our results also indicated that BCR-induced PtdIns(3,4,5)P_3 generation, whilst not essential for BCR-induced apoptosis may play role in coupling the BCR to apoptotic, ERK-MAPKinase dependent signals.

Our results also described the coupling of the BCR in both immature and mature B lymphocytes to sphingosine kinase, a lipid kinase whose activity has not previously been shown in B lymphocytes, but is known to activate survival signals in other cell types. Although the BCRs of both immature and mature B lymphocytes were shown to be coupled to this kinase, the kinetics of activation were shown to differ slightly. BCR-ligation of both mature and immature B cells induced a short, transient, activation of sphingosine kinase, perhaps suggesting that sphingosine kinase activation may induce a activation/proliferative signal, which requires additional co-stimulation for its sustained activation and proliferative effects. Accordingly, CD40 co-stimulation of WEHI-231 cells was shown to enhance and prolong sphingosine kinase activation.

Our results also indicated that BCR-ligation down-regulates ERK-MAPKinase, but not Ras activity. We have therefore investigated the role of Rap1 and RKIP, molecules known to competitively compete with Ras for Raf activity. Initial studies have suggested that Rap1 is not involved in this process, whilst results examining the role of RKIP are still inconclusive. Future aims are therefore to further characterise the mechanisms employed by BCR-ligation in WEHI-231 cells in the downregulation of the prolifeartive ERK-MAPKinase signals and how these are restored by CD40 co-stimulation.

CHAPTER 1 - B Lymphocytes.

1.1 The immune system.

The immune system has evolved to protect the host from pathogens such as parasites, viruses or bacteria. The cells that mediate this immune response include phagocytes, antigen-presenting cells including dendritic cells and lymphocytes which work together in a specific, yet complex, manner. Phagocytic cells can be divided into two families, monocytes/macrophages and granulocytes, the latter including neutrophils, basophils/mast cells and eosinophils. These cells can internalise microorganisms before exposing them to an array of killing mechanisms. These may include the generation of reactive oxygen intermediates such as superoxide anions ($\bullet O_2^-$), nitric oxide formation, proteolytic enzymes such as lysozyme and acid hydrolyases, cationic proteins or intracellular pH changes. These cells form the basis of the innate immune response which is the first line of defence against infection but is non-specific in nature. Lymphocytes also play an important role in the immune response, but unlike phagocytic cells, lymphocytes specifically recognise individual antigens and form part of the adaptive immune response. Lymphocytes can be divided into two categories - T lymphocytes and B lymphocytes. B lymphocytes fight extracellular pathogens by the releasing antibody, a molecule that recognises and binds to a specific target molecule termed the antigen. T lymphocytes are involved in a wider range of activities. These include the control of B lymphocyte development and proliferation, interacting with phagocytes to aid in the destruction of their internalised pathogens and the direct destruction of host cells that have become infected by viruses or other pathogens. The latter function is mediated by cytotoxic (Tc) $CD8^+$ T lymphocytes whilst the other described functions are attributable to helper (TH) $CD4^+$ T lymphocytes. One interesting feature of the adaptive immune response is that, unlike the innate immune response, it improves with each successive encounter with the same pathogen. The adaptive immune response is therefore important for the generation of long-life immunity to a particular infection.

During the induction of an immune response there is considerable interaction between both the innate and adaptive immune responses. For example, some phagocytes, such as tissue macrophages, can internalise antigen and present them to T lymphocytes in a form they can recognise in a process called antigen presentation. As a consequence the activated T lymphocyte can release an array of cytokines to aid in phagocyte activation and the consequent destruction of its internalised pathogen. In addition some phagocytes use antibodies released by B lymphocytes to allow them to recognise pathogens more effectively. For example, if an antibody binds to a pathogen, it can link to a phagocyte via the Fc portion of the antibody. This allows the pathogen

to be internalised and destroyed by the phagocyte, with the antibody acting as an opsonin.

Both T and B cells have become major targets of biomedical research. This has generated a wealth of literature in both the development, and effector functions of these cell types. However, it is clear that much work still has to be done in these fields. One particular area that is attracting much interest is the molecular and biochemical mechanisms underlying B cell development/differentiation and particularly maturation-state dependent cellular responses. This is of particular relevance in the latter stages of B lymphocyte development because the signals transduced following antigen encounter are mediated by the same receptor, but result in differential biological responses. The study of these differential responses will further our knowledge not only of B lymphocyte development, but also the role of B lymphocyte dysfunction in a number of disease states including autoimmune disease.

1.2 B cell development.

The B cell has a major role in the humoral immune response to infection by producing antigen specific antibodies. Unlike birds, in which primary B-cell lymphopoiesis occurs in the lymphoepithelial organ, the bursa of Fabricius, mammals do not have a specific organ for B-cell lymphopoiesis. Consequently, B cells develop from pluripotent stem cells in the fetal liver from 8-9 weeks of gestation in humans and by about 14 days in the mouse (1). In later embryonic stages, the site of B-cell production moves to the bone marrow where it continues into adult life. In terms of kinetics, studies have shown that approximately 2 x 10^7 murine B cells are produced each day. However, since the murine spleen only contains 7.5 x 10^7 cells, it is clear that a large percentage of these developing cells must die. Like thymocytes developing in the thymus, the majority (75%) of B cells maturing in the bone marrow undergo a process of negative selection. This allows for the development of B lymphocytes that can interact with foreign antigen, whilst at the same time avoiding the generation of B cells that bind self-antigen. After leaving the bone marrow, B cells enter the periphery, passing through the blood into the secondary lymphoid organs - spleen, lymphocyte nodes and mucosal-associated lymphoid tissues. Within these secondary lymphoid organs, B lymphocytes localise into discrete clusters or follicles in association with follicular dendritic cells and specialised stromal cells. This microenvironment provides essential survival signals required for the survival of these peripheral B lymphocytes.

During their normal development, B lymphocytes progress through orderly stages of differentiation from initial lineage commitment in the bone marrow to the formation of fully mature immunocompetent B cells in the periphery (**figure 1**). The

starting point of B lymphocyte development is the haematopoietic stem cell (HSC) found in the fetal liver and after birth, and into adult life, in the bone marrow. In addition to generating both T and B lymphocytes these cells can differentiate into erythroid or myeloid cells (2). Commitment of HSCs to the B lymphocyte lineage has been shown to be dependent on the expression of the paired box transcription factor Pax5 (2). Indeed, studies have shown that when a mixed population of wild-type and Pax5-/- HSCs are starved of IL-7, the wild-type cells differentiate into B lymphocytes, whilst the Pax5-/- cells survive and obtain a myeloid cell phenotype. Thus, present models suggest that Pax5, whilst promoting the expression of B lymphocyte specific genes also acts to suppress promiscuous transcription of genes from other haematopoietic lineages.

Following commitment to the B lymphocyte lineage, each precursor cell passes through a number of developmental "compartments" in which they lose or gain specific surface molecules depending on their maturational stage which, phenotypically, can be used to define discrete stages of development (3) (**figure 2**). The stages of B lymphocyte differentiation can also be divided into two quite distinct stages; antigen-independent and antigen-dependent phases (4) (**figure 1**).

Antigen-independent differentiation occurs in the bone marrow and ultimately leads to the production of immature B cells with surface membrane immunoglobulin, that is both functional and specific, coupled to di-sulphide linked Igα (CD79a)-Igβ (CD79b) heterodimers which act as signalling elements. The processes involved in the expression of this "mature" receptor complex, or B-cell receptor (BCR), are initiated early in B lineage commitment (**figure 3**) and have been extensively studied in murine models.

The differentiation of HSCs to immature B lymphocytes begins with the production of an early pro-B cell and is accompanied by the ordered expression of surrogate, and finally, mature BCRs (**figure 4**) by following a defined pattern of immunoglobulin heavy and light chain rearrangements under the control of the recombination-activating genes, RAG1 and RAG2, which are highly expressed at the pro- and pre- B cell stages of differentiation (5). Thus, RAG activity ultimately leads to the production and cell surface expression of functional immunoglobulin.

The immunoglobulin heavy chain variable region is encoded by V and J gene segments with additional diversity provided by the D gene segment. Rearrangement of the immunoglobulin heavy gene begins at the early pro-B cell stage with the rearrangement of D gene segments to J gene segments (D_HJ_H) producing late pro-B cells. Although no functional immunoglobulin protein is expressed in late pro-B cells, these cells do express components of the mature BCR on their cell surface. Recent studies (6) have shown the expression of Igα-Igβ heterodimers on the surface of late

pro-B cells in association with calnexin. Further rearrangement to the immunoglobulin gene occurs at the late pro-B cell stage with the joining of the V_H gene to the already rearranged D_HJ_H gene segments. If this rearrangement produces a productive heavy chain rearrangement, these cells are thought to divide several times before reaching the pre-B cell stage. The use of Igβ-/- mice has shown that expression of this molecule on the cell surface of pro-B cells is essential for the continuing development of these cells. Studies have shown that mice deficient in Igβ exhibit a complete block in B cell development before V_H to D_HJ_H rearrangement. It has therefore been suggested that signalling through the Igα/Igβ-calnexin receptor on pro-B cells may be required for successful initiation of V_H to D_HJ_H gene rearrangement (7). Further evidence to support this comes from recent observations investigating the potential signalling qualities of Igα-Igβ molecules expressed on pro-B cells from RAG-2-/- mice. Ligation of Igβ on these cells was shown to mimic pre-BCR like signals which induce early B cell differentiation (6). These studies showed that developmentally arrested pro B cells from RAG-2-deficient mice could be induced to differentiate to the preB cell stage when treated with anti-Igβ antibody. These results suggest that crosslinking of Igβ on pro-B cells activates differentiation signals similar to those initiated by the preB cell receptor during normal development. As RAG-2-/- pro-B cells express all the necessary machinery for signal transduction, except the expression of μ heavy chain, and observations that Igβ-deficient mice have a developmental block at the pro-B cell stage, these results suggest an essential role for Igα-Igβ molecules as signal transducers of the pre-BCR and that signals delivered by Igα-Igβ molecules in pro-B cells can drive B cell differentiation to the pre-B cell stage (6).

As previously mentioned, progression to the pre-B cell stage results in the surface expression of μ chain. This process is known to be dependent on the cell surface association of the μ heavy chain with the light chain surrogate proteins VpreB and λ5 (8) along with Igα-Igβ molecules. The expression of these molecules, termed the "pre-B cell receptor" has shed new light on the developmental progression of pre-B cells. Genetic studies, creating mice deficient in either the membrane form of μ heavy chain or the λ5 protein, showed that B lymphocyte development was blocked at the pre-B cell stage (9). Surface expression of this pre-BCR has been shown to be important for allelic exclusion at the heavy chain locus (10). This is because this receptor instructs the cell to stop further V_H gene rearrangements (11). Furthermore, studies using a membrane μ-chain mutant have shown that expression of this mutant prevents the association of Igα/Igβ with this molecule, whilst also preventing allelic exclusion and developmental progression to the pre-B cell stage (7).

Maturation of pre-B cells to immature B cells involves further gene rearrangements to the immunoglobulin light chain to generate conventional light chains (κ or λ), with appropriate constant and variable regions, which associate with the already expressed heavy chain (10). This leads to the expression of a BCR consisting of μ heavy chain, conventional light chains and also the associated, intracellular Ig-α/Ig-β molecules, and is the first BCR to exhibit antigen specificity and also expresses a more diverse repertoire of specificity than the pre-BCR (10, 12).

Following the stable expression of IgM on the B cell surface, immature B cells enter into the antigen-dependent phase of development where they can respond to specific antigens due to the expression of mature mIgM on their cell surface. Therefore, up until this point, no selective processes have been employed to prevent the maturation of B cells with BCRs capable of recognising self-antigen. Immature B cells consequently go through a number of complex checkpoints mediated by the antigen specificity of their BCR and involves both positive and negative signals regulating both survival, death and also the reactivation of the immunoglobulin V(D)J gene recombinase system. The reactivation of the RAG gene system is believed to allow further light-chain rearrangements in immature B cells that recognise self-antigen whilst in the bone marrow, thus allowing them to alter their antigen specificity (13). This forms part of clonal selection allowing for the removal of immature B cells that possess self antigen receptors and would therefore mount an auto-immune response if they were allowed to escape to the periphery, however, clonal selection and the phenomenon of allelic exclusion specifically result in the generation of antigen monospecificity, that is, one B lymphocyte is specific for one antigen.

The process of negative selection of B lymphocytes is still an area under intense investigation but is believed to occur by three distinct, yet inter-linked, molecular mechanisms, deletion, anergy and receptor-editing (14). Recent reports have indicated that immature B cells isolated from the bone-marrow undergo programmed cell death, or apoptosis, within 16 hours when cultured *in vitro* with anti-BCR antibodies (14). However, although BCR-ligation of immature B cells *in vitro* undoubtedly initiates an apoptotic mechanism, the *in vivo* response to self-antigen presented in the bone marrow may be very different. Initial studies trying to address these areas suggested that highly purified immature B cells initiate an apoptotic programme upon BCR-ligation. In contrast, unfractionated bone-marrow B cell cultures induced receptor editing instead of apoptosis (14). This suggested that the environment of the cell, in this case the bone-marrow, provides signals blocking apoptosis and inducing RAG activation. The process of receptor editing during negative selection allows potentially self-reactive immature B cells to rearrange their light chains and subsequent antigen specificity (13). However, these cells are not always rescued from apoptosis and many

undergo unsuccessful receptor editing and fail to produce a functional BCR and subsequently die by apoptotic mechanisms (10).

Recent studies by Sandel *et. al.*, 1999 (14) have confirmed a role for the cells microenvironment in determining the response to antigen encounter. By labelling transitional B cells (immature B cells that have just entered the periphery from the bone marrow) using carboxy-fluorescein diacetate succinimidyl ester (CFSE) in conjunction with propidium iodide staining, the authors were able to monitor the fate of these transitional cells when mixed with bone marrow or disassociated spleen following receptor ligation. Interestingly, labelled transitional cells co-cultured with bone marrow did not undergo apoptosis, but rather induced RAG expression following BCR-ligation which would allow for further light chain rearrangement and hence alter the specificity of the cells BCR. In contrast, labelled cells co-cultured with dissociated spleen undergo apoptosis. This suggests that the bone marrow microenvironment provides signals that block BCR-induced apoptosis and promote receptor editing due to RAG induction. However, in the periphery, transitional B cells do not receive these signals and undergo apoptosis as a default programme. The authors suggest that the protective effect mediated by co-culture with the bone marrow environment may be due to the presence, and cell-cell contact, with as yet unidentified Thy-1.2 expressing bone marrow stromal cells. Subsequently, the authors suggest that these cells may be capable of altering BCR signals in favour of receptor editing rather than apoptosis, or to allow self-reactive immature B lymphocytes to survive long enough in the bone marrow to produce a non-autoreactive BCR. Additional work by the same authors (8) has suggested that immature B lymphocytes undergo a "window of opportunity for re-direction" following BCR-ligation. In summary, the author suggests that following the induction of an apoptotic programme after just 10-20 minutes of BCR-ligation, the cells undergo a 10-12 hour window in which their fate can be re-directed. For example, in the bone marrow this may result in RAG expression and hence receptor editing. If no additional signals are initiated within this 10-12 hour window then these cells irreversibly undergo apoptosis.

Additionally, treatment of immature B cells with anti-IgM results in the loss of surface IgM by capping and endocytosis and may also play a role by which self-reactive B cells are rendered tolerant, or anergic, during development. This mechanism allows self-reactive cells to initiate receptor-editing. Here, cells that have not been deleted are rendered anergic due to BCR internalisation and are believed to re-enter the pre-B cell compartment where they undergo a second round of light chain rearrangement (8). Therefore, this process is thought to break allelic exclusion and allow the cell to undergo immunoglobulin gene rearrangement and subsequently express a completely new immunoglobulin receptor.

As an environment, the bone marrow does not allow immature B cells to encounter all self-antigens. Therefore, B cells maintain an immature B cell phenotype for a number of days after leaving the bone marrow for the periphery. These "transitional-immature" B cells therefore remain sensitive to deletion if they encounter antigen (8). Surviving cells then enter the periphery as transitional (T1) cells expressing low levels of IgD ($IgM^{high}IgD^{low}$). It has been suggested that the temporary maintenance of high-level mIgM expression retains the transitional cells sensitivity to antigen and thus deletion by apoptosis if it recognises self antigen present only in the periphery (10). Therefore, because the default programme for these cells following antigen exposure is apoptosis, these cells require T cell-dependent survival signals if they are to survive

Following their exit from the bone marrow into the periphery, B lymphocytes that have successfully traversed the immature-transitional (T1) stage of development migrate to the spleen, arriving at the marginal zone sinusoids before moving into the outer zones of the periarteriolar lymphoid sheath (PALS). Few of these T1 B cells will develop into fully mature B cells. Typically, of the 2×10^7 IgM+ B cells that develop daily in murine bone marrow only 10% will reach the spleen and only 1-3% will survive and enter the mature B cell pool (15). The precise mechanisms leading to the selection of T1 cells destined for the mature B cell pool are still unclear. However, recent studies have suggested that cells at this stage of development require constant BCR-derived signals to progress to the next stage of development (11, 16). These BCR-derived signals almost certainly provide a positive signal and a number of studies provide evidence suggesting that lack of these signals, or excessively strong BCR-derived signals, leads to apoptosis within 3-4 days (17, 18). In addition, stimulation of these T1 B cells by cytokine or co-receptor ligation is thought to help shape the level of BCR stimulation which will in turn decide the fate of the cell (17). B lymphocytes which survive this passage develop into transitional (T2) cells with a characteristic $IgM^{high}IgD^{high}$ phenotype (15, 16). These cells then migrate to the primary follicles of the spleen where they become mature $IgM^{low}IgD^{high}$ B lymphocytes

In contrast to what occurs with immature B lymphocytes, BCR-ligation leads to the activation of mature B lymphocytes and if the appropriate accessory signals are present, an immune response. Subsequently, BCR-ligation and hence cellular activation by antigen, initiates one of two responses in mature B lymphocytes. Firstly, following antigen stimulation these cells may become IgM or IgG antibody-secreting plasma cells, devoted to antibody production in the primary immune response. Secondly, mature B cells primed with antigen may undergo isotype switching, V region somatic mutation and affinity maturation to become memory B cells which can in turn become IgG secreting plasma cells (4). Memory B cell formation is crucial for the

induction of the secondary immune response, and the ability to generate these cells forms the basis of vaccination.

The formation of memory B cells occurs in germinal centres (GCs) which are formed during primary immune responses to T cell-dependent antigens in lymphoid follicles (**figure 5**). The initial stages of germinal centre formation involve the exponential growth of B cell blasts activated by T-cell-dependent antigens in areas rich in follicular dendritic cells (FDCs). As well as acting as antigen-presenting cells (APCs) to $CD4^+$ T cells (17), FDCs also maintain the expression of unprocessed antigen on their cell surface, which is later recognised by newly-formed memory B lymphocytes (4). Following their expansion the B cell blast population migrates from the centre of the follicle to form the dark zone of the GC. Here, the B-cell blasts continue to proliferate, lose membrane-Ig expression and are now termed centroblasts. The dividing centroblasts are then thought to activate a hypermutation programme and give rise to daughter centrocytes which are non-dividing. These centrocytes then migrate to the light zone of the GC which is rich in FDCs. The level of B cell apoptosis in the light zone of the GC is very high because the centrocytes present in the light zone are intrinsically hard-wired to undergo apoptosis unless they are rescued. Rescue of centrocytes from apoptosis in the light zone has been shown to occur by two mechanisms. Firstly, centrocytes which express high-affinity receptors for Ag expressed on FDCs are positively selected. Secondly, CD40 ligation on the surface of centrocytes has also been shown to rescue these cells from apoptosis, suggesting a role for helper T-cell interactions (4, 17). Positively selected centrocytes then go on to establish the memory B-cell pool, providing the precursors for plasma cells with improved antibody affinity (19).

1.3 Positive versus negative signalling via the BCR.

Ligation of the BCR with antigen plays a crucial role in determining the fate of B lymphocytes according to their maturational state. Hence, immature B lymphocytes have been shown to be clonally deleted following BCR-ligation, whilst mature B lymphocytes become activated by the same receptor at the intracellular level. Although many of the signalling events regulating cell cycle progression and commitment to apoptosis or cell survival and proliferation appear to be similar to those described in other systems, the precise combination of early BCR-coupled signalling events integrated to dictate a particular response are yet poorly defined. Therefore in recent years research into dissecting the signalling events involved in both positive and negative BCR signalling has been intense. These studies have aimed to address key differences in transcriptional events following BCR-ligation, but have also addressed areas such as the quality and quantity of BCR-ligation as possible modulators of cell

fate. This section aims to summarise the main signalling events activated by BCR-ligation and also discuss current concepts behind differential signalling by the BCR in both negative and positive responses. Firstly however, I shall describe the current models of regulation of cell progression and apoptosis.

1.4 Cell cycle.

Under normal physiological conditions, cell growth is regulated by a number of cell cycle checkpoints to prevent abnormal cell cycle entry. These checkpoints ensure that aberrant cell cycle progression under conditions of cell damage results in the induction of apoptotic programmes aimed at deleting these cells. Cell growth/proliferation is therefore a balance of appropriate signals between growth promoting, and growth inhibitory pathways.

Cell cycle progression is tightly regulated at two major checkpoints. Firstly, at the G_1/S boundary, where cells commit to DNA synthesis, and secondly, at the G_2/M boundary where cells commit to mitotic division (20) (**figure 6**). Cell cycle entry is promoted by the actions of the G_1 cyclin dependent kinases (CDKs) which associate with cyclins, proteins expressed at specific stages of the cell cycle. These kinases include Cdk4 and Cdk6 which associate with the cyclins D1, D2, D3, and also Cdk2 which associates with cyclin E (21). The Cdk-cyclin complexes promote G1/S phase progression by reversing the G_1 phase block enforced by the retinoblastoma protein, pRb^{105}. The G_1 block enforced by hypophosphorylated Rb results from its ability to suppress the activity of the transcription factor, E2F, by sequestering this protein away from genes required for S-phase entry (21). However, following the phosphorylation of Rb by cyclin-CDK complexes, E2F is released from Rb and can interact with the necessary genes required for S-phase entry (20). Disruption of the Rb protein, either through gene mutation/deletion or hyperphosphorylation, is also present in a range of human cancers (22).

The ability of cyclin-CDK complexes to promote S-phase entry is tightly regulated within the cell by two structurally distinct families of CDK inhibitors (20). Firstly, the WAF1 family (p21, p27 and p57) of proteins and secondly the INK4 (inhibitor of CDK4) family (p15, p16, p18 and p19). The activity of the WAF1 family member $p27^{Kip1}$ has been shown to play an important role in regulating cell growth in fibroblasts. In serum starved fibroblasts levels of $p27^{Kip1}$ are high, yet decrease substantially upon growth-factor stimulation (21). In addition, one member of the INK4 family, INK4a, has been shown to have loss of function in a wide range of tumour types, and pathogenically, this is thought to be exceeded in frequency only by p53 inactivation (22). These two families act to prevent cell cycle progression in response to cellular stress or damage and promote growth-arrest and or apoptosis.

Subsequently, these molecules form a link between growth promoting pathways and the induction of apoptosis again confirming significant cross-talk between the signalling pathways regulating these two opposing responses.

The ability of the p53 protein to induce G_1 arrest in cells with DNA damage, thus preventing the replication of faulty cells, is well documented. It is believed that this allows cells time to repair damaged DNA before further replication. However, deletion or genetic mutation to p53 plays a role in the development of most human cancers. When activated, p53 has been shown to increase both the expression and activity of $p21^{Waf1}$ (20). In addition to inducing G_1 growth arrest, p53 activity is also known to induce apoptosis in a range of cell types including T and B lymphocytes (23). It has been suggested that the downstream activation of $p21^{Waf1}$ by p53 has a anti-apoptotic function because a decrease or loss of p21 gene expression induces growth arrest in cells that would normally undergo apoptosis (24). This suggests that p53, whilst directly linked to cell cycle arrest may also play additional roles in the induction of apoptosis.

1.5 Apoptosis - programmed cell death.

The process of programmed cell death, or apoptosis, plays an essential role in both developing or adult creatures. Unlike necrosis, which occurs after acute cellular injury and results in the cell releasing its cellular content into the surrounding area which may cause a damaging inflammatory response, cells initiating apoptotic processes die in a orderly, tidy manner, without affecting neighbouring cells. Morphologically, apoptotic cells can be identified due to the processes of membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation (25). These changes are common to all apoptotic cells, independent of the trigger signal, suggesting that a common signalling pathway is induced leading to cell death.

The caspases, a family of evolutionary conserved cysteine proteases, have been shown to play crucial roles in the induction of apoptotic processes (26). Normally caspases exist as inactive precursors, however, upon the initiation of apoptotic signals, these proteases are cleaved forming active enzymes (27). Subsequently, caspase activation has been shown to involve the activation of a proteolytic cascade (26). Recently, mitochondrial function has been shown to play a key role in the induction of this cascade (**figure 7**). Induction of apoptosis has been shown to induce 'permeability transitions' in the mitochondrial membrane suggesting that mitochondrial constituents may leak into the cytoplasm during apoptosis (28). Cytochrome C release from the mitochondria has been shown to play crucial role in the induction of caspase activity (29). A number of proteins have been shown to regulate cytochrome C release from the mitochondria and hence regulate apoptosis. In particular, certain members of

the Bcl-2 gene family are targeted to the mitochondria under apoptotic conditions and have been shown to play key roles in the release of cytochrome C. (30). In particular the Bid protein, a pro-apoptotic member of the Bcl-2 family, has been implicated in the regulation of cytochrome C release. Caspase-8 activity has been shown to cleave Bid releasing a C-terminal fragment (tBID) which is targeted to the mitochondria, inducing cytochrome C release (30). It has been suggested that the release of cytochrome C may be due to the heterodimerisation of tBid with other Bcl-2 family members such as Bcl-X_L which may result in the inhibition of this anti-apoptotic protein (30). Other pro-apoptotic members of the Bcl-2 family are also believed to function by homo- or hetero-dimerisation which is believed to neutralise anti-apoptotic members of the Bcl-2 family which normally act to prevent cytochrome C release from the mitochondria (31). However, the mechanisms underlying these processes are still unclear. One member of the Bcl-2 family which has attracted much attention in recent years is the pro-apoptotic protein, Bad. Bad has been shown to exert its apoptotic effect by forming heterodimers with Bcl-2 or Bcl-X_L. Phosphorylation of this pro-apoptotic protein by survival signals has been shown to result in the block of Bad-mediated apoptosis. This has been shown to be due to the phosphorylation of Bad at Ser^{112} and Ser^{136} by the anti-apoptotic protein kinase, Akt (32). Serine phosphorylation is believed to promote the association of Bad with the phospho-serine binding protein, 14-3-3. This results in the release of Bcl-X_L from Bad which promotes cell survival by blocking the caspase protease cascade (33).

1.6 BCR structure.

The B cell antigen-receptor (BCR) (reviewed in (34)) is a multiprotein structure containing a clonatypic antigen binding component (sIg) produced from the rearrangement of immunoglobulin heavy and light chain genes, noncovalently associated with disulfide linked Igα (CD79α) and Igβ (CD79β) heterodimers (34) and plays a vital role in mediating the response of B lymphocytes to foreign antigen. There are five classes of immunoglobulin, IgM, IgD, IgG, IgA and IgE, and although all of these can be expressed as B cell antigen receptors, only IgM and IgD are expressed on mature naive B cells. Structurally, mIg consists of homodimeric heavy chains, each covalently associated with a light chain, forming a symmetrical four chain structure with two antigen binding sites. Studies have shown mIg to consist of extracellular, transmembrane and cytoplasmic regions (**figure 8**). The precise length of the cytoplasmic regions have been subject to much debate with two algorithms being suggested. Firstly, work by Kyte and Doolittle suggested that the cytoplasmic tails of mIgM and mIgD immunoglobulin classes were composed of only three amino acids. The other immunoglobulin classes, mIgG, mIgA and mIgE were believed to consist of cytoplasmic tails of up to 28 amino acids (34). However, conflicting studies by Klein,

Kane and Delisi predicted that the cytoplasmic tails of mIgM and mIgD were much longer, consisting of eleven amino acids (34). However, even these longer cytoplasmic domains are clearly still too small to possess any intrinsic catalytic activity, suggesting that BCR transmembrane signalling is probably mediated by the interaction of mIg with accessory signal transducing molecules.

The discovery of Igα and Igβ in the late 1980s provided a link between the BCR and the initiation of intracellular signalling mechanisms. Encoded by the mb1 and B29 genes respectively (35), Igα (34kDa) and Igβ (38kDa) exist as disulphide linked heterodimers with extracellular and intracellular domains (4). Like mIgM and mIgD, Igα and Igβ do not contain any intrinsic catalytic activity. However, the presence of the Igα and Igβ heterodimers is essential for BCR-mediated signalling events. The discovery of an amino acid sequence involved in signalling in T-cell and Fc receptors (36) termed the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tails of Igα and Igβ provided a mechanism for the initiation of signalling events following BCR-ligation. Interestingly, ITAM sequences are also found in the accessory chains. The ITAM motif consists of two tyrosine domains separated by 13 amino acids forming the sequence ...**Y**XX[L/V]$X_{7\text{-}11}$**Y**XX[L/V]... where Y is tyrosine, L is leucine, V is valine and X represents any amino acid. Subsequently, aggregation of mIg molecules due to the binding of multivalent antigens promotes the phosphorylation of the ITAMs (four per BCR) by Src-family protein tyrosine kinases (PTKs). Generally, phosphorylation of the ITAMs occurs mainly at the membrane proximal tyrosine residue. However, phosphorylation of both tyrosine residues creates a binding site for the tandem Src-homology (SH)2 domains of a related PTK, Syk (36).

In addition to Igα and Igβ, other molecules have been shown to associate with the BCR. These include a group of ubiquitously expressed cytoplasmic proteins called BCR-associated proteins or BAPs (34). BAP32, 37 and 41 (numbers referring to the molecular weight of these proteins (kDa)) have been shown to associate with mIgM, whilst BAP29 and 31 appear to preferentially associate with IgD. The role of these proteins in BCR signalling are still poorly defined. Reports have suggested a transmembrane role for the BAPs in which they associate with the BCR via transmembrane domains (35) whilst other studies suggest these proteins are purely cytoplasmic and are not involved in transmembrane signalling events (34). Additionally, kinetic experiments have shown that PTK activation following mIgD ligation lasts longer than that activated via mIgM, and it has been suggested that this may be due to the differential expression of BAP molecules with these different immunoglobulin isotypes (35) and the differential recruitment of scaffold proteins.

1.7 BCR signalling.

The genes responsible for important biological responses in B lymphocytes such as growth and apoptosis are only now beginning to be understood. Identification of these genes has helped in the dissection of signalling pathways activated following BCR-ligation. The recruitment and activation of PTKs is the first signalling event mediated by BCR-ligation in both positive and negative responses (37). Presently, three distinct types of PTK are known to become activated following BCR engagement (7). These include the Src-PTKs (Lyn, Blk, and Fyn), Syk and Btk. The current model for induction of BCR-mediated signalling events involves the phosphorylation of key tyrosine residues on the ITAMs of the Igα and Igβ cytoplasmic tails by Blk, Fyn and Lyn PTKs following BCR-ligation. The phosphorylated ITAM creates a binding site for Syk via its tandem, tyrosine binding, SH2 domains. Syk then becomes tyrosine phosphorylated and activated along with Btk (probably due to direct phosphorylation by the Src-PTKs) (38). This simple model, however, does not explain recent findings suggesting that both Lyn-/- and Syk-/- B cells can still signal through their BCR (17). This suggests that the BCR can activate both Lyn- and Syk-independent pathways. Therefore differential activation of PTKs could play key roles in directing BCR-mediated signalling cascades down distinct paths and ultimately, differential physiological outcomes.

Early studies investigating downstream signalling elements from the PTKs have provided evidence to suggest that the signals transduced via the BCR in both immature and mature B lymphocytes are very similar. Current models of B lymphocyte signalling suggest that the Ras-MAPKinase, PI(3)K and PLC pathways are common to both BCR-mediated signalling events in immature and mature B lymphocytes (**figure 9**). These signalling cascades therefore provide the core pathway for both the deletion, or activation of B lymphocytes. It is now clear that secondary pathways modulate these core signals to enhance or attenuate BCR-mediated signalling events. This can occur through maturation-state dependent changes in protein expression, including catalytic enzymes, substrates, adaptor/linker proteins or co-receptor expression. Subsequently, enhancement of enzyme expression would serve to amplify the signal cascade, whilst expression of a particular adapter/linker protein could re-direct the signalling cascade in a new direction.

The core signalling elements of BCR stimulation include the activation of the Ras-MAPKinase cascade. Activation of this pathway is believed to play important roles in the activation of transcriptional events (38). Activation of the Ras-MAPKinase pathway has been implicated in both positive and negative BCR stimulated events. ERK-MAPKinase has been shown to play a key role in the ability of the BCR to stimulate the proliferation of resting splenic B cells (39). In contrast to this, ERK-

MAPKinase activation has been shown to correlate with BCR-induced apoptosis in the immature B lymphocyte cell line, WEHI-231, (40).

A wide range of adaptor or linker proteins are utilised by BCR-ligation to recruit specific signalling molecules in both immature and mature B lymphocytes. These adaptor proteins possess no intrinsic enzymatic activity, but function to direct protein-protein interactions between enzymatically active intermediates. Grb2, which consists of two SH3 and a central SH2 domain, is the prototype adaptor molecule utilised in the activation of Ras in many signalling cascades. In growth factor mediated responses, the association of Grb2 with the phosphorylated receptor via its SH2 domain and its binding of the guanine nucleotide exchange factor (GEF), Sos, through SH3-interactions, allows the Grb2-Sos complex to be located near the cell membrane and, in turn, near the substrate of Sos, Ras (41). However, in B lymphocyte signalling, another linker protein, Shc, which interacts with Igα and Igβ ITAM motifs and ITAM-associated PTKs promotes the association of Shc-Grb2-Sos complexes close to the BCR and Ras (41). Recent data (41) also suggests, however, that BCR-induced Ras activation also occurs in a Shc-independent manner due to the utilisation of an additional adaptor/linker protein, termed B cell linker protein (BLNK). This highlights the ability of different adaptor molecules to modulate BCR-mediated responses.

BLNK, sometimes called SLP-65, is a 68kDa protein which becomes rapidly tyrosine phosphorylated following BCR-ligation or treatment of B lymphocytes with pervanadate (36). In addition, BLNK is known to translocate to the cell membrane following BCR-ligation and it is known to associate with Grb2 and Sos to form a BLNK/Grb2/Sos complex. This complex, independent of Shc, brings Sos into close contact with Ras and represents an additional mechanism leading to the activation of the Ras/Raf/MAPKinase cascade in B lymphocytes.

Other adaptor/linker proteins known to regulate MAPKinase activity include the Crk family members which have been shown to negatively regulate Ras activation. The Crk family consists of three members, CrkL, Crk I and Crk II, and CrkL and Crk II have been shown to bind to C3G, the GEF for the Ras-related GTPase, Rap1. Activation of Rap1 is believed to negatively regulate receptor activated responses by down-regulating ERK-MAPKinase activation (**figure 10**). Structural similarities between the effector binding domains of Ras and Rap1 allow active Rap1 to bind to downstream effectors of the Ras pathway such as Raf-1. This process may be enhanced due to the cellular localisation of these two GTPases. Whilst Ras is predominantly membrane bound, Rap1 is located in endocytic and lysosomal vesicles, and this may function to enhance the ability of Rap1 to sequester Ras effectors away from Ras itself.

However, a number of recent studies have suggested that the Crk family of adaptor molecules may also play key roles in the activation of a number of downstream

effector molecules and that, in some cases, Rap1 activation may actually occur in a Crk/C3G independent manner. Studies by York *et. al.*, 1998 (42) have shown that formation of a stable complex between Rap1 and B-Raf that promotes the activation of ERK-MAPKinase in nerve growth factor treated PC12 cells, suggesting that Rap1 activity can positively regulate receptor-mediated responses. To add further complexity to Rap1 mediated activation of the MAPKinase family, studies by Blaukat *et. al.*, 1999 (43) have shown that the adaptor proteins p130Cas and Crk are both required in Pyk2 mediated activation of the JNK pathway. In addition, a recent study by McLeod *et. al.*, 1998 (44) has provided evidence for a Crk/C3G independent activation of Rap1 which appears to be dependent on a novel PLCγ pathway.

Activation of the PI(3)K pathway by BCR ligation has been shown to be dependent on both PTK activation and direct binding by Ras (36). The role of PI(3)K in B lymphocyte signalling is still unclear. However, evidence exists for its role in both positive and negative BCR-mediated effects. Inhibition of PI(3)K has been shown to block BCR-induced growth-arrest in the human RL B cell line suggesting a negative role for PI(3)K in B cell proliferation (17). However, inhibition of PI(3)K in normal or neoplastic B lymphocytes that are in cell-cycle induces apoptosis, suggesting a positive role for PI(3)K in regulating B lymphocyte growth (17). PI(3)K activity leading to the generation of PtdIns $(3,4,5)P_3$ has also been shown play a role in the activation of other key signalling cascades activated following BCR-ligation including phospholipase C (PLC) activity.

PLC activation is the final element of the core signalling pathways induced by BCR-ligation. This was one of the first pathways shown to be activated by the BCR and is responsible for the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns $(4,5)P_2$) to form diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (38). The generation of these two second messengers have been shown to be important for the respective activation of PKC and the release of Ca^{2+} from internal stores into the cytoplasm (45). Activation of PLC has been shown to play positive and negative roles in B lymphocyte biology. Artificial elevation of intracellular Ca^{2+} and PKC activation by calcium ionophores and phorbol esters to mimic PLC activity, has been shown to induce proliferation and the rescue of splenic B cells from Fas-induced apoptosis, whilst inducing growth-arrest/apoptosis in several B lymphoma cell lines (38).

The translocation of protein to the cell membrane is not only important in Ras activation, but has also been shown to be required for the activation of PLC. Previous studies have suggested that both Btk, via a PtdIns $(3,4,5)P_3$ dependent mechanism and Syk are required for the activation of PLC (36).

PtdIns $(3,4,5)P_3$ mediated activation of PLC is believed to occur by at least two distinct mechanisms (7). Firstly, PtdIns $(3,4,5)P_3$ by binding to the PH domain of Btk

has been shown to mediate the activation of this protein kinase. Active Btk is then known to phosphorylate and activate PLC (7). Secondly, PtdIns $(3,4,5)P_3$ may lead to the direct activation of PLC due to the binding of the PH domain of PLC to PtdIns $(3,4,5)P_3$ thereby recruiting PLC to the plasma membrane.

BCR-induced activation of Syk has long been known to play an important role in the activation of PLC. However, although the SH2 domain of PLC can bind to tyrosine-phosphorylated Syk *in vitro*, reconstitution of BCR signalling components into a non-lymphoid cell line have indicated that additional factors are required to initiate a BCR-mediated calcium response (41). Recent studies have shown that BCR-induced Syk activation leads to the phosphorylation of the linker protein, BLNK. Subsequently, membrane recruitment and activation of PLC by BLNK has been shown to be the essential intermediate required for full activation of PLC (46). Indeed, studies using the chicken DT40 cell line have shown that disruption to the BLNK gene abolishes PLC phosphorylation (36).

The role of PLC activity, and hence intracellular Ca^{2+} mobilisation has been shown to play key roles in the induction of differential signalling patterns in both immature and mature B lymphocytes. Studies in the early 1990s revealed that immature B lymphocytes mobilise intracellular Ca^{2+} following BCR-ligation but fail to generate IP_3 to the levels that are seen in mature B cell following similar stimulation (47). This suggests Ca^{2+} stores in immature B-cell are more sensitive to IP_3 than mature B cells or that key signalling intermediates are missing in immature B cells.

Although intracellular Ca^{2+} is known to regulate both proliferative and apoptotic signals, it is also known to regulate additional biological events in both immature and mature B lymphocytes. Studies by Benschop *et. al.*, 1999 (47) have provided evidence suggesting that BCR-mediated Ca^{2+} events lead to receptor editing in immature B cells and upregulation of the activation markers CD86 (required for presenting antigen to T-cells), CD69 and MHC class II in mature B cells. Interestingly, this study also suggested that induction of receptor editing in immature B cells require much lower increases in intracellular Ca^{2+} than the induction of activation marker expression in mature B cells. This therefore suggests that immature and mature B cells differentially activate distinct sets of genes following BCR-ligation and have different thresholds for the activation of these processes.

In addition to regulating Ca^{2+} flux via IP_3 production, PLC activity also induces PKC activation due to the production of DAG. The PKC family of enzymes can be divided into three isotype families based on their enzymatic activities (48). The first of these isotype groups is the conventional, or classical, PKCs. These include PKCα, PKCβ and PKCγ and have shown to be both $Ca2^{+}$ and phorbol-ester sensitive. The novel PKCs which include PKCε, PKCδ, PKCθ and PKCη are $Ca2^{+}$-insensitive,

but still phorbol-ester sensitive. The final PKC family are the atypical PKCs, which include PKCι and PKCζ and have been shown to be both $Ca2^+$ and phorbol-ester insensitive.

Important downstream targets of PKC in B lymphocytes are still being investigated. Recent work by King *et. al.* (49) has suggested a role for PKC in modulating B lymphocyte biological events (also reviewed in (8)). The results from this study suggest that PKC-β is partially responsible for BCR-induced proliferative signals in mature B lymphocytes and that uncoupling of this PKC-pathway in immature B lymphocytes leads to apoptosis following BCR ligation. Interestingly, BCR-induced apoptosis in immature B lymphocytes was also shown to be rescued by PKC-β activation in this study. In addition, prolonged phorbol-ester treatment to deplete mature B lymphocytes of DAG-sensitive PKC isoforms has been shown to induce apoptosis, and not activation, following BCR-ligation of mature B cells (50). In addition to studying the role of PKC in both immature and mature B lymphocyte biology, the generation of PKC knockout mice has helped explain the role of this enzyme in B lymphocyte development at the molecular level. PKC-β-deficient mice were subsequently shown to exhibit a phenotype similar to *xid* mice (49), which results from a point mutation in the PH domain of Btk and results in a slight reduction in peripheral B cell numbers, with those that are present, skewed towards an immature B cell phenotype (7). As pro-B to pre-B transition in *xid* mice is normal, it is clear that these mice exhibit developmental defects during the antigen-dependent phases of B cell development. Likewise, PKCβ-deficient mice also experience reduced responses to T cell-dependent antigens, suggesting a role for PKCβ in mature B lymphocyte activation (49). These results also suggest that other isoforms of PKC are unable to replace PKCβ in BCR signalling events. However, it should be noted here that whilst DAG-sensitive PKCs may play a role in the rescue of primary immature B lymphocyte from apoptosis, these signals are known to induce apoptosis in a number of cell lines such as DT40 and WEHI-231 cells (49).

Additional roles for PKC in modulating the biological response to BCR-ligation in immature and mature B lymphocytes have been reported by Carmen *et. al.*, 1996 (51). These authors reported that BCR-ligation induced activation of the early G1 kinase, cdk4 and its regulatory sub-unit cyclin D2 in both immature and mature B lymphocytes. However, unlike mature B lymphocytes, BCR-ligation failed to induce cyclin E and cdk2 expression, which is required for G_1/S transition, in immature B lymphocytes. This provides interesting data to explain the differential effects of BCR-ligation in immature and mature B lymphocytes. In addition, the results in this study reported that LPS and PMA/ionomycin, which activate Ca^{2+} sensitive PKC isoforms,

induced cyclin E and cdk2 expression in immature B lymphocytes as well as promoting RNA and DNA synthesis. These results confirm a role for PKC in mediating proliferative signals in mature B lymphocytes and again suggest this pathway may be redundant in immature B lymphocytes.

In addition to the three core signalling events activated following BCR-ligation in B lymphocytes, the role of novel, maturation state-dependent lipid signalling pathways is also being investigated by a number of groups. For example studies by Gilbert *et. al.* (52) presented data suggesting that the BCR on mature B lymphocytes is coupled to a novel PI-hydrolysing PLD under proliferative conditions. In contrast, the BCR is not coupled to PtdCho-PLD activity seen under proliferative conditions in other cells such as T lymphocytes. Interestingly, PtdCho-PLD was activated in mature B lymphocyte by ATP stimulation, which inhibits murine B lymphocyte activation suggesting that PtdCho-PLD is associated with anti-proliferative signals in mature B lymphocytes. In addition, the authors presented data suggesting that phosphatidic acid generated by the PI-PLD was converted to PKC-activating forms of DAG, unlike the phosphatidic acid generated by ATP-stimulated PtdCho-PLD. These results provide further evidence to suggest a role for PKC in BCR-mediated proliferative responses in mature B lymphocytes. The authors also addressed whether PtdCho-PLD was responsible for the BCR-mediated signals leading to growth-arrest/apoptosis in immature B lymphocytes, however PtdCho-PLD did not appear to play an early (<1 hour) role in the induction of these negative signals.

There is evidence however, for the role of other lipid second messengers in transducing apoptotic or growth arrest promoting signalling pathways. The involvement of lipid mediators in B lymphocyte signalling is now well established. The involvement of cytosolic phospholipase A_2 in immature B lymphocyte responses is perhaps the most striking because its expression also appears to be tightly regulated at a developmental level. Studies (53) have shown that anti-Ig treatment of WEHI-231 cells leads to the activation of $cPLA_2$ which hydrolyses phosphatidyl choline to generate lyso-phosphatidlycholine and arachidonic acid (AA), a known lipid second messenger with roles in apoptotoic cell responses. In contrast, anti-Ig treatment of mature splenic B lymphocytes, inducing cell activation and proliferation, did not stimulate $cPLA_2$ activity. Indeed, western blot analysis indicated that $cPLA_2$ was not expressed at the protein level in mature splenic B lymphocytes, indicating that $cPLA_2$ expression may play important roles in the mechanisms underlying B lymphocyte maturation.

Other lipid second messengers known to play a role in lymphocyte apoptosis include ceramide. Indeed, recent reports (54) have indicated that ceramide production following anti-Ig treatment correlates with the induction of apoptosis in the WEHI-231 cell line. In addition, resistance to anti-Ig mediated apoptosis in a WEHI-231 cell subline appears, in-part, to be due to insufficient ceramide production (54).

Interestingly, ceramide generation is believed, in part, to be closely regulated by AA. Indeed, TNFα-induced arachidonic acid release has been shown to precede activation of the enzyme responsible for ceramide generation, sphingomyelinase (SMase) (55), whilst AA has also been shown to stimulate SMase activity in a cell free system (56).

Another hypothesis that aimed to address the physiological differences between immature and mature B lymphocyte signalling events concerned the expression of IgD on the cell surface of mature, but not immature, B lymphocytes. Early suggestions put forward the idea that IgM was involved in negative signalling events whilst IgD was involved in B cell activation (57). However, recent evidence suggests that apoptosis can be triggered in immature/transitional B lymphocyte when either mIgM or mIgD are cross-linked with high avidity (58). This same study provided evidence to suggest that mIgD derived signals were ineffective in reversing mIgM-induced apoptosis. However, other studies have suggested that increasing expression of mIgD does confer some resistance to deletion (58). Therefore the role of mIgD in the development of B lymphocyte remains unclear, however, it appears that intrinsic, maturation-dependent differences in sIg-mediated signalling events play a vital role in determining the response to sIg ligation in immature/mature B lymphocytes.

Recent advances in the understanding of BCR mediated signalling events following antigen binding have suggested a role for glycosphingolipid- and cholesterol-rich plasma membrane microdomains, or lipid rafts, in the initiation of these events (59). Subsequently, it has been suggested that the BCR complex is rapidly recruited to these lipid rafts upon antigen binding. In addition, these rafts were shown to contain the Src family kinase Lyn, but not the transmembrane phosphatase, CD45R. This suggests that lipid rafts may function to concentrate activated BCRs and bring them into close contact with downstream signalling enzymes and adaptor proteins utilised by BCR signalling pathways.

1.8 Role of co-receptors in B lymphocyte signalling.

Previous discussions have addressed the differential biological responses generated by the BCR following receptor ligation in either immature or mature B lymphocytes. This may be due to the differential expression of signalling proteins at different maturational stages, or recruitment of adaptor/linker proteins, but also the surface expression of a number of co-receptors which have been shown to modulate, either positively or negatively, signals generated by the BCR. Like adaptor/linker proteins, these co-receptors, which include CD19, CD22 and FcγRIIB function by recruiting signalling enzymes which modulate BCR signalling events.

1.8.1 CD19

CD19, a B lymphocyte restricted glycoprotein, is expressed throughout B cell development until plasma cell differentiation (10, 60) and forms part of a complex also containing CD21 and CD81. Co-ligation of CD19 with the BCR is known to reduce the threshold for B cell activation and CD19-/- mice have been shown to be hypo-responsive to BCR stimulation (41). Although CD19 is expressed throughout the B cell lineage, deletion of CD19 appears to have little effect on B cell development until the mature B cell stage of development. Studies have shown that lack of, or overexpression of CD19 leads to decreases in the number of conventional B lymphocytes in the periphery (17, 61). In the latter case, the reduction in peripheral B lymphocytes can presumably be explained by the hyperresponsiveness of immature B lymphocytes to antigen receptor ligation and hence enhanced negative selection in the bone marrow. However, in normal physiological situations, co-ligation of CD19 with the BCR substantially increases mature B cell activation leading to increased release of intracellular calcium, increased DNA synthesis, increased ERK-MAPKinase activation and antibody production (62). Additional evidence for CD19-mediated regulation of the threshold for BCR signalling comes from studies using double HEL-transgenic mice. These mice express a BCR specific for HEL (anti-HEL) and also soluble HEL (sHEL). Mature B lymphocytes from these mice are anergic in nature and do not produce auto (anti-HEL) antibodies. However, overexpression of CD19 in these mice has been shown to induce autoantibody production which is believed to be due to CD19-mediated lowering of the threshold for BCR-mediated signalling (17).

CD19- and CD21-deficient mature B cells have also been reported to have lowered responses to T-cell dependent antigens and subsequent lack of germinal centre formation and decreased affinity maturation of serum antibodies (60) suggesting that CD19-CD21 signalling is important for T dependent B cell responses. This is because CD19-CD21 signalling not only lowers the threshold for BCR-mediated B lymphocyte activation, but also signalling via CD21 has been shown to be co-stimulatory for anti-CD40 stimulated splenic B cell growth and these two signals result in enhanced B lymphocyte differentiation (60). In addition, studies have also shown that CD40 ligation also results in tyrosine phosphorylation of CD19 and activation of PI(3)K (63) suggesting that CD40 may also regulate CD19 signalling and hence alter the threshold for activation by the BCR.

How co-ligation of CD19 with the BCR augments B cell activation remains unclear, however, new insights into CD19 signalling mechanisms have helped unravel this process. Upon BCR-ligation, CD19 becomes tyrosine-phosphorylated which provides binding sites for PI(3)K, the GEF Vav, Shc and the Src-family PTKs. The phosphorylation of the cytoplasmic tail of CD19 is believed, in part, to be due to the

actions of Lyn, which is activated following BCR ligation and subsequently phosphorylates and binds to CD19. Studies using CD19-deficient mice have shown that lack of CD19 leads to a reduction in the tyrosine phosphorylation of multiple effector molecules downstream of the BCR following BCR-ligation (61). In addition, *in vitro* kinase assays have shown that the kinase activity of purified Lyn was significantly increased when co-incubated with purified CD19. These results suggest that CD19 functions to amplify PTK activity downstream of BCR-ligation and that loss of this PTK-amplification may account for the hyporesponsiveness of BCRs in CD19 deficient mice.

The signalling mechanisms behind the ability of CD19 to augment BCR-mediated intracellular calcium and ERK-MAPKinase signals are also becoming clear. As previously mentioned, BCR-mediated phosphorylation of CD19 enhances Vav binding, which, coupled to its co-localisation with BCR-activated PTKs, leads to its enhanced tyrosine phosphorylation and hence activation. Vav acts as a GEF for the Rho-GTPases which in turn can activate PI(5)K which has been shown to result in PLC activation, IP_3 generation and hence calcium mobilisation (36, 60). Additional studies have accounted for the ability of CD19 to augment BCR-mediated ERK-MAPKinase activation (62). This study provided data to suggest that co-ligation of CD19 and BCR lead to the synergistic enhancement of MEK1 activity. Interestingly, these studies also presented data suggesting that BCR-mediated ERK-MAPKinase activation was both Ras- and PKC-dependent. However, CD19 mediated enhancement of MEK1 activity was shown to be independent of both Ras and PKC suggesting a novel route of MEK1 activation.

1.8.2 CD22

Another co-receptor restricted to the B cell lineage which has been shown to modulate BCR-mediated signalling events is CD22. The surface expression of CD22 has been shown to remain low during early stages of B lymphocyte development, but increases as B lymphocytes mature in the periphery (17). Like CD19, CD22 rapidly associates with the BCR and becomes tyrosine phosphorylated by Lyn after BCR-ligation. This creates phosphotyrosine motifs that recruit multiple SH2 domain containing effector molecules. However, unlike CD19, CD22 has been shown to be a negative regulator of BCR signalling events, increasing the threshold for BCR activation. This occurs under physiological conditions where the BCR and CD22 molecules are in close association with each other. Other studies have shown that CD22, when ligated separately from the BCR, actually acts to promote BCR-mediated responses. This has been proposed to be due to the ability of CD22 to sequester inhibitory signalling elements such as the tyrosine phosphatase, SHP-1, away from the

BCR (64). However, the physiological relevance of these studies are unclear. Therefore, it is the negative, and more physiological role of CD22 in B cell signalling that has attracted much of the attention over the last few years.

BCR-mediated phosphorylation on immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of CD22 recruits intracellular signalling molecules including SHP-1, Lyn, PI(3)K and PLCγ1(60). It is the active recruitment of the protein tyrosine phosphatase SHP-1 to the BCR-CD22 complex that permits BCR-mediated signalling events to become suppressed. Studies using CD22-/- mice have shown CD22-/- B cells exhibit increased calcium responses following BCR-ligation compared to wild-type B cells confirming that CD22 acts to suppress BCR-mediated signalling events (41). Phenotypically, CD22-/- mice exhibit normal early B cell development and normal numbers of B cells in the spleen (60). However, peripheral B cells were shown to express decreased levels of mIgM and increased levels of MHC class II, similar to pre-activated B cells (17). These results, and observations suggesting that SHP-1 deficient mice deliver similar phenotypic abnormalities, confirm a negative role for CD22 (increasing BCR threshold for activation), mediated by SHP-1, in modulating BCR-mediated signalling events.

What signalling mechanisms are negatively regulated by CD22 when complexed with the BCR are still unclear. Studies by Tooze *et.al.*, 1997 (65) provided good evidence to suggest that CD22 was an important regulator of BCR-mediated MAPKinase activation in mature splenic B lymphocytes. Interestingly, cross-linking of CD22 with the BCR appeared to selectively suppress BCR-mediated ERK-MAPKinase and to a lesser extent JNK activity, whilst p38-MAPKinase activity was unchanged. This suggests that CD22/SHP-1 are capable of selectively inhibiting some pathways, but not others, and this could re-direct the balance of BCR-mediated signalling events.

Although CD22 appears to be able to modulate BCR-mediated responses, it is itself under intense regulation. A recent study by Greer *et. al.*, 1999 (66) provided evidence to suggest that the transmembrane protein tyrosine phosphatase, CD45, played an important role in regulating CD22 phosphorylation. Indeed, CD45-deficient B cells were shown to have increased tyrosine phosphorylation of CD22, but also increased levels of SHP-1 associated with CD22 after BCR-ligation. Therefore, CD45 appears to play a role in regulating the level of CD22 phosphorylation following BCR-ligation.

1.8.3 FcγRIIB

B lymphocytes can receive signals that can modify the activation signals delivered through the BCR. These include the CD22 molecule described above, but also the Fc receptor, FcγRIIB, which contains immunoreceptor tyrosine-based

inhibitory motifs (ITIMs) in its cytoplasmic tail. It widely reported that the activation of naive mature B lymphocytes can be inhibited by co-ligation of the BCR with the Fc receptor.

FcγRIIB mediated inhibition of BCR-mediated signalling events, like CD22, involves the recruitment of phosphatases. FcγRIIB recruits SHP-1 and SHP-2, however, unlike CD22, FcγRIIB also recruits the lipid phosphatase SHIP. SHIP catalyses the hydrolysis of PtdIns $(3,4,5)P_3$ to PtdIns $(3,4)P_2$. FcγRIIB mediated recruitment of SHIP therefore attenuates BCR-mediated activation of both Btk and Akt as both of these enzymes require PtdIns $(3,4,5)P_3$ levels (41, 67). In addition, recent studies have suggested that SHIP plays a negative role in BCR-mediated signalling by acting as an adaptor molecule (68). This report suggests that FcγRIIB mediates the inhibition of ERK-MAPKinase by the recruitment of SHIP which in turn acts as a binding site for p62dok and RasGAP. The linker protein Shc is also believed to play key roles in FcγRIIB mediated suppression of ERK-MAPKinase (17, 69). Thus, it is thought that increases in the association between SHIP and Shc following BCR and FcR cross-linking reduce Shc/Grb2 interactions and hence Ras activation.

1.8.4. CD40

CD40, and its ligand CD40L (CD154) are known to play crucial roles in the regulation of the humoral immune response including cell activation, proliferation, immunoglobulin isotype switching and germinal centre B cell formation (70).

In addition to having roles in mediating B lymphocyte biology during normal development, recent studies have also suggested an important role for CD40 in antitumour strategies against B lymphocyte malignancies (reviewed in (71)). These studies have indicated that CD40 stimulation leads to the up-regulation of adhesion/costimulatory molecules on the cell surface of tumour cells which aids the immune recognition of these malignant cells. In addition, CD40 stimulation of tumour cells has also been shown to induce the release of anti-tumour cytokines by the tumour itself. However, limitations to the effectiveness of CD40 as a viable therapy against some B lymphocyte malignancies have been discovered due to the anti-apoptotic/proliferative signals delivered by CD40-ligation in some low-grade B lymphocyte malignancies. Therefore current research into CD40-mediated tumour therapy is focused on avoiding pro-tumourigenic effects of CD40 stimulation whilst understanding the role of CD40 in tumour recognition and the stimulation of the immune system.

1.8.4.1 CD40 structure and expression.

Since the identification of CD40 almost 15 years ago and the discovery that it plays crucial roles in T cell-mediated B cell activation, much has been learnt about the signalling pathways activated by the receptor following ligation with CD40L or soluble CD40 antibodies (anti-CD40). The CD40 receptor is a 48-k.Da. transmembrane glycoprotein and is a member of the TNF receptor (TNFR) superfamily (72) (**figure 11**). Structurally, human CD40 consists of a 193 amino-acid extracellular domain, a 22 amino-acid transmembrane domain and a 62 amino-acid cytoplasmic domain. The extracellular domain consists of four homologous, repeating, cysteine-rich extracellular domains characteristic of TNFR family motifs (73). It has been shown that murine CD40 shares approximately 60% homology to human CD40, with the greatest homology (78%) to the human form in the cytoplasmic domain (74). This cytoplasmic domain contains no sequence of known protein tyrosine kinase activity. Indeed, the cytoplasmic tail of human CD40 contains no tyrosine residues, whilst only one exists in the murine form. Nevertheless, the cytoplasmic domain of CD40 is constitutively phosphorylated which is believed to be due to serine and threonine residues, in which Thr-234 has been shown to be crucial for signal transduction (74).

Although CD40 expression was originally thought to be a B lymphocyte specific receptor it is now known to be widely expressed on human and mouse dendritic cells, monocytes, macrophages, mouse fibroblasts and human smooth muscle cells (75). Although CD40 has been reported to be expressed on virtually all B lymphocytes, the level of CD40 expression varies depending on the maturational stage of the cell. Studies by Hasbold *et. al.* (76) provided data indicating that murine CD40 is expressed at low levels on 30-40% of pre-B cells, at intermediate levels on 80% of immature B cells and on essentially all mature B cells, suggesting that CD40 expression may be more important in later stages of B lymphocyte maturation.

1.8.4.2 CD40 signal transduction.

Like both the p55 and p70 isoforms of the TNF receptor, CD40 has no intracellular kinase domain and no consensus sequence for binding kinases (72). However CD40 ligation is known to rapidly activate the protein tyrosine kinases Lyn and Syk (77) whilst also inducing the tyrosine phosphorylation of phosphotidylinositol 3-kinase (PI3-K), phospholipase C-γ2 (72) and also activating serine/threonine kinases (70). However, like all signalling mechanisms, it is important to be careful when comparing data from different cell types. Consequently, studies highlight quite distinct differences in proximal CD40 signalling events in B lymphocytes depending on

whether the cells used were resting, activated or EBV-transformed (74, 77). In addition, some studies suggest that instead of phosphorylating and activating a number of protein tyrosine kinases, CD40 engagement de-phosphorylates the Src family PTKs or syk (17).

As well as regulating protein tyrosine kinase activity, CD40 is known to associate with intracellular proteins termed TNF receptor-associated proteins (TRAFs). TRAF2, TRAF3 and TRAF 5 are known to associate with a specific region in the cytoplasmic domain of CD40 (73) (**figure 11**). TRAF3 or CD40 receptor-associated factor (CRAF1) is specific to CD40 signal transduction and does not bind to other membrane proteins such as p70 TNFRII (74). One fascinating feature of the TRAFs is their ability to bind both membrane elements (such as CD40) and also DNA. This latter function is due to the five zinc ring finger motifs encoded in the N-terminal domain of TRAF 2 and 3. In addition, TRAF proteins share homology in a C-terminal TRAF binding domain. This allows the various TRAFs to form homo- or heterodimers following CD40 ligation (73). These complexes (much like the STAT family of proteins) may then translocate to the nucleus to function as transcriptional activators. Indeed, TRAF2 is predicated to play a crucial role in CD40-mediated activation of the transcription factor NF-κB.

Whilst we understand the importance of CD40 signalling in B lymphocytes we are still a long way from understanding the precise signalling mechanisms utilised by this receptor. What is clear however, is that CD40 signalling in B lymphocytes involves both nuclear and cytoplasmic elements, with additional differential roles determined by a cells activation or developmental state. In addition the role of CD40-induced signalling pathways in the regulation of apoptosis is currently attracting much attention. A recent review by Craxton *et. al.* (17) highlights the role of CD40 in both the induction or prevention of apoptosis in B lymphocytes which appears to be closely regulated by the developmental stage of the cell.

1.9 Aims and objectives.

The aims and objectives of this investigation were to:

I. determine the signalling mechanisms involved in the deletion of immature B lymphocytes following BCR-ligation.

II. investigate the signalling mechanisms involved in the rescue of immature B lymphocyte from deletion by co-stimulation with anti-CD40.

Figure 1. Stages of B lymphocyte Maturation. B lymphocyte maturation starts in the bone marrow with the commitment of haematopoietic stem cells to the B lymphocyte lineage. These cells develop in an antigen-independent manner. Pro-B cells express a functionally rearranged immunoglobulin heavy chain in addition to the surrogate light chains $V_{pre\text{-}B}$ and λ_5 which are important for allelic exclusion. Cells then enter the pre-B cells stage and express cytoplasmic μ chains. Light chain rearrangement occurs, leading to the expression of mIgM on the cells surface. The B cell now enters the antigen-dependent stage of B lymphocyte development as an immature B cell where the B lymphocyte repertoire is determined by the selection of B lymphocyte expressing non-self antigen receptors. Those cells which pass this section process will respond to subsequent antigen exposure (specific to their BCR) by differentiating into either a plasma cell or memory cell depending on the nature of T cell help.

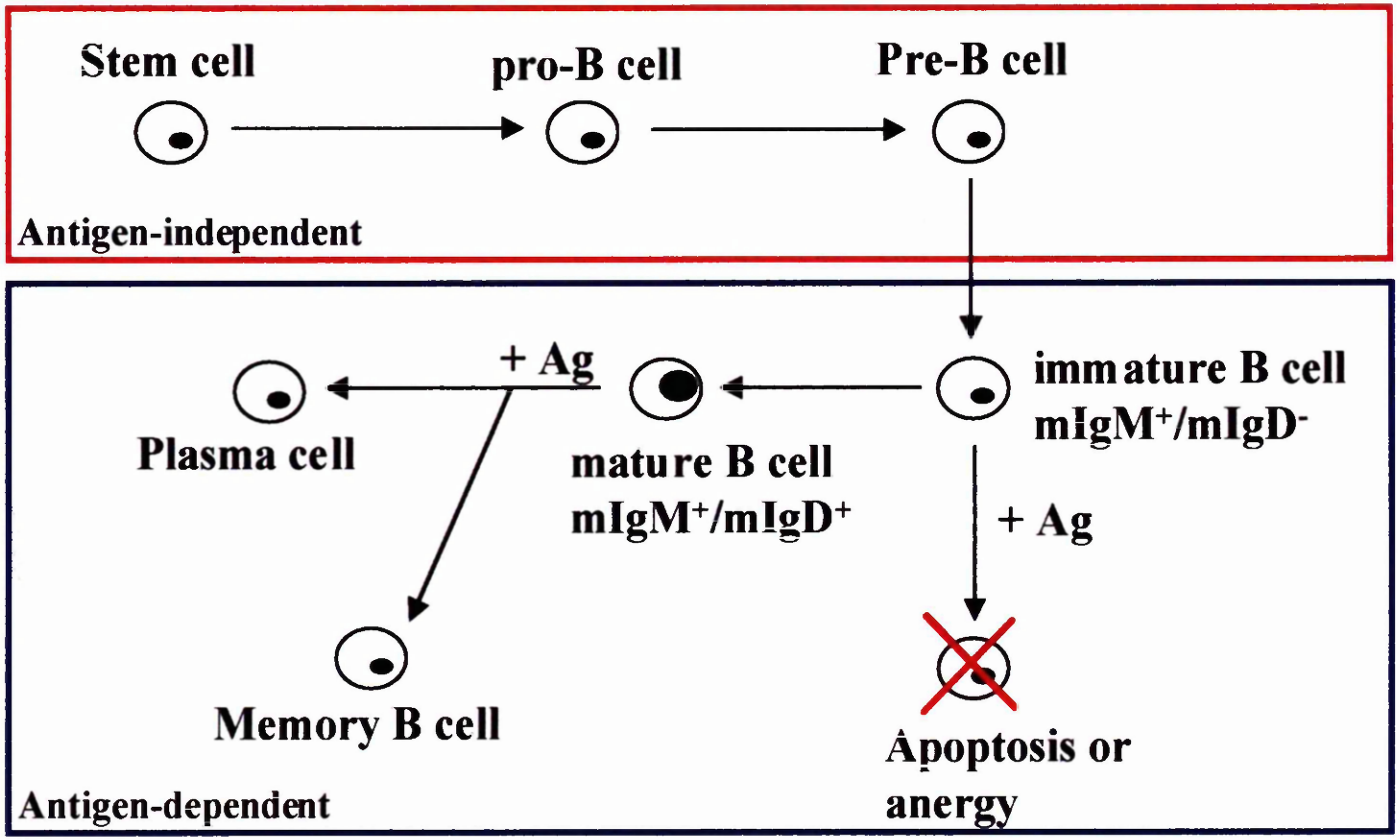

Stem cell
pro-B cell
Pre-B cell
Antigen-independent
+ Ag
immature B cell
mIgM+/mIgD-
Plasma cell
mature B cell
mIgM+/mIgD+
+ Ag
Memory B cell
Apoptosis or
anergy
Antigen-dependent

Figure 1

Figure 2. B lymphocyte development is associated with differential expression of a number of cell surface markers. During development numerous markers are expressed on the cell surface which can be used to determine the developmental stage of the B lymphocyte.

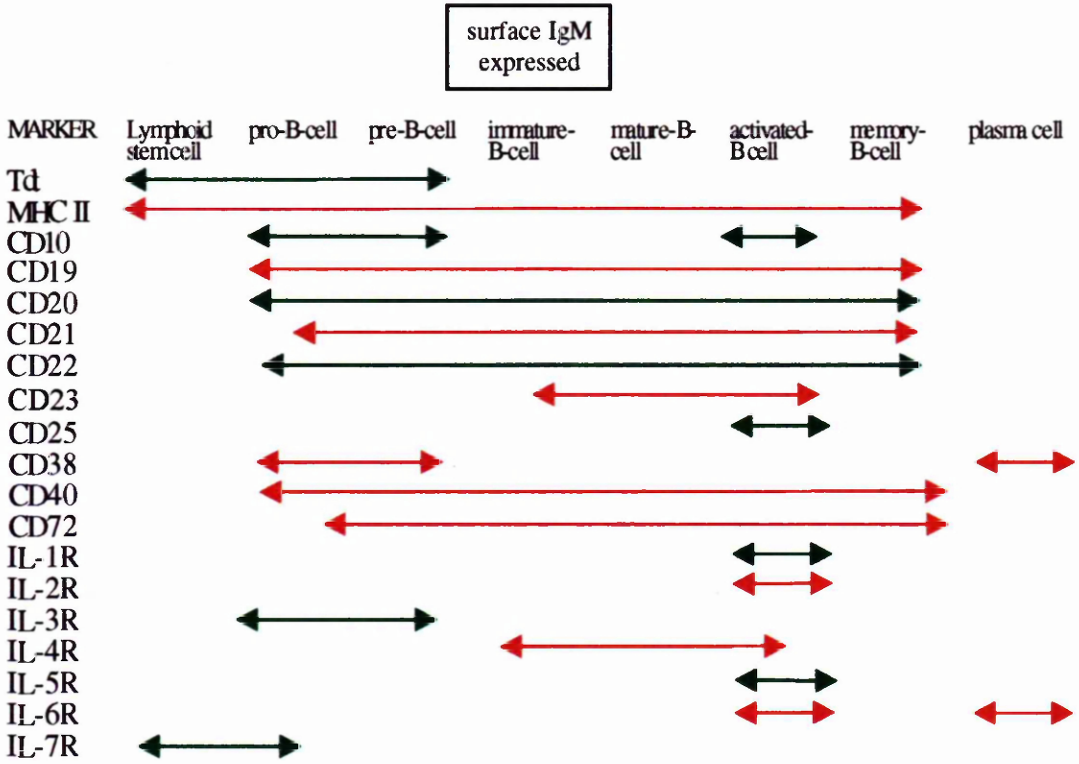

surface IgM expressed
MARKER
Lymphoid stemcell
pro-B-cell
pre-B-cell
immature-B-cell
mature-B-cell
activated-B cell
memory-B-cell
plasma cell
Td
MHC II
CD10
CD19
CD20
CD21
CD22
CD23
CD25
CD38
CD40
CD72
IL-1R
IL-2R
IL-3R
IL-4R
IL-5R
IL-6R
IL-7R

Figure 2

Figure 3. B lymphocytes react differently to self-antigen and express BCRs of different maturity depending on their developmental stage. Immature B cells are the first cells of the B lymphocyte lineage to express fully functional BCRs. Self-antigen block the developmental progression of immature B cells and initiates receptor editing. Transitional B cells response to self-antigen exposure by the rapid induction of apoptotic machinery. Mature B cells that have successfully traversed the selection process respond to antigen exposure by initiating activation programmes.

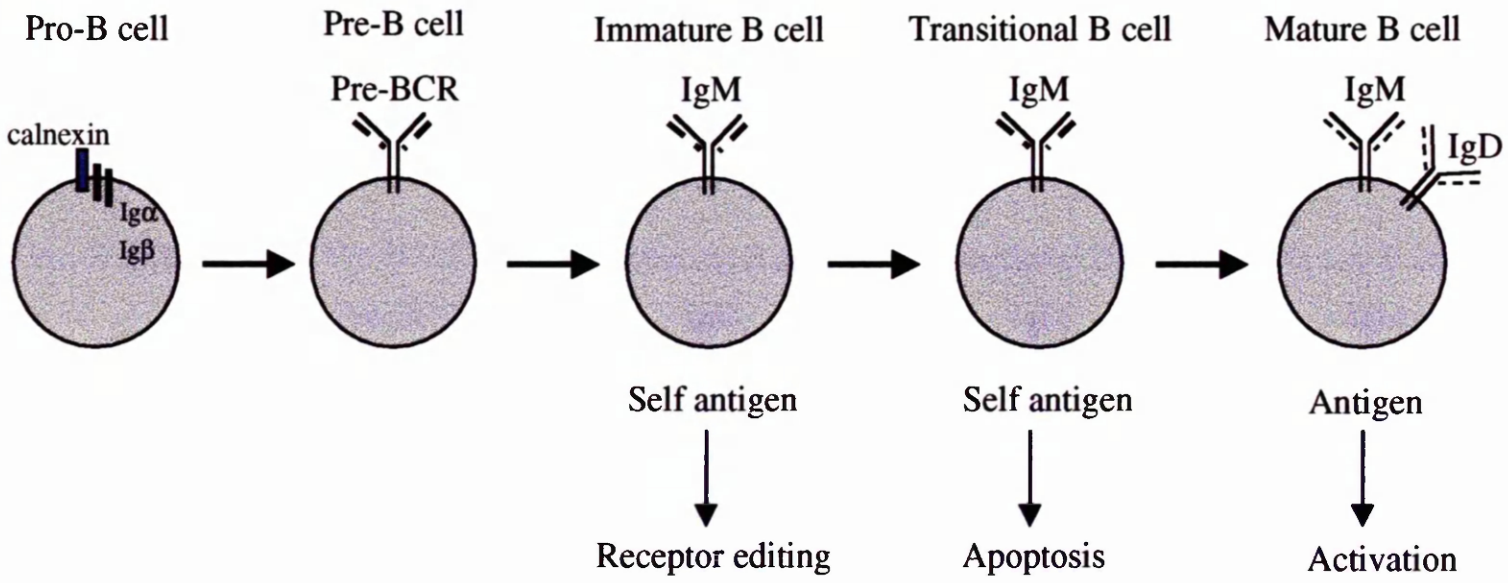

Pro-B cell
Pre-B cell
Immature B cell
Transitional B cell
Mature B cell
calnexin
Igα
Igβ
Pre-BCR
IgM
IgM
IgM
IgD
Self antigen
Self antigen
Antigen
Receptor editing
Apoptosis
Activation

Figure 3

Figure 4. B lymphocyte development is accompanied by the ordered expression of different "surrogate" and then finally, a fully mature BCR. The various stages of B lymphocyte development can be defined by the rearrangement of the immunoglobulin heavy and light chain genes. Genetic mutations to specific genes have been used to generate knockouts which result in the developmental arrest of B lymphocyte maturation. Those commonly used are shown.

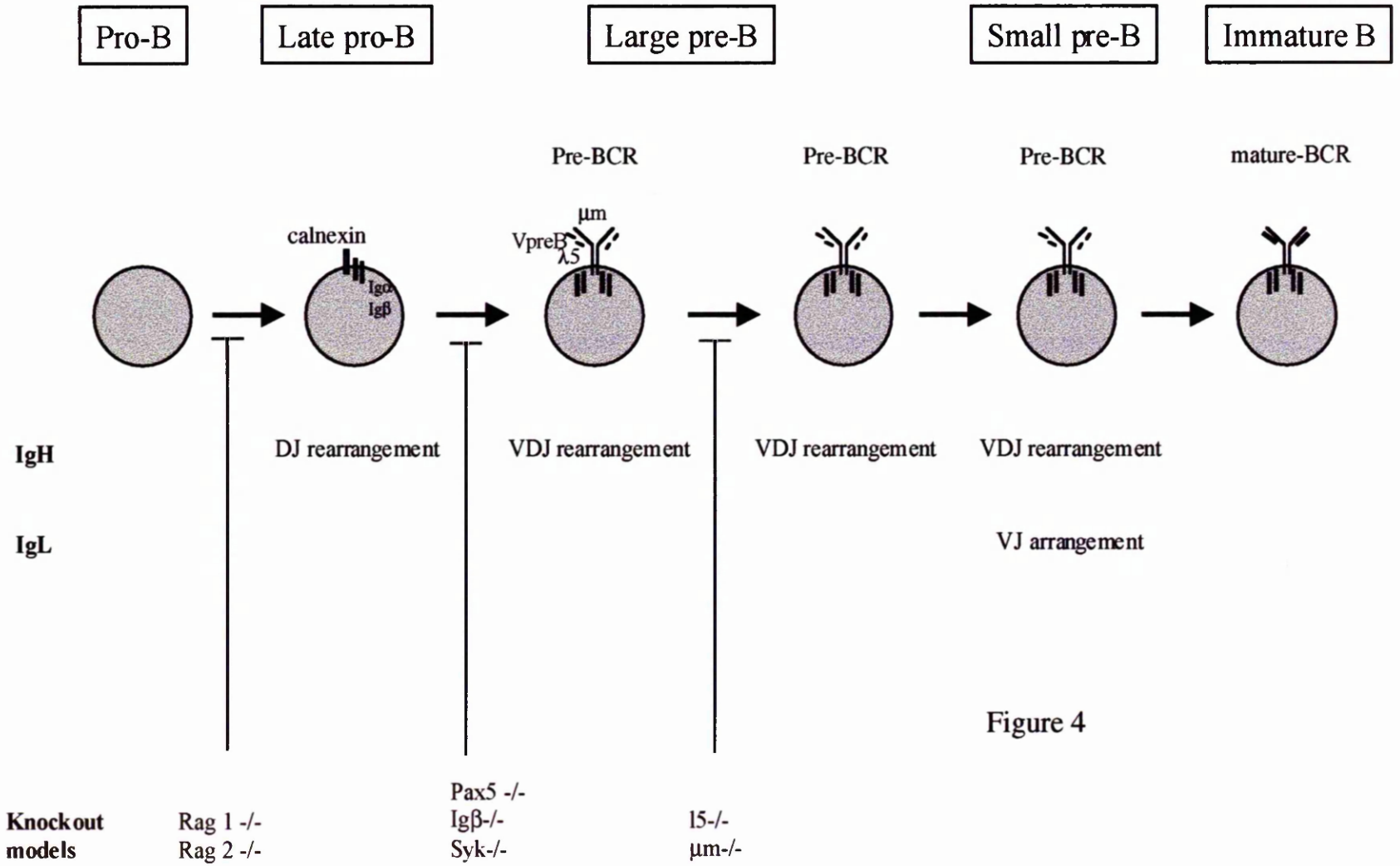


Figure 4

Figure 4

Figure 5. Germinal centre formation. During T-dependent activation resting naive B cells (1) migrate from the follicular mantle into B cell follicles. Here they differentiate into proliferating centroblast (2) where they form the germinal centre dark zone. This is the region where somatic mutation in the variable region of the immunoglobulin gene occurs. These centoblasts then differentiate into centrocytes (3) and undergo positive selection, depending on the affinity of their mutated antigen receptors, in the light zone of the germinal centre. Those cells with low affinity and autoreactive antigen receptors undergo spontaneous apoptosis. Positively selected centrocytes may undergo CD40-mediated isotype switching, become protected from Fas-induced apoptosis. and differentiate into either plasma cells (4) or memory B cells (5).

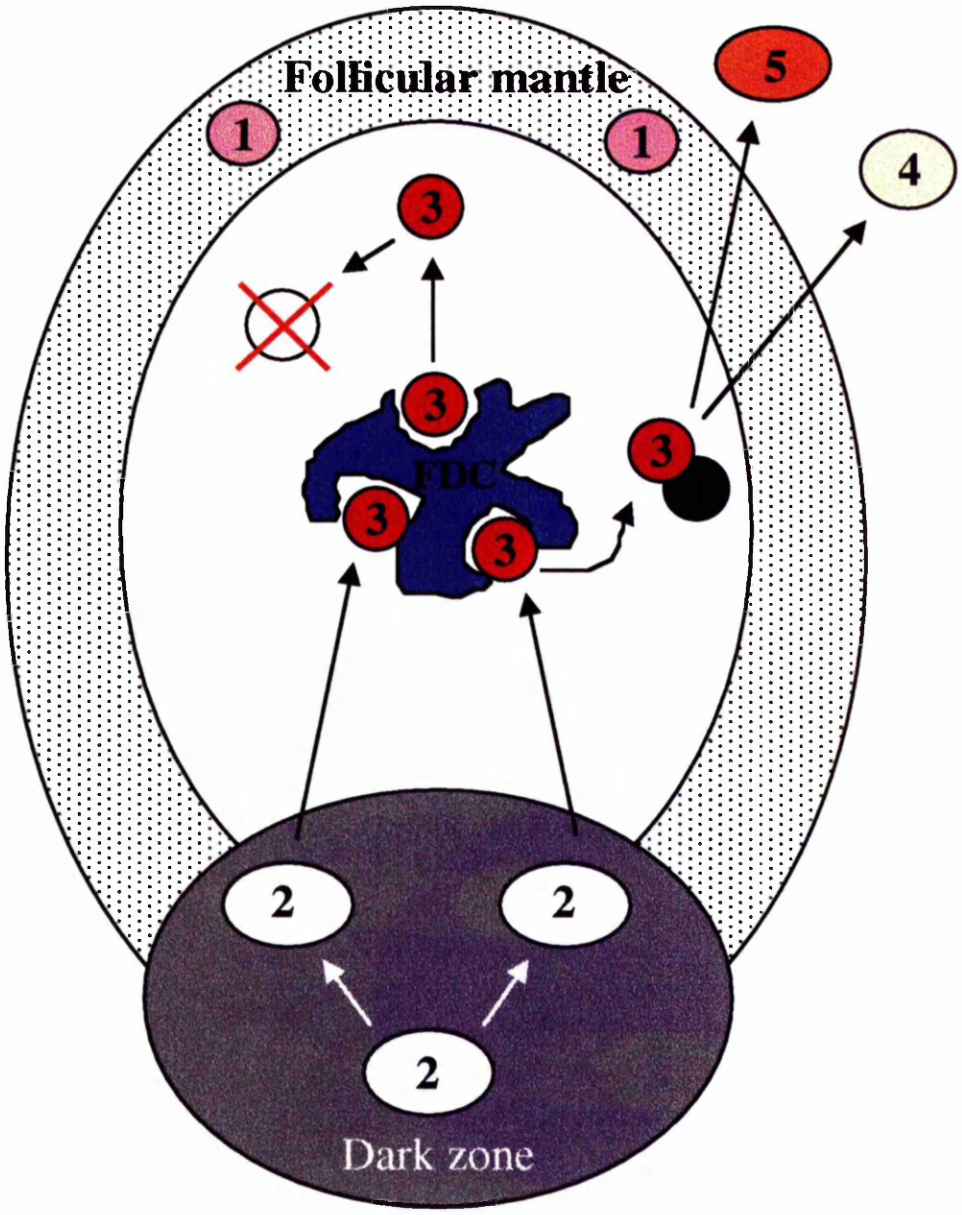


Figure 5

Figure 6. The cell-cycle of a cell proceeds in a logical number of stages. Replication of DNA occurs in the S (synthesis) phase of the cell cycle The interval before this is called the G_1 phase, and the phase between the end of DNA synthesis and mitosis (M) is called the G_2 phase. These two G (or gap) phases allow the cell to determine if they are the right size to commence DNA replication and also determine whether DNA replication has occurred without any errors.

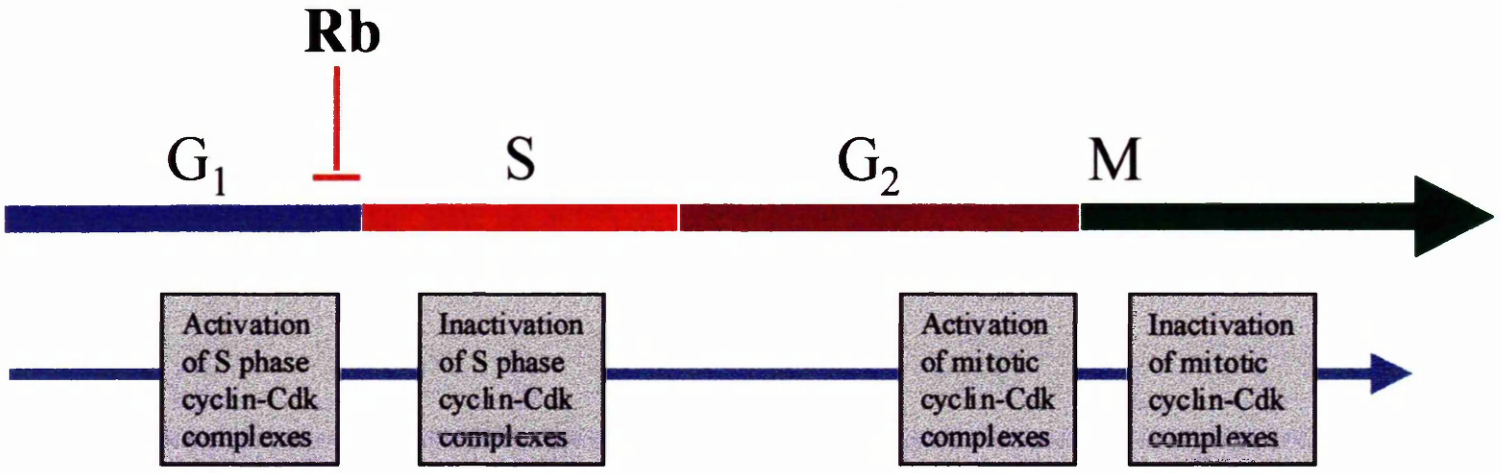

Rb
G1
S
G2
M
Activation of S phase cyclin-Cdk complexes
Inactivation of S phase cyclin-Cdk complexes
Activation of mitotic cyclin-Cdk complexes
Inactivation of mitotic cyclin-Cdk complexes

Figure 6

Figure 7. Role of the mitochondria in the initiation of apoptosis. Death signals can be transmitted to the nucleus through pro-apoptotic members of the Bcl-2 family, including Bid, Bax and Bad. This results in cytochrome c release, and apaf-1-caspase 9 activation. In addition, cytochrome c release can be induced by caspase-8 activity, mediated by death signals such as those generated through the tumour-necrosis-factor (TNF) receptor. Activation of death signals through the TNFR can also bypass the mitochondrial step and directly activate caspase-3. Anti-apoptotic members of the Bcl-2 family, such as Bcl-x_L can prevent apoptosis by preventing the release of cytochrome C from the mitochondria.

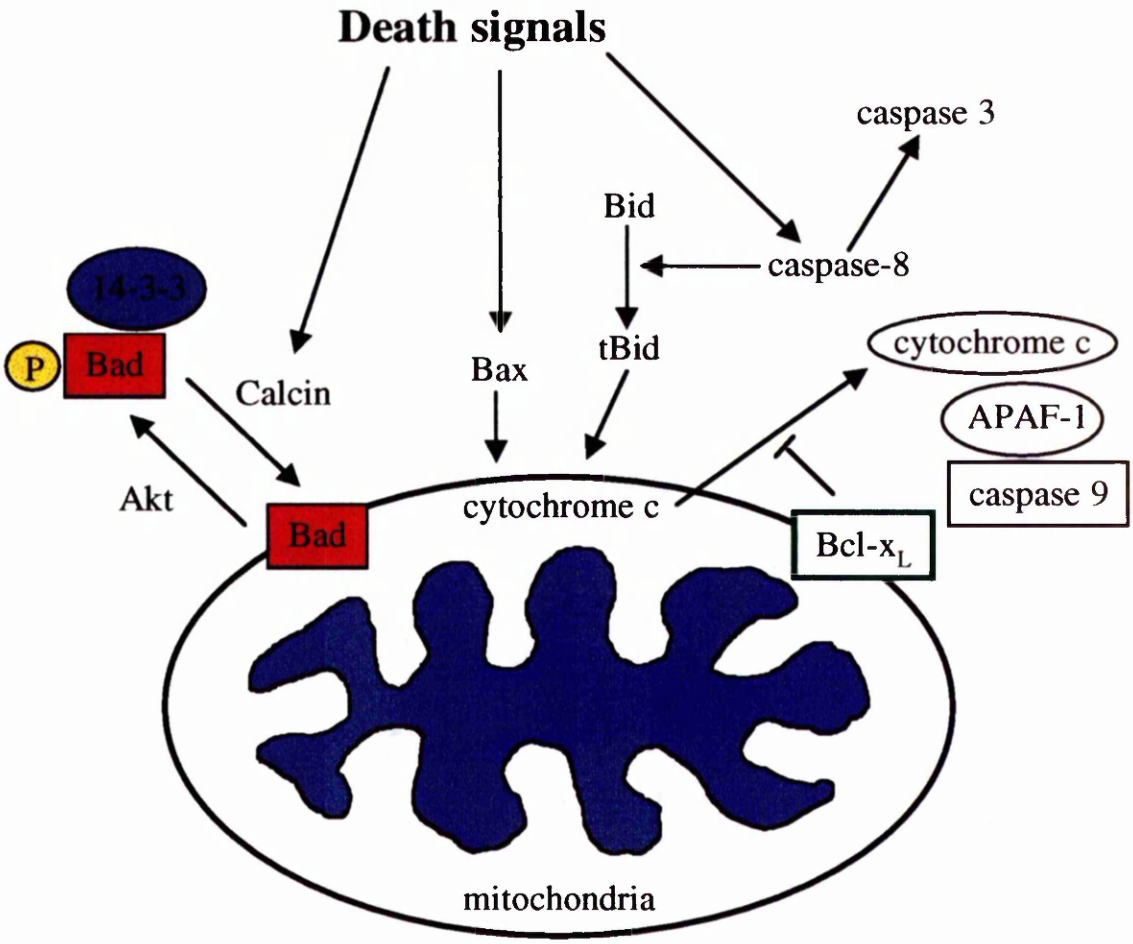

Death signals
caspase 3
Bid
caspase-8
14-3-3
P
Bad
Calcin
Bax
tBid
cytochrome c
APAF-1
caspase 9
Akt
Bad
cytochrome c
Bcl-x$_L$
mitochondria

Figure 7

Figure 8. Structure of the mature BCR. Diagram shows the association of mIg with disulfide linked heterodimers Igα and Igβ. The Igα and Igβ heterodimers contain ITAM motifs which act as the signalling elements of the mature BCR.

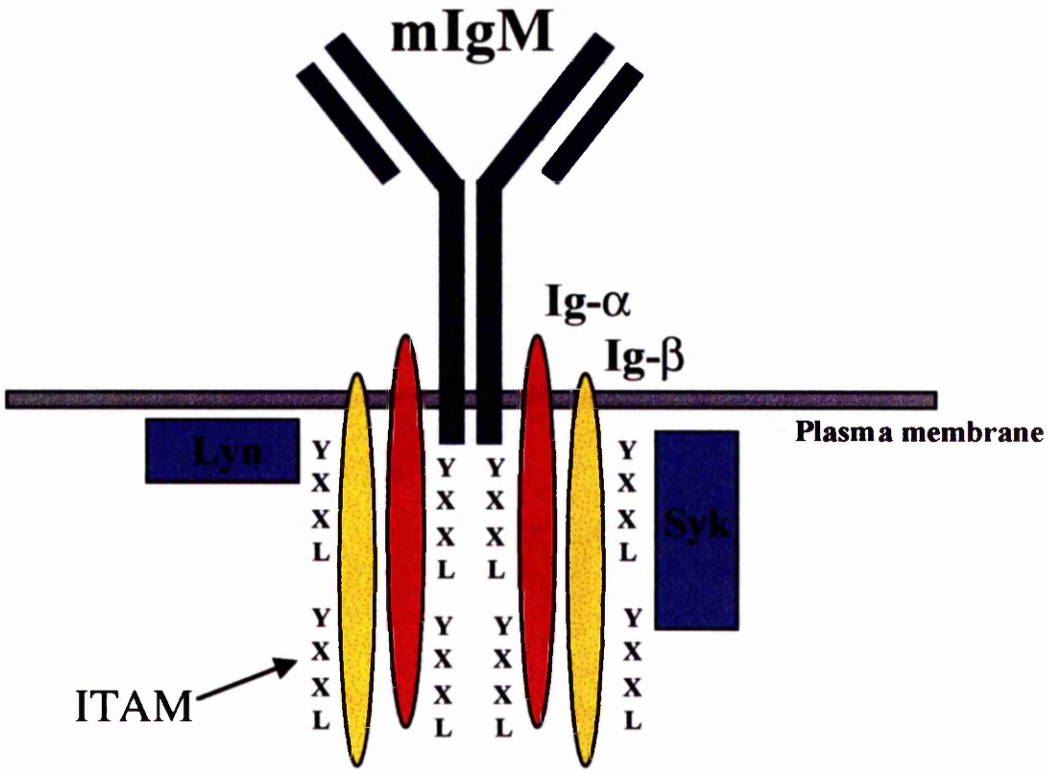


Figure 8

Figure 9. Signalling pathways activated upon BCR-ligation in immature B lymphocytes. Three classical signalling pathways are activated upon BCR-ligation in immature B lymphocytes. These include the Ras/Raf/MAPKinase leading to the activation of ERK-MAPKinase. Activation of the PI(3)K pathway leading to the generation of PtdIns(3,4,5)P_3 and activation of the PLC pathways leading to DAG and IP_3 generation. These pathways are also known to be activated by receptor-ligation in mature B lymphocytes.

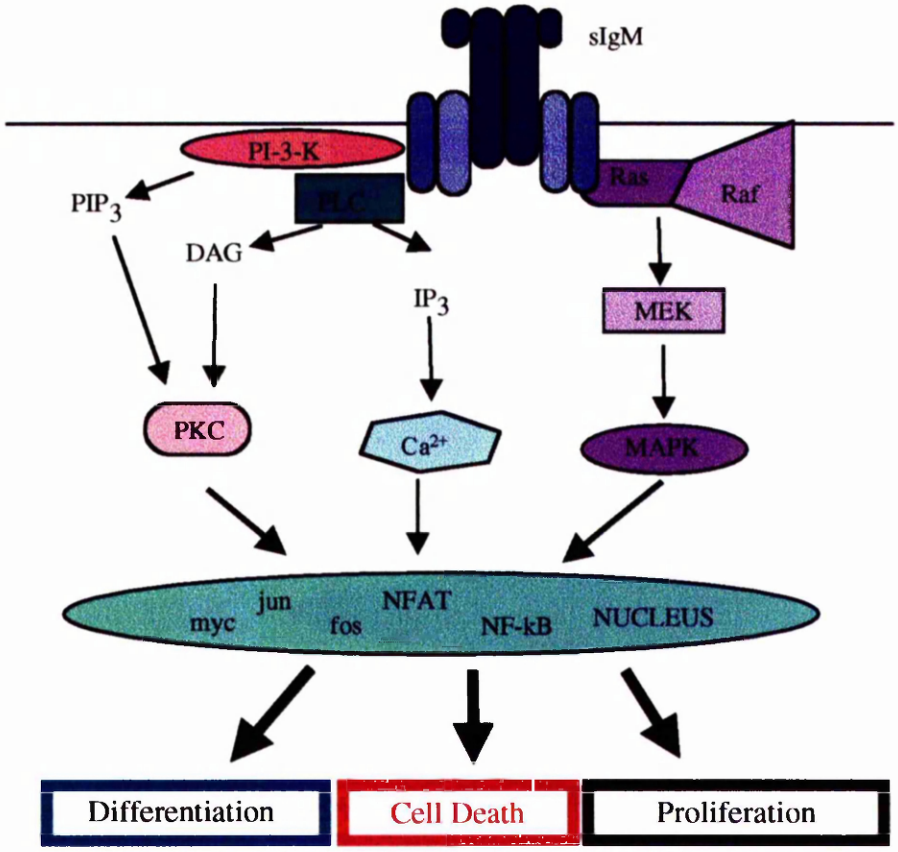

sIgM
PI-3-K
PIP3
PLC
DAG
IP3
Ras
Raf
MEK
PKC
Ca2+
MAPK
myc
jun
fos
NFAT
NF-kB
NUCLEUS
Differentiation
Cell Death
Proliferation

Figure 9

Figure 10. Activation of Rap1 by BCR-ligation. BCR-ligation induces the tyrosine phosphorylation of the membrane associated docking proteins $p130^{Cas}$ and $p120^{Cbl}$. Crk proteins can then bind to these tyrosine phosphorylated proteins via their SH2 (tyrosine-binding) domains. This subsequently recruits the guanine nucleotide exchange factor, C3G, to the cell membrane where it associates with and activates Rap1.

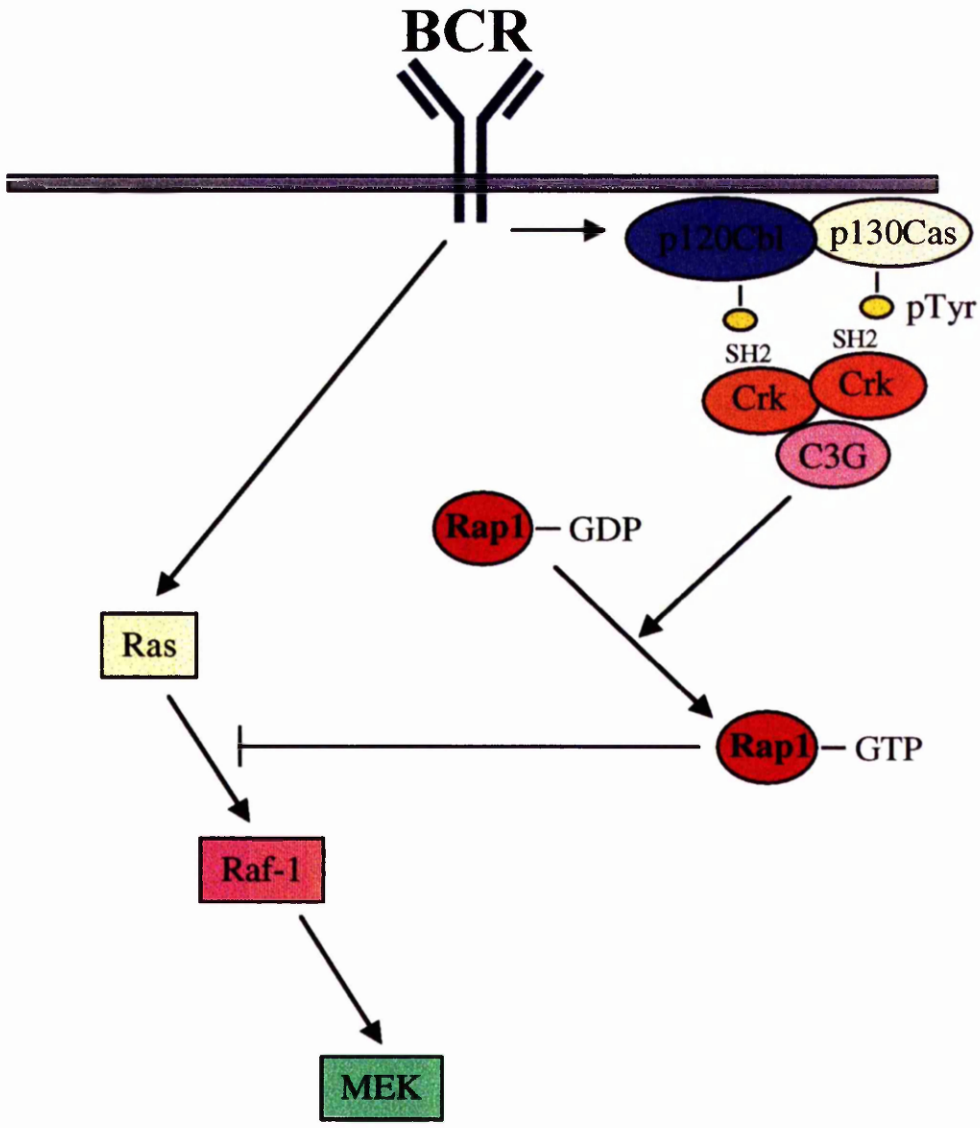

BCR
p120Cbl
p130Cas
pTyr
SH2
SH2
Crk
Crk
C3G
Rap1
GDP
Ras
Rap1
GTP
Raf-1
MEK

Figure 10

Figure 11. Schematic representation of the structure of the CD40 receptor. Figure shows the binding of TRAF2, TRAF3 and TRAF5 to C-terminal TRAF binding domains on the intracellular tail of CD40. Arrows represent signal transduction pathways leading to the activation of NFκB and regulation of protein tyrosine kinases.

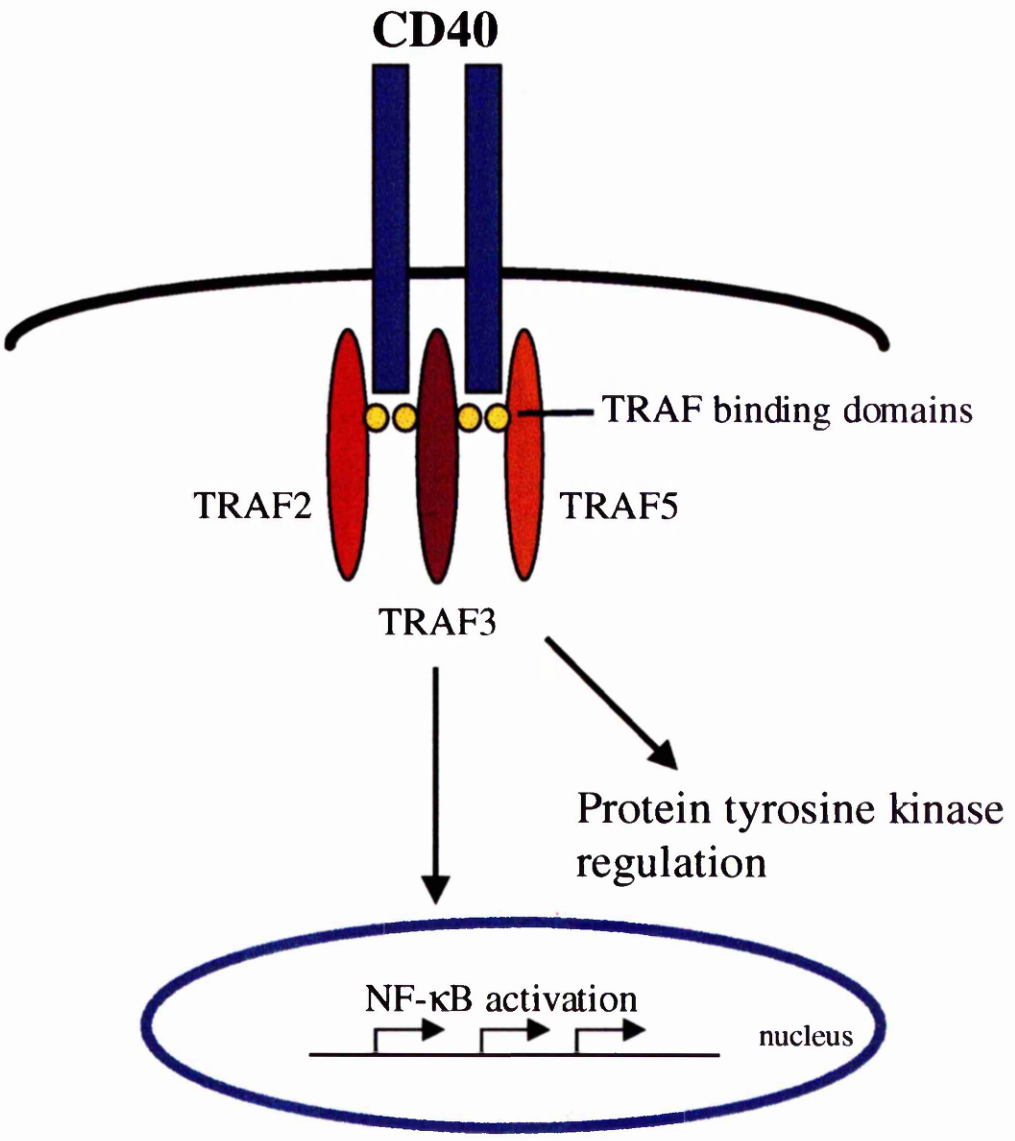

CD40
TRAF binding domains
TRAF2
TRAF5
TRAF3
Protein tyrosine kinase regulation
NF-κB activation
nucleus

Figure 11

CHAPTER 2 - Materials and Methods.

2.1 Cells, reagents and antibodies.

The murine B cell lymphoma, WEHI 231 was cultured in RPMI-1640 medium containing 5 % foetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (RPMI complete) at 37°C in 5 % CO_2. RPMI complete media for WEHI-231 B cells was additionally supplemented with 2-mercaptoethanol (50 μM). All cell culture reagents were obtained from GIBCO (Paisley, Scotland).

PD98059, H-89 (dihydrochloride), Ly 294002, L-a-Phosphatidylinositol-3,4,5-trisphosphate (Dialmitoyl-Heptaammonium Salt (PtdIns(3,4,5)P_3)), rolipram and propidium iodide were obtained from CALBIOCHEM (Cambridge, MA, USA). U0126 was obtained from PROMEGA (UK). SB230580 and N,N-Dimethyl-D-erythro-sphingosine (DMS) were obtained from ALEXIS BIOCHEMICALS. Cilostamide was obtained from ICN BIOCHEMICALS (Aurora, Ohio). Wortmannin and IBMX were obtained from SIGMA CHEMICAL COMPANY (Poole, Dorset, UK). [6-^{3}H]thymidine (5 Ci/mmol) and L-[3-^{3}H]serine (33.0 Ci/mmol) were obtained from AMERSHAM INTERNATIONAL (Buckinghamshire, UK). Carboxy-fluorescein diacetate succinimidyl ester (CFSE) was obtained from Molecular Probes (Leiden, Netherlands).

All other reagents were obtained from Sigma Chemical Company (Poole, Dorset, UK).

Phospho-specific p44/p42 MAPKinase (ERK-MAPKinase/pERK), p44/42 MAPKinase (wERK), phospho-specific SAPK/JNK (pJNK), SAPK/JNK (wJNK), phospho-specific p38 MAPKinase (pp38), p38 MAPKinase (wp38) and anti-rabbit immunoglobulin-horseradish peroxidase antibodies were obtained from NEW ENGLAND BIOLABS (Hitchin, HERTS. UK). Anti-p85a (PI(3)K) and anti-PAC-1 were obtained from SANTA CRUZ BIOTECHNOLOGY (Santa Cruz, CA, USA).

2.2 Purification of anti-CD40 antibody

Equal volumes of saturated ammonium sulphate solution and tissue culture supernatant (FGK 45) were mixed and the immunoglobulin fraction allowed to precipitate overnight at 4°C. The precipitated immunoglobulin was harvested by centrifugation at 10,000 rpm and then dialysed exhaustively in malonate buffer (per litre: malonic acid sodium salt (6.5g), malonic acid (1.2g), betaine (1g). A column consisting of a glass wool-stoppered 20ml syringe was then filled with 5ml S sepharose fast-flow. The antibody solution was loaded onto the column which was then washed

with malonate buffer until the eluant A_{280} reached a steady state (consistently about 0.05). The antibody was then eluted using the malonate buffer containing 0.5M NaCl in 1ml fractions, the profile of the elution was followed and the amount of protein estimated by measuring A_{280} (an OD of 1.4 being approximately equivalent to 1 mg/ml of protein).being followed using A_{280}. The antibody solution was then extensively dialysed against PBS, filter sterilised and stored at -20°C.

2.3 Purification of anti-Ig (anti-mouse μ-chain) antibody

Equal volumes of saturated ammonium sulphate solution and tissue culture supernatant (B.7.6) were mixed and the immunoglobulin fraction allowed to precipitate overnight at 4°C. The precipitated immunoglobulin was harvested by centrifugation at 10,000 rpm and then dialysed exhaustively in PBS. A column, consisting of a glass wool-stoppered Pasteur pipette containing 1 ml of or Protein G-sepharose (capacity: 35 mg/ml)) was prepared, and this was connected to a reservoir made from a 50 ml syringe. PBS (20 ml) was then run through the column to wash away the ethanol from the protein-G storage buffer. Tissue culture supernatant was then run through the column, and the flow-through retained. The column was washed with PBS until the level of eluted protein (as measured at A_{280}) had reached a steady state. The bound antibody was eluted using 1 ml aliquots of 0.2M acetic acid which were collected in vials containing an equal volume (100 μl) of 1M Tris buffer (pH 8.8). The elution was followed and the amount of protein estimated by measuring A_{280} (an OD of 1.4 being approximately equivalent to 1 mg/ml of protein). The appropriate fractions were then pooled and dialysed in PBS overnight, filtered sterilised and stored in 1 ml aliquots at 4°C or -20°C .

2.4 Measurement of DNA synthesis.

For measurement of DNA synthesis, WEHI 231 cells (10^4 cells/ well) were cultured in triplicate in round bottom microtitre plates in RPMI-1640 medium supplemented with glutamine (2 mM), sodium pyruvate (1 mM), 1% non-essential amino acids, 2-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 mg/ml) and 5% FCS, in the presence of the appropriate agonist in a total volume of 200μl. Cells were cultured at 37°C in a 5% (v/v) CO_2 atmosphere at 95% humidity for 48. [^{3}H]thymidine (0.5 μCi/ well) was added 4 hours prior to harvesting of the cells with an automated cell harvester (Skatron). Incorporated label was estimated by liquid scintillation counting and is represented as DPM ± SEM.

2.5 Cell Stimulation and Lysate Preparation

WEHI 231 cells (10^7 cells) were stimulated as required in RPMI-1640 medium supplemented with glutamine (2 mM), sodium pyruvate (1 mM), 1% non-essential amino acids, 2-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 mg/ml) and 5% FCS. Reactions were terminated by the addition of 2 x ice-cold modified RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM sodium chloride, 2% (v/v) NP 40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA) plus 10 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonylfluoride, chymostatin (10 μg/ml), leupeptin (10 μg/ml), antipain (10 μg/ml), and pepstatin A (10 μg/ml)) and lysates solubilised for 30 minutes on ice before centrifugation at 12, 000 rpm for 15 minutes. The resulting supernatants (whole cell lysate) were stored at -20°C before being used for immunoprecipitation or western blot analysis.

2.6 Immunoprecipitation

Whole cell lysates (100-300 μg total protein) were normalised to 1 μg/μl in RIPA buffer. Lysates were then precleared with 10 μl protein-G-sepharose (50% slurry) for 30 minutes at 4°C with constant rotation. Lysates were then centrifuged at 13,500 rpm for 5 minutes at 4°C and the supernatant transferred to fresh tubes. Proteins to be analysed were immunoprecipitated from precleared lysate by the addition of the appropriate antibody (1-2 μg). Samples were incubated overnight at 4°C with constant rotation. The immunocomplex was captured with 10 μl protein-G-sepharose (50% slurry) for 2 hours at 4°C with constant rotation. Lysates were then centrifuged at 13,500 rpm for 5 minutes at 4°C and the immunocomplex washed twice in ice-cold modified RIPA buffer. The beads were then resuspended in 30 μl 2 x loading buffer (100 mM Tris (pH 6.8), 4% (v/v) bromophenol blue, 0.2% SDS and 20% (v/v) glycerol) + 5% 2-Mercaptoethanol (2-Me), boiled for 5 minutes at 100°C and stored at -20°C or pulse centrifuged at 10,000 rpm and subjected to SDS-PAGE electrophoresis as described below.

2.7 SDS-Polyacrylamide Gel Electrophoresis and Western Blotting.

2.7.1 SDS-Polyacrylamide Gel Electrophoresis.

Gel apparatus and glass plates were cleaned with 70% ethanol and assembled according to manufacturers instructions.

Polyacrylamide gels were prepared as follows (enough for two gels):

10% Polyacrylamide running gel - 5.9 ml dH_20, 5.0 ml 30% acrylamide mix (BIO-RAD), 3.8 ml 1.5 M Tris (pH 8.8), 150 µl 10% SDS, 150 µl 10% ammonium persulphate and 6 µl TEMED.
5% Polyacrylamide stacking gel - 5.0 ml dH_20, 0.83 ml 30% acrylamide mix (BIO-RAD), 0.63 ml 1.0 M Tris (pH 6.8), 50 µl 10% SDS, 50 µl 10% ammonium persulphate and 5 µl TEMED.

The running gel was then poured into the gel apparatus, overlaid with 70% ethanol and allowed to set for approximately 30 minutes. The layer of ethanol was removed and the gel border washed with dH_20. The stacking gel was then poured, a well comb was inserted and the gel allowed to set for approximately 30 minutes. The comb was removed and the wells washed with dH_2O. The gel apparatus was then placed in an electrophoresis tank which was filled with electrophoresis buffer (25 mM Tris, 250 mM glycine and 0.1% SDS (w/v)).

Whole cell lysates (of known protein concentration) were denatured in the appropriate volume of 2x sample buffer + 5% 2-Me by heating for 5 min at 100°C. Samples were loaded into individual wells with molecular weight rainbow markers (10 µl) also loaded into one or two wells. The samples were electrophoresed into the stacking gel at 150V and then resolved in the separating gel at 200V in ice-cooled PAGE apparatus.

2.7.2 Transfer of resolved proteins to PVDF millipore membrane.

SDS-PAGE-resolved proteins were transferred to PVDF membrane using a Bio-Rad Transfer equipment. The gel was placed onto two sheets of transfer-buffer soaked filter paper within a transfer cassette and any air bubbles were removed with a buffer-wetted gloved finger. PVDF membrane (pre-activated in methanol for 30 seconds and then washed in transfer buffer for 5 minutes) was placed on the gel, and any air bubbles were removed. Two sheets of buffer-soaked filter paper were placed on top of the gel/ filter sandwich. The transfer cassette(s) was then placed in the transfer tank and subjected to 200 mA (constant current) for 3 hours or 50 mA (constant current) overnight.

2.7.3 Western Blot analysis of protein expression.

Following transfer, PVDF membranes were washed once in PBS/Tween-20 (0.1%) and blocked with PBS/Tween-20 (0.1%)/10% non-fat milk for 2 hours. Membranes were then incubated with the appropriate primary antibody overnight (4°C) or 2 hour at room temperature. All antibody solutions were diluted in PBS/Tween-20 (0.1%)/10% non-fat milk. Following primary antibody incubation, PVDF membranes were washed (3 x 15 minutes) with PBS/Tween-20 (0.1%). PVDF membranes were then incubated with the appropriate secondary antibody for 1 hour at room-temperature. PVDF membranes were then washed (5 x 10 minutes) with PBS/Tween-20 (0.1%) and protein bands visualised using the ECL system (Amersham International plc) on Kodak X-ray film (Kodak, UK).

2.8 Flow cytometry analysis of DNA content and cell cycle analysis.

WEHI-231 cells (approximately 5 x10^5) were harvested and washed once in PBS/2mM EDTA. Cells were resuspended in 200 µl ice-cold hypotonic buffer (0.1% (w/v) sodium(tri) citrate, 0.1% Triton X-100 (v/v) and 50 µg/ml propidium iodide (PI)) and incubated at 4°C for 10 minutes, and then at room temperature for 35 minutes. Cells were analysed for PI fluorescence at an excitation wavelength of 488nm on a BECTON DICKINSON FACSCalibar™ using Cell Quest for analysis.

Cell cycle analysis was used to determine the % of cells which were either apoptotic, in G_1 arrest, in S phase or in G_2/M of the cell cycle (**figure 12**). Briefly, FL-2 voltage settings were altered until the large G_1 peak (representing 2N DNA levels) sat between 100-200. A marker (40 units wide) was set 20 units either side of the peak. The 2N DNA peak was doubled (representing 4N DNA) and markers set 30 units either side of this number. Markers were also set below the 2N peak (representing sub-diploid DNA or apoptotic cells) and also between the 2N and 4N peaks, representing cells in the S phase (those with DNA levels between 2N and 4N).

In addition to PI staining, merocyanine 540 dye was also used as a indication of apoptosis. Briefly, 5 x 10^5 cells were stimulated as required. Cells were harvested and resuspended in 200 µl PBS + 40 µg/ml merocyanine 540. Cells were incubated in the dark for 30 minutes at room temperature and then analysed by for merocyanine fluorescence (FL-1) on a BECTON DICKINSON FACSCalibar™ using Cell Quest for analysis. Apoptotic cells were taken to be represented by the merocyanine high population which represent cells with increased membrane potential and permeability.

2.9 Cell proliferation by CFSE dye analysis.

WEHI-231 cells were washed in sterile PBS (approximately 1.25 x 10^7/ml). Cells were then incubated in 2.5 mM CFSE (diluted in PBS) at approximately 3.5 x 10^7 cells/ml for 10 minutes at 37°C in the dark. Cells were then washed twice in RPMI-1640 + 5% FCS. Cells were then stimulated as required (cell sample of approximately 10^4 cells was taken to determine the intensity of dye loaded into cells at time zero). After cell stimulation, cells were washed twice in FACS buffer (PBS/2% FCS), resuspended in FACS FLOW (approximately 3.5 x 107 cells/ml). At least 10^4 stained cells were analysed (FL-1) using a BECTON DICKINSON FACScan™ using Lysis II software for analysis. Data files were converted on CELL QUEST software and the number of cell divisions per stimulation determined as follows. Briefly, cells analysed at time zero were measured for dye intensity on a histogram which gave a reading of total dye uptake. Markers were set so that each marker range equalled half of the previous setting (eg. 8000, 4000, 2000, 1000 etc.) as each cell division results in daughter cells with half the dye intensity. The percentage of cells within each marker range for a particular stimulation was then determined.

2.10 PI(3)K assay.

WEHI-231 cells were washed in phosphate free RPMI-1640 and then resuspended (2 x 10^7/ml) in phosphate free RPMI-1640 containing 5% dialysed (against phosphate free RPMI-1640) FCS in the presence of 0.5mCi/ml [^{32}P]PO_4 (HCl free) for 90 minutes at 37°C. Following labelling, cells were washed in phosphate free RPMI-1640 and then stimulated in RPMI-1640 supplemented with glutamine (2 mM), 2-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 mg/ml) and 5% FCS. Cell stimulation over a 0-60 minute time course was initiated by the addition of 50 μl of stimulus (x 2 concentration) to glass vials containing cells (50 μl/5 x 10^6 total cells) and vials were incubated in at 37°C for the appropriate time. Cells stimulated over a longer time course were incubated at 5 x10^5/ml (5 x 10^6 total) with the appropriate stimuli. Reactions were terminated by the addition of 500 μl ice cold methanol, followed by 350 μl PBS and 500 μl chloroform (final ratio of 0.9:1:1 (water:methanol:chloroform)). In the case of longer time courses, cells were harvested and resuspended in 100 μl RPMI-1640 supplemented with glutamine (2 mM), 2-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 mg/ml) and 5% FCS prior to reaction termination as described above. After the termination of the reaction and lipid extraction, cell samples were vortexed and centrifuged at 1000 rpm for 5 minutes at 4°C to achieve phase separation.

Whatman silca gel (A-150) TLC plates were impregnated with potassium oxalate by developing in a TLC tank overnight with a solvent system containing 1.2% potassium oxalate in methanol/water (2:3 v/v). Plates were then air dried and activated by heating at 100°C for 15 min and allowed to cool on the bench. An aliquot (450 µl) of the lower chloroform phase was removed from each sample to a clean glass vial, spiked with unlabelled PtdIns(3,4,5)P_3 (2 µg) and dried by vacuum centrifugation. Each dried sample was redissolved in chloroform (30 µl) and applied to a pre-absorbent strip of the TLC plate along with a further wash of the vial. The plate was then developed in a paper-lined tank pre-equilibrated with the solvent chloroform:acetone:methanol:acetic acid:water (80:30:26:24:14 v/v/v/v/v) for approximately 15 hours. PtdIns(3,4,5)P_3 was located by exposure of the plate to iodine vapour and the radioactivity associated with each sample was then determined by PhosphoImager analysis (STORM) or scraping and liquid scintillation counting.

Although both analysis methods were shown to produce similar trends between replicates, Phosphor-Imager analysis, measured by band volume, was open to possible errors due to uneven back-ground levels between plates. Therefore, liquid scintillation spectrometry was used as a more accurate measure to quantify PtdIns(3,4,5)P_3 generation. **Figure 13** shows the Phosphor-Image of a TLC plate and in particular the separation of different lipid species following TLC including PtdIns(3,4,5)P_3.

2.11 Sphingosine-1-phosphate assay.

WEHI-231 cells were washed in phosphate free RPMI-1640 and then resuspended (2 x 10^6/ml) in phosphate free RPMI-1640 containing 5% dialysed (against Earles salts) FCS in the presence of 20µCi/ml [^{3}H]serine overnight (approximately 15 hours) at 37°C. Following labelling, cells were washed in ice-cold RPMI-1640, 10mM HEPES, 5% FCS and then stimulated in RPMI-1640 supplemented with glutamine (2 mM), 2-mercaptoethanol (50 µM), penicillin (100 U/ml), streptomycin (100 mg/ml) and 5% FCS plus 0.1 mM L-canaline and the pyridoxal phosphate analogue 4-deoxypyridoxine (0.5 mM) to inhibit the pyridoxine-dependent sphingosine-1-phosphate lyase. Cell simulation over a 0-60 minute time course was initiated by the addition of 50 µl of stimulus (x 2 concentration) to glass vials containing cells (50 µl/5 x 10^6 total cells or 1 x 10^7 total primary B lymphocytes) and vials were incubated in at 37°C for the appropriate time. Cells stimulated over a longer time course were incubated at 5 x10^5/ml (5 x 10^6 total) with the appropriate stimuli.

Reactions were terminated by the addition of 350 µl ice-cold HCl (0.1M), followed by 350 µl ice-cold methanol and 500 µl chloroform (final ratio of 0.9:1:1 (aqueous:methanol:chloroform)). In the case of longer time courses, cells were

harvested and resuspended in 100 µl RPMI-1640 supplemented with glutamine (2 mM), 2-mercaptoethanol (50 µM), penicillin (100 U/ml), streptomycin (100 mg/ml) and 5% FCS prior to termination of the reaction as described above. After the termination of the reaction and lipid extraction, cell samples were vortexed and centrifuged at 1000 rpm for 5 minutes at 4°C to achieve phase separation.

Whatman silca gel (G-60) TLC plates were pre-run in solvent (chloroform:methanol:acetic acid:water (90:90:15:6 v/v/v/v)) in a TLC tank for approximately 2 hours and plates were then air dried. An aliquot (450 µl) of the lower chloroform phase was removed from each sample to a clean glass vial, spiked with unlabelled SPP (1 µl of 10 mM stock) and dried by vacuum centrifugation. Each dried sample was redissolved in chloroform (30 µl) and applied to a pre-absorbent strip of the TLC plate along with a further wash of the vial. The plate was then developed in a paper-lined tank pre-equilibrated with the solvent mix: chloroform:methanol:acetic acid:water (90:90:15:6 v/v/v/v)) until the solvent front reached the top of the plate. SPP was located by exposure of the plate to iodine vapour and the radioactivity associated with each sample was then determined by scraping and liquid scintillation counting.

In order to examine the separation of lipid species under these conditions, a untreated cell sample was analysed by TLC under the above conditions along with lanes loaded with SPP standard. Lipids were visualised using iodine vapour which showed a band corresponding to the Rf value of SPP standards present in the cell sample. The whole lane was then scraped, in 0.5cm segments, to determine both the number and separation of lipid species (**figure 14**). These results indicated that SPP ran with an approximate Rf value of 0.7 with clear separation from other contaminating lipids so that SPP bands could be excised accurately and analysed.

2.12 Cytosolic Phospholipase A_2 assays

Cytosolic phospholipase A_2 ($cPLA_2$) activity in whole cell lysates or mitochondrial fractions was determined using a commercial $cPLA_2$ assay KIT (CAYMAN CHEMICAL COMPANY, Ann Arbor, MI) based on spectrophotometric detection (A_{414}) of free thiol by Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid); DTNB) following hydrolysis of the arachidonyl thioester bond at the *sn*-2 position of the $cPLA_2$ substrate, arachidonyl thio-phosphatidylcholine. A role for $iPLA_2$ activity was excluded by the use of the selective inhibitor bromoenol lactone and the requirement for calcium for PLA_2 activity.

In addition, in some experiments $cPLA_2$ activity was assessed by measurement of [^{3}H]arachidonic acid release as described previously [Gilbert, 1996 #2818]. This activity was blocked by the inhibitor arachidonyl trifluoromethyl ketone (selective for

iPLA$_2$ and cPLA$_2$) excluding a role for sPLA$_2$. Briefly, prior to each experiment, cells were washed, resuspended in fresh isotope-free medium and cultured for a further hour at 37°C. The cells were then washed three times in Hanks buffered saline, pH 7.4, containing 2% (w/v) bovine serum albumin and 10 mM glucose (HBG), resuspended in this buffer at 10^7 cells/ml and equilibrated for 30 minutes at 37°C. Cells (10^6/assay) were then stimulated with the appropriate agent for the indicated time at 37°C. Reactions were terminated by the addition of 1 ml ice-cold methanol/15 µl glacial acetic acid followed by a further 0.5 ml methanol and 0.75 ml chloroform and the cells extracted for 30 minutes on ice. Phases were split by the addition of chloroform and water and the chloroform phase was then dried under vacuum. For measurement of [^{3}H]arachidonate levels, 30 µl samples (prepared in chloroform;methanol (2:1)) were spotted onto Silica Gel 60 thin layer plates and developed in hexane: diethylether : formic acid (80: 20: 2, by volume). After exposure to iodine vapour, arachidonate was identified by comparison with standards, the plate was sprayed lightly with water, and the corresponding silica gel scraped from the plate and assayed for radioactivity.

2.13 Measurement of intracellular PGE_2.

Prostaglandin E_2 (PGE_2) activity in whole cell lysates or cell supernatant was determined using a PGE_2 competitive binding immunoassay assay KIT (R & D SYSTEMS EUROPE, Abingdon, UK) based on spectrophotometric detection (A_{405}) as described in kit protocol. Briefly, 50 µl whole cells lysate (protein levels standardised to 1 µg/µl) or 100 µl cell supernatant was added to 100 µl assay buffer, followed by 50 µl PGE2 conjugate (conjugated to alkaline phosphatase) and 50 µl PGE2 antibody solution. Samples and appropriate controls were incubated at room temperature for two hours. 200 µl pNPP substrate was added to all wells and samples incubated for one hour at room temperature. The optical density of each well was then determined using a microplate reader set to 405 nm with wavelength correction set at 570 and 590 nm.

2.14 Measurement of Ras activity.

Ras activity of cell samples was determined using a Ras Activation Assay Kit (UPSTATE BIOTECHNOLOGY, Lake Placid, NY) according to manufacturers protocol. Briefly, cells were stimulated as required (1 x 10^7/sample), harvested and lysed in 200 µl 5x Mg^{2+} lysis buffer. Cell lysates were diluted to approximately 1 µg/µl with 5x Mg^{2+} lysis buffer and precleared with 10 µl glutathione agarose for 30 minutes at 4°C with constant rotation. Samples were then centrifuged at 13,500rpm for

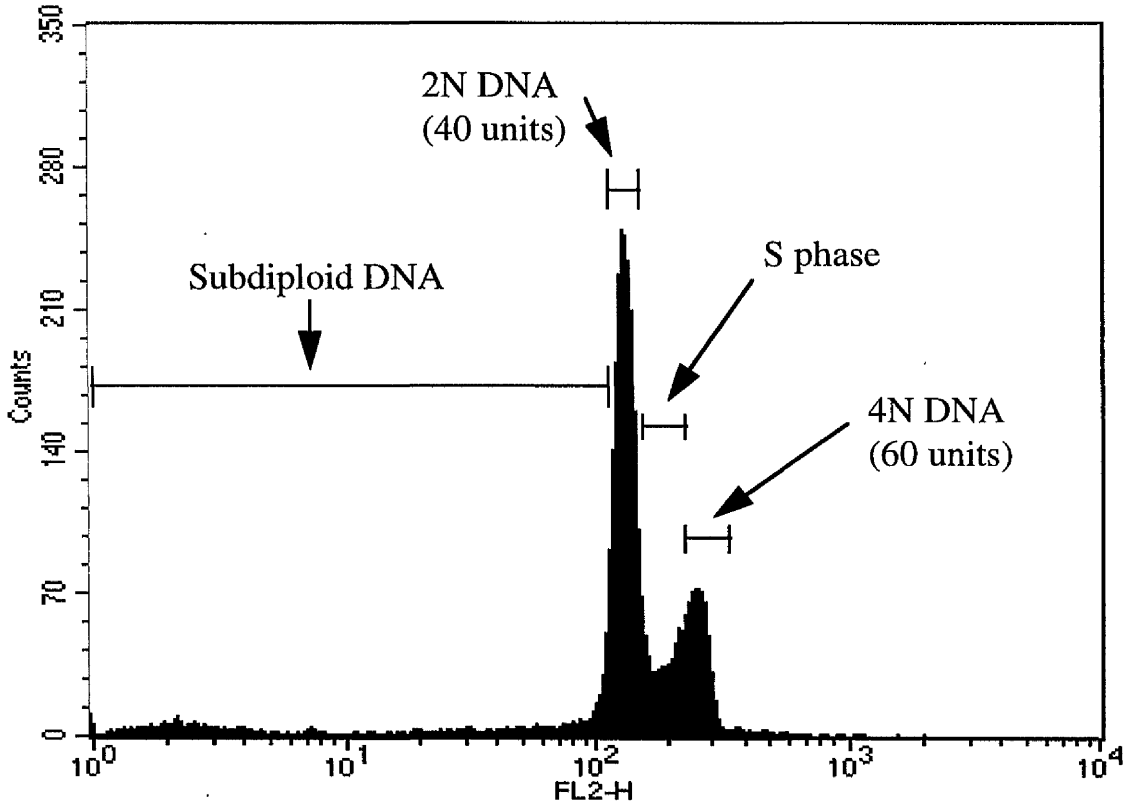

2N DNA
(40 units)
S phase
Subdiploid DNA
4N DNA
(60 units)
Counts
0
70
140
210
280
350
10^0
10^1
10^2
10^3
10^4
FL2-H

Figure 12

Figure 13. Lipid separation of PtdIns(3,4,5)P_3 by thin-layer chromatography (TLC). Lipids were extracted from WEHI-231 cells as described in materials and methods. Organic phase was dried under vacuum-centrifugation. Lipids were resuspended in $ChCl_3$ and applied to G40 Silica TLC plates. Samples were run as described in Materials and Methods and analysed on a Storm Phosphor-Imager (Molecular Dynamics) following a 4 hour exposure.

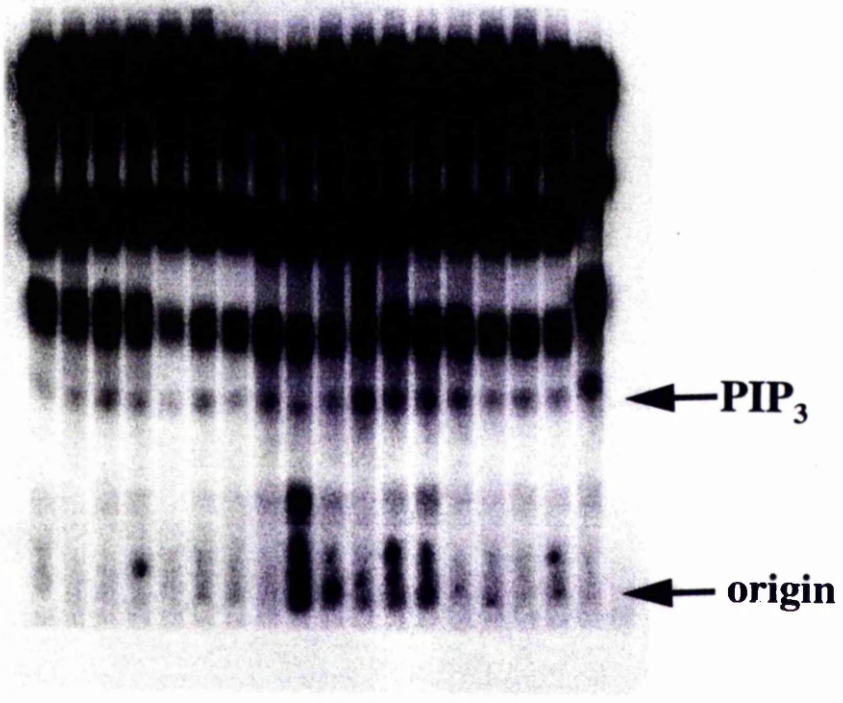


Figure 13

Figure 14. Separation by TLC of lipid fractions from [^{3}H]serine labelled cells clearly separates SPP from other lipid species. WEHI-231 cells ($1x10^6$/ml) were lysed and lipids extracted from the sample as described in materials and methods. Lipid samples were dried under vacuum-centrifugation and subject to TLC analysis. One complete lane was scraped in 0.5cm segments and [^{3}H]serine levels quantified by liquid scintillation counting. SPP standards were also run on adjacent lanes and location of SPP, visualised by iodine staining, is marked.

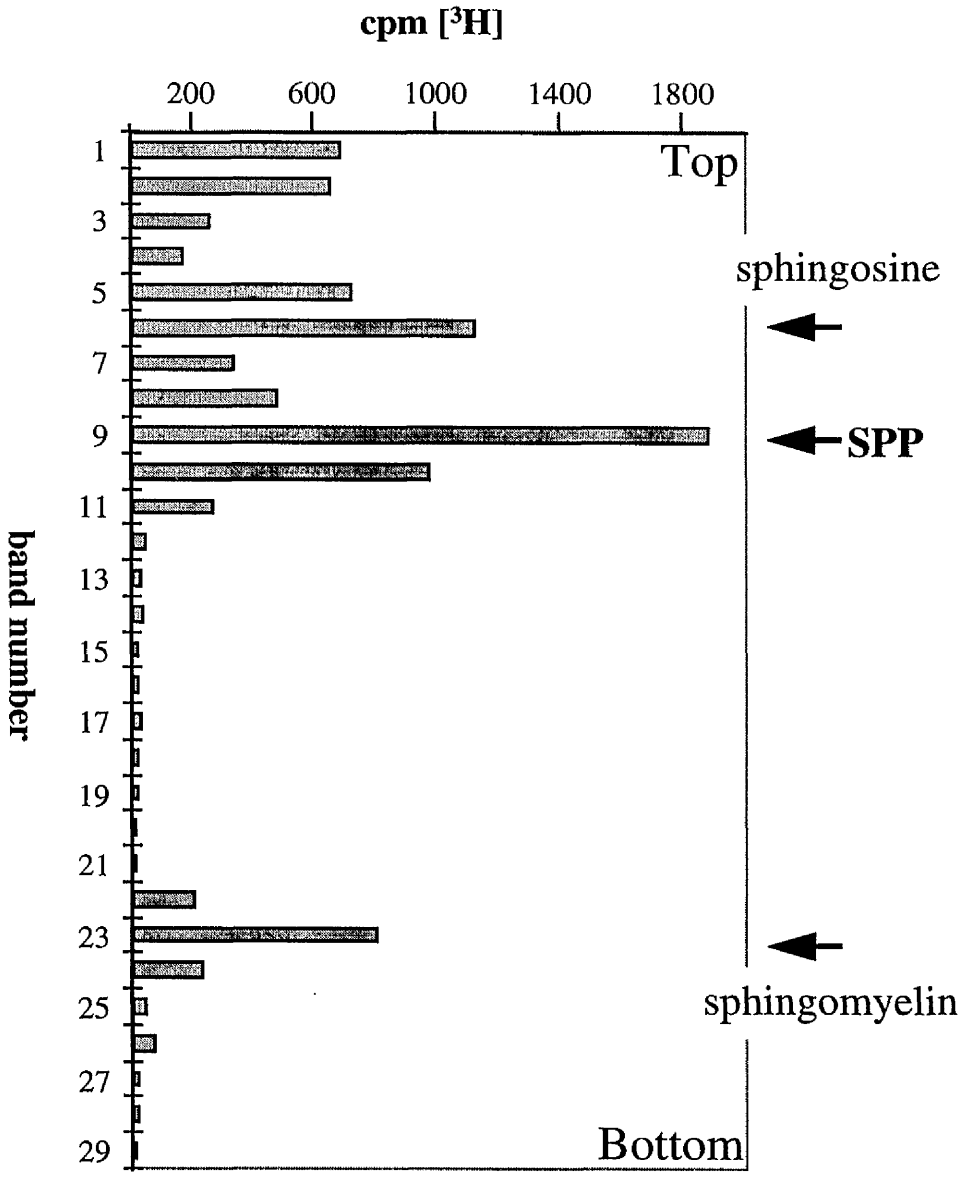

cpm [3H]
200
600
1000
1400
1800
band number
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
Top
sphingosine
SPP
sphingomyelin
Bottom

Figure 14

CHAPTER 3 - Characterisation of a model system of immature B lymphocyte apoptosis and repertoire selection.

3.1 Apoptosis of Immature B lymphocytes.

During the generation of a functional B lymphocyte repertoire it is necessary to regulate the maturation of immature B cells to prevent the emergence of cells that bind self-antigen and which are therefore potentially auto-reactive. This major check-point in B lymphocyte development occurs not only in the bone marrow, but also in the periphery in cells termed as 'transitional immature B lymphocytes' (8). These transitional B lymphocytes remain sensitive to deletion upon leaving the bone marrow as some self-antigens are only encountered when these cells exit the bone marrow into the periphery. Thus, in contrast to mature B lymphocytes which generally undergo a process of activation following antigen encounter, immature/transitional B lymphocytes undergo a process of negative selection which may involve deletion, anergy or replacement of the self-reactive receptor with a non-self reactive BCR. Additional factors such as the cells microenvironment or co-stimulation by IL-4 or CD40 are also believed to play important roles in determining cell fate, the latter suggesting that these cells are responsive to some form of T cell help (50). The ability of T cell-derived signals to rescue B lymphocytes from apoptosis is well documented. For example, many reports have described the role of anti-CD40 antibodies in the rescue of self-reactive immature B lymphocytes from deletion, mimicking the ability of T cell help to bypass tolerance induction in immature B lymphocytes (78). This rescue provides a mechanism for these cells to undergo further rounds of immunoglobulin gene rearrangement in the hope of generating a non-self BCR. In addition, a combination of signals delivered by BCR-ligation and CD40, presumably from CD40L expressed on T-cells, is also known to override apoptotic signals delivered by the BCR and maintain germinal centre B cell survival. The rescue from apoptosis by CD40 in germinal centres has been shown to be an essential process in the development of high affinity antibody producing memory B cells (73).

Antigen-induced apoptosis in both immature thymocytes and mature T lymphocytes is thought to involve Fas:FasL interactions (79) with TCR ligation inducing the upregulation of Fas and FasL surface expression and cell death being initiated with FasL interacting with Fas in an autocrine manner. However, Fas-FasL induced cell death appears to be more important at the mature T cells stage as mutations to Fas lead to the uncontrolled proliferation of mature T cells but does not affect early T cell development (80). In addition, mature B lymphocytes are also susceptible to Fas-mediated apoptosis, which is believed to be mediated by FasL-expressing T -cells. The apoptotic process activated in immature B lymphocytes following BCR ligation has

been suggested to be Fas-independent. Evidence for this (80) comes from studies showing FasL is expressed at low levels on the WEHI-231 cell line, which is widely used in the study of negative selection of B lymphocytes, and that these cells do not appear to upregulate FasL mRNA when activated via their BCR.

Over the years much progress has been made in determining the mechanisms of BCR-induced cell death in immature B lymphocytes. However, early studies using primary cell-cultures proved difficult to work with. This was because murine primary immature B cells are relatively difficult to purify in large numbers because of low yields per animal, but also because these cells are largely resistant to long-term culture *in vitro* (81). The introduction of immortalised cell-lines has helped resolve this problem. In particular, the study of BCR-induced cell death in immature primary B lymphocytes has been helped to a large extent by the murine B lymphoma cell line, WEHI-231 which has proved a popular model for the study of the biochemical and molecular mechanisms underlying immature B lymphocyte cell death.

3.2 The WEHI-231 B cell lymphoma: a cell line model for immature B cell selection.

The murine B cell lymphoma cell line WEHI-231 is used as a model for immature B lymphocyte clonal deletion because it has a cell surface phenotype of an immature B lymphocyte (mIgM^{+}, mIgD$^{-\,(\text{or low})}$, FcR^{low}, Fas^{low} and MHC class II^{low}) and undergoes growth arrest and apoptosis following BCR ligation (54, 82, 83). In addition, co-ligation of CD40 can rescue these cells from apoptosis, although the biochemical and molecular mechanisms responsible for this are still unclear.

BCR-induced apoptosis in the WEHI-231 cell line has been shown to include the regulation of the c-myc oncogene, in addition to p53 and its transcriptional target gene p21$^{WAF1/CIP1}$, a cyclin-dependent kinase (CDK) inhibitor. Overexpression of c-myc is known to induce apoptosis in certain cell types. However, studies have shown that BCR-mediated apoptosis in WEHI-231 cells correlates with a decrease in c-myc mRNA and protein expression (84). This decline has been shown to result from decreases in the binding of NF-κB/rel to DNA, activity vital for maintaining c-myc levels and the prevention of cell death (23). Indeed, the transcription factor NF-κB has been shown to be important for B lymphocyte activation and development, and inhibition of NF-κB activity with protease inhibitors, which prevents receptor mediated breakdown of IκB, has been shown to induce apoptosis in a number of B lymphocyte cell lines (85). Other studies have shown that anti-CD40 stimulation is able to rescue WEHI-231 cells from apoptosis by inducing NF-κB activity, thereby maintaining c-

myc RNA levels. This provides further evidence for the role of this transcription factor in regulating the fate of immature B lymphocytes (84).

Studies from the same group have also highlighted a role for the tumour-suppressor gene p53 in WEHI-231 cell death. p53 protein is known to play important roles in G_1 to S-phase cell-cycle progression. Wu *et. al.* showed that anti-Ig treatment of WEHI-231 cells increased protein expression of both p53 and $p21^{WAF1/CIP1}$ (23). Interestingly, $p21^{WAF1/CIP1}$ is known to be a downstream transcriptional target for p53 and anti-Ig mediated increases in $p21^{WAF1/CIP1}$ protein levels were delayed relative to p53 increases. As p21 protein is known to convert active cyclin-dependent kinases into their inactive forms, increases in p21 protein levels following anti-Ig treatment may play an important role in the induction of growth-arrest in WEHI-231 cells.

Other effectors implicated in BCR mediated apoptosis in WEHI-231 cells include the caspases. Although the role of caspases in the negative selection of immature B lymphocytes has not been widely studied, a recent report indicates the role of caspase-7, but not caspase 2 or 3, in the induction of apoptosis in WEHI-231 cells following BCR-ligation (86). Interestingly, this report also suggested that BCR-mediated growth arrest was caspase-7 independent, which indicated that BCR-mediated growth arrest and apoptosis may be regulated by different biochemical pathways.

3.3 CD40.

Following antigen encounter, not all immature B lymphocytes undergo apoptosis. Consequently, some cells undergo receptor editing whilst some enter an anergic state. This may reflect the influence of additional, external, signals. One such signal that has been shown to play an important role in immature B lymphocyte signalling is that via CD40, which has been implicated in the rescue of these cells from apoptosis in the periphery due to T-cell dependent interactions. Therefore, the ability of immature B cells to respond to T-cell derived signals may induce the recruitment of antigen-reactive immature B cells into an ongoing immune response where T-cell help is already triggered. Thus, if only antigen triggered T cells are recruited into this scenario, and the interaction between immature B cell and T cell are antigen dependent, then autoimmune reactions should not develop. This risk is further reduced because T cell selection is much more vigorously regulated in T cells than in B cells. Therefore the chance of a self-reactive T cell recruiting and driving the proliferation of immature B cells is very slim.

Although the ability of CD40 to rescue both immature B lymphocytes from BCR-mediated apoptosis (8, 83, 87) and prevent the spontaneous death of germinal-centre cells is well documented the signalling pathways employed by CD40 ligation in these situations are not.

One group of molecules that has attracted attention in this field is the Bcl family of proteins. In particular, studies have highlighted the role of the anti-apoptotic protein Bcl-x_L. Studies by Choi *et. al.* (83) showed the ability of CD40 ligation to up-regulate the expression of Bcl-x_L protein in the WEHI-231 cell line within just 6 hours (also reviewed by Craxton *et. al.* (17)). In addition to new protein production (Bcl-x_L), anti-CD40 mediated rescue from BCR-induced growth-arrest/apoptosis has been shown to overcome other BCR-induced signalling events. For example, anti-CD40 treatment prevents BCR-mediated increases in the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (Craxton *et. al.*(17)) and subsequent decrease in Cdk2 activity which is required for cell cycle progression in the late G_1 phase.

3.4 Aims of project.

The aims of this research project are to further elucidate the signalling pathways activated in immature B lymphocyte apoptosis. In addition, further studies aim to identify proximal signalling pathways activated by CD40 ligation and how these prevent BCR-mediated apoptosis.

This chapter aims to characterise the biology of BCR-induced growth-arrest and apoptosis in the WEHI-231 cell line model. In particular, it aims to address:

1. the kinetics of BCR-mediated signalling events and the ability of co-treatment with anti-CD40 to overcome these inhibitory signals.
2. the morphological events associated with the induction of growth-arrest/apoptosis including cell size and granularity and how co-stimulation with anti-CD40 can modulate these events.

3.5 Results.

3.5.1 Ligation of the BCR on WEHI-231 cells induces growth-arrest.

Treatment of WEHI-231 immature B lymphocytes with Fab_2' fragments of anti-Ig antibodies (anti-Ig) is known to inhibit DNA synthesis and induce apoptosis (88). As shown in **figure 15A**, treatment of WEHI-231 cells with anti-Ig causes a dose-dependent increase in growth arrest, with a 50% decrease in DNA synthesis occurring at a concentration between 0.01 and 0.1 μg/ml. In addition, co-stimulation of WEHI-231 cells with anti-CD40 is known to reverse anti-Ig mediated growth arrest (83). Therefore, **figure 15A** also shows that co-stimulation with anti-CD40 restores DNA synthesis levels, measured by thymidine uptake, back to basal levels. **Figure 15B** shows that anti-CD40 co-stimulation rescues WEHI-231 cells from anti-Ig mediated growth arrest in a dose-dependent manner, with maximal effects observed at a concentration of 10 μg/ml.

3.5.2 Fas-mediated growth-arrest and apoptosis.

In addition to examining the effect of BCR ligation on DNA synthesis, the susceptibility of WEHI-231 cells to other agents was also examined. Both TNF-α and Fas are known to induce growth-arrest and apoptosis in a wide range of cells types including human cancer cell lines (HeLa) or T lymphocytes (Jurkat). Previous studies have shown splenic B lymphocytes and immature B lymphoma cells lines to be resistant to anti-Fas induced apoptosis (80), yet the former is sensitive to T-cell mediated FasL-induced death. In addition, recent reports (89) have suggested that transitional B lymphocytes may be susceptible to Fas-mediated cell death subsequent to signalling via the BCR. We therefore investigated the effect of anti-Fas stimulation, or TNF-α treatment, to examine whether WEHI-231 cells may actually represent a late-immature or transitional B lymphocyte phenotype. The results obtained (**figure 16A**) confirm that the growth-arrest inducing pathway activated by Fas:FasL interactions in many T lymphocytes is not functional in WEHI-231 cells. Indeed, treatment of WEHI-231 cells with anti-Fas antibodies appeared to induce a slight proliferative response relative to control cells. Treatment with recombinant TNF-α caused no change in DNA synthesis levels relative to control cells indicating that TNF-α plays no role in WEHI-231 growth-arrest mechanisms, results consistent with data from Scott *et. al.* (80). However, anti-Fas treatment does induce an apoptotic response in WEHI-231 cells (**figure 16B**) suggesting that WEHI-231 express the necessary machinery required for Fas-mediated cell death. Although the ability of Fas to induce apoptosis, but not

growth arrest would appear contradictory, these results may reflect that anti-Fas stimulation only results in the death of a particular population of WEHI-231 cells. This may mimic the ability of Fas to only induce apoptosis in cells that have been "primed" for Fas-mediated cell death. Indeed, Fas stimulation of "un-primed" may be proliferative in nature. Therefore the slight increase in proliferation of anti-Fas treated cells, compared to control cells, may result from the balance between apoptosis and the induction of proliferative signals.

3.5.3 Kinetics of anti-Ig-mediated growth arrest and CD40-mediated rescue.

As previous studies (8) have suggested that the negative signals generated following BCR-ligation in immature B lymphocytes progress in an ordered kinetic manner, we decided to examine the kinetics of both growth-arrest induction and rescue from this by co-stimulation with anti-CD40. Whilst cells stimulated with anti-Ig for the whole 48 hours showed, as expected, almost complete growth arrest, those stimulated for only the last 24 hours also showed high (approximately 70%) levels of growth arrest (**figure 17A**). Interestingly, stimulation of cells with anti-Ig for the last 1- 6 hours of culture was sufficient to cause some 30% reduction in DNA synthesis. These results indicate that early BCR-mediated signals are sufficient to induce a substantial commitment to growth arrest which are confirmed/enhanced by one or more additional late (6-24 hours) signals mediated by BCR ligation.

Interestingly, the kinetic pattern of these results correlate with recent findings within our group (Katz *et. al.* Journal of Immunology - in press). These studies show that BCR-ligation of WEHI-231 cells promotes the induction of an early (within 5 hours), but substantial, decrease in mitochondrial transmembrane potential, $\Delta\psi_m$, followed by a profound dissipation of $\Delta\psi_m$ which was maximal by 20-24 hours. In contrast, mature splenic B cells, which proliferate rather than undergo apoptosis following BCR-ligation, did not exhibit this decrease in $\Delta\psi_m$ within 5 or 24 hours. In addition, BCR-ligation was also shown to induce a decrease in cellular ATP which occurred after the onset of mitochondrial membrane disruption suggesting that ATP depletion resulting from mitochondrial disruption may play a role in BCR-mediated growth arrest/apoptosis in WEHI-231 cells.

In addition, and consistent with other findings (87) we found that anti-CD40 could mediate strong rescue signals even when added up to 8 hours post anti-Ig ligation, suggesting that CD40-ligation can effectively rescue cells that have already initiated growth-arrest promoting signals (**figure 17B**). Interestingly, further findings within our group (Katz *et. al.* Journal of Immunology - in press) have shown that

costimulation with anti-CD40 acts to stabilise $\Delta\psi_m$ (albeit at an intermediate $\Delta\psi_m$) and prevent the profound dissipation of $\Delta\psi_m$ observed in anti-Ig-treated cells over a 24 hour period. These results suggest that BCR-mediated decreases in mitochondrial transmembrane potential may be reversible and can be stabilised by costimulation with anti-CD40.

3.5.4 Ligation of the BCR in WEHI-231 cells induces apoptosis.

The above experiments (**figures 15A and 17A**) confirmed that BCR-ligation induced growth-arrest in WEHI-231 cells, a process that could be reversed by co-stimulation with anti-CD40. However, these studies give no indication as to the levels of apoptosis induced by these agents. We therefore measured apoptosis by determining the number of cells within a certain population that expressed sub-diploid DNA measured by FACS analysis using the DNA-binding dye propidium iodide (PI). **Figure 18** shows the effect of BCR-ligation and also co-stimulation with anti-CD40 on WEHI-231 cells. The results confirm the ability of WEHI-231 cells to readily undergo apoptosis following BCR-ligation. This phenomenon was also shown to be dose-dependent with 10 μg/ml anti-Ig inducing maximal apoptosis. In addition, they also confirm the ability of co-treatment with anti-CD40 antibodies to reverse anti-Ig mediated apoptosis (**figure 18**). Cells were also treated with a combination of anti-Ig plus the glucocorticoid, dexamethasone (**figure 18**), which is known to induce a strong apoptotic response as a positive control for the assay.

The kinetics of induction of apoptosis in WEHI-231 cells following BCR-ligation has been shown in a number of reports. Studies by Bras *et. al.* (86) have shown that 24 hours post anti-Ig treatment WEHI-231 cells enter a state of growth arrest with increased numbers of cells in G_0/G_1. However, by 48 hours, a large proportion (approximately 30%) of the cells are apoptotic. These results are similar to findings in our own group (Caroline Lord; unpublished observations) and indicate that the induction of apoptosis in WEHI-231 may require prior G_1 arrest.

3.5.5 Cell Division Analysis of WEHI-231 cells.

To investigate whether co-stimulation of anti-Ig treated cells with anti-CD40 treatment of WEHI-231 cells restores basal proliferation or promotes increased cell proliferation of a subset of WEHI-231 cells, we quantified the number of cell divisions under-taken by cells following stimulation by use of the cell-permeable dye CFSE. Initial observations indicated that cell cycle progression, measured by the number of total cell divisions relative to untreated/basal (control) cells, varied according to cell

treatment (**figure 19**). Compared to control cells, anti-Ig stimulation was shown to increase the number of cells entering four or five rounds of cell division whilst blocking those entering a sixth. In comparison, and in agreement with previous data (**figure 15A**), cell division analysis indicated that anti-CD40 co-stimulation could release anti-Ig treated cells from growth arrest restoring levels of cell division to that of untreated (basal) cells.

3.5.6 Rescue from BCR-induced apoptosis in WEHI-231 cells by anti-CD40 co-stimulation is accompanied by morphological changes in the cell population.

Morphological changes associated with the induction of DNA synthesis and apoptosis were also examined. Untreated (control) WEHI-231 cells grow, in culture, in close proximity to each other, forming either a loose monolayer on the flask/plate floor (**figure 20A**) or as large clumps of cells in suspension in the media. However, ligation of the BCR, causes clear morphological changes in the cell population with some cells forming small, distinct clumps whilst others clearly undergo apoptosis and its associated morphological events such as membrane blebbing. In stark contrast, co-treatment of anti-Ig treated cells with anti-CD40 causes substantial morphological changes in the cell population. Associated with both the rescue from growth-arrest (**figure 15A**) and apoptosis (**figure 18**) is a large increase in cell-cell contact, with the formation of large near spherical clumps of cells.

In addition to gross morphological changes to the whole cell population, BCR-ligation and/or co-stimulation resulted in changes in cell size at the single cell level. The induction of apoptosis by BCR-ligation was shown to correlate with a reduction in cell size. **Figure 20B** shows the effect of BCR-ligation on cell size (FCS) and granularity (SSC) measured by FACs analysis. Untreated cells can be divided into two populations, a substantial population of large, relatively non-granular cells and a lesser population of smaller, yet equally granular cells. However, upon BCR-ligation, the balance of these populations shifts towards the smaller sized cells, whilst anti-CD40 co-stimulation restores the balance back to the population of cells that are large in size.

3.6 Discussion.

The results obtained in this chapter not only confirm the WEHI-231 cell line to be a viable model for the study of both BCR-mediated growth-arrest and apoptosis but also as a model for the examination of CD40-mediated rescue from growth-arrest and apoptosis. How CD40 functions *in vivo* in the protection of immature B lymphocytes remains unclear. CD40 has similarly been shown to play an important role in the prevention of spontaneous apoptosis of isolated germinal centre B cells (78), which may play important roles in the development of high-affinity antibody generating memory B cells. The role of CD40 in re-directing immature B cell fate, like that of GC B cells, is likely to occur in the periphery and not the bone marrow. Indeed, it has been suggested that CD40 expressed on activated T cells rescues the deletion of antigen-reactive cells re-programming them towards a proliferative response. This would have the effect of recruiting antigen-specific immature B cells into a immune response where T cell help already exists (8).

In addition to reversing anti-Ig mediated death signals, the results in this section suggest that anti-CD40 stimulation promotes cell-cell contact resulting in cell clumping. These observations are similar to another study in which anti-CD40 treatment resulted in cellular clumping in addition to rescue from apoptosis. Results from Sumimoto *et. al.* (90) using the human B lymphoma cell line, DND-39, described an associated increase in cell aggregation during rescue from BCR-mediated apoptosis by anti-CD40 antibody co-stimulation. These increases in cell aggregation were thought to be due to the ability of anti-CD40 antibodies to activate the adhesive functions of LFA-1/ICAM-1 and disruption of cell adhesion by co-treatment with anti-LFA-1 and anti-ICAM-1 antibodies prevented the rescue of cells from apoptosis. Whether CD40-induced increases in cell-cell contact are responsible for mediating some of anti-CD40's anti-apoptotic properties in WEHI-231 cells is still unclear.

We have also shown that the kinetic length of antigen exposure can determine the level of commitment into growth arrest. Consequently, we have also shown that where we appear to get a final commitment of cells into growth arrest, then this appears to be the final time point where these cells can be effectively rescued by anti-CD40 treatment (**figures 17A and 17B**). The progression of anti-Ig stimulated immature B lymphocytes through a series of "temporal windows" resulting, finally, in the induction of apoptosis has also been described in other reports (8). This orderly progression through initiation of an apoptotic programme, followed by a delay in which the fate of the cell can be re-directed is believed to allow external signals such as those delivered through CD40 to redirect the fate of the cell. Additionally, this "temporal window" is believed to allow self-reactive immature B lymphocytes time to initiate receptor editing

in order for them to alter their receptor specificity and re-enter the B lymphocyte pool before further maturation. However, failure to successfully rearrange their BCR, or receive external help, will ultimately result in deletion of these cells from the B lymphocyte pool. The ability of WEHI-231 cells to develop through similar "temporal windows" in which short term BCR-ligation initiates a negative signal, which, if no additional signal is forth-coming (anti-CD40), ultimately leads to cell death confirm that this cell line represents a good model for the study of immature B lymphocyte deletion or activation. Dissection of the biochemical and molecular events at these stages would allow for a greater understanding of immature B cells biology and the development of immunocompetent mature B lymphocytes in the periphery.

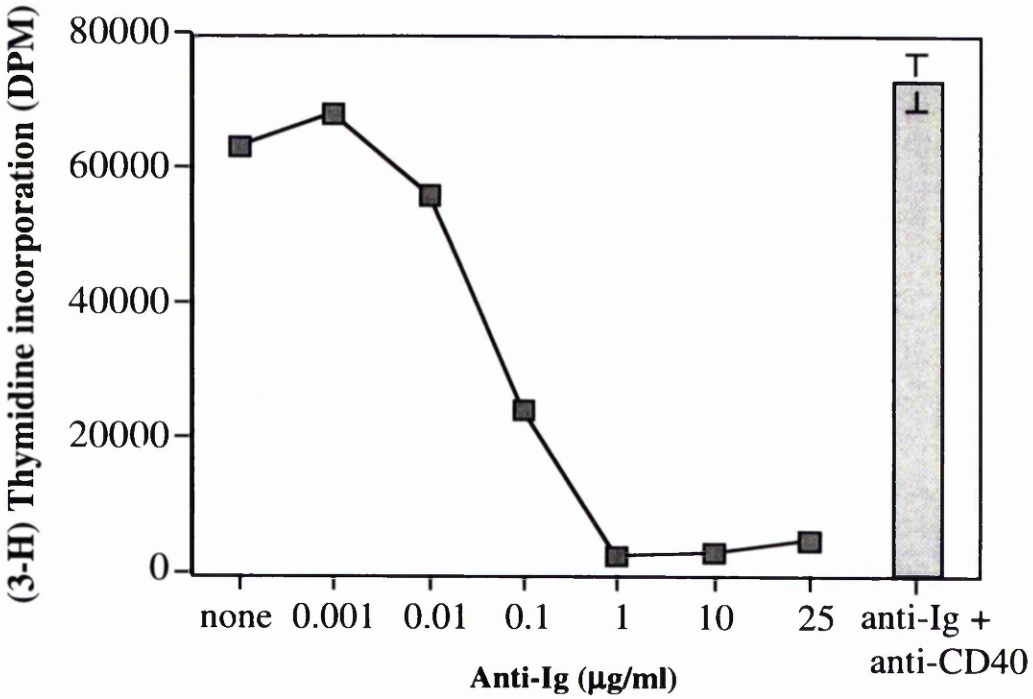
As previous results have suggested, the kinetics of commitment to growth-arrest correlate with decreases in the mitochondrial membrane potential observed in WEHI-231 cells following BCR-ligation. Previous studies (53) have suggested a role for cytosolic PLA_2 in the induction of apoptosis in WEHI-231 cells. Interestingly, recent work in our group (Katz *et. al.* Journal of Immunology - in press) has shown the collapse of $\Delta\psi_m$ can be mimicked by addition of exogenous arachidonic acid (AA), the product of cytosolic PLA_2 activity. These results suggest that BCR-mediated induction of cytosolic PLA_2 activity and generation of arachidonic acid may play a key role in the collapse of $\Delta\psi_m$ and commitment to apoptosis in WEHI-231 immature B cells. Indeed, these studies also indicated that BCR-ligation induced the translocation of cytosolic PLA_2 to the mitochondria which was reversed with co-stimulation with anti-CD40.

Therefore, the observation that even short exposures to anti-Ig are capable of inducing moderate levels of growth-arrest (**figure 17A**) may reflect the ability of BCR-ligation to induce a rapid (1-5 hours) decrease in $\Delta\psi_m$ via cytosolic PLA_2 activation. Furthermore, longer incubations (8-24 hour) incubations with anti-Ig (**figure 17A**), which commit the majority of cells to growth-arrest, were seen to correlate with a later, more pronounced decrease in $\Delta\psi_m$ (Katz *et. al.* Journal of Immunology - in press) and leakage of ATP suggesting that the greater the decrease in $\Delta\psi_m$, the greater the level of commitment to growth-arrest/apoptosis. The ability of anti-CD40 co-stimulation to rescue WEHI-231 cells from BCR-induced growth-arrest up to approximately 8 hours post anti-Ig treatment (**figure 17B**) suggests that the early decrease in $\Delta\psi_m$ mediated by BCR-ligation is reversible and is stabilised by anti-CD40 co-stimulation. However, if the $\Delta\psi_m$ is allowed to drop below a critical threshold before CD40 co-stimulation is received, then the ability to stabilise $\Delta\psi_m$ and restore depleted ATP levels may be lost, and hence account for the inability of late (>8 hour) anti-CD40 additions to rescue anti-Ig treated cells from growth arrest. The signalling pathways utilised by CD40 under these conditions are not known and warrant further investigation.

As described above, studies within our group have suggested a key role for cytosolic PLA_2 in mediating BCR-induced death signals in WEHI-231 cells. However, the signalling cascades utilised by the BCR in the induction of cytosolic PLA_2 activity and hence cell death are still unknown. Activation of cytosolic PLA_2 is known to require upstream input from calcium and also MAPkinase activity. Therefore, we have investigated the ability of BCR-ligation to modulate MAPkinase activity in order to determine if MAPkinase signalling in immature B lymphocyte plays a role in the induction of death signals.

Figure 15. Anti-Ig treatment causes growth arrest in WEHI-231 cells. **A**. WEHI-231 cells (10^4/well) were cultured in the presence of various concentrations of anti-Ig, or, for the combination of both anti-Ig and anti-CD40 (both at 10 μg/ml). Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values are the means ± SD of quadruplicate wells. **B.** WEHI-231 cells (10^4/well) were cultured in the presence of anti-Ig (10 μg/ml) with or without increasing doses of anti-CD40 (0.1-10 μg/ml). Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values are the means ± SD of quadruplicate wells.

(A)


(3-H) Thymidine incorporation (DPM)
80000
60000
40000
20000
0
none
0.001
0.01
0.1
1
10
25
anti-Ig + anti-CD40
Anti-Ig (µg/ml)

(B)

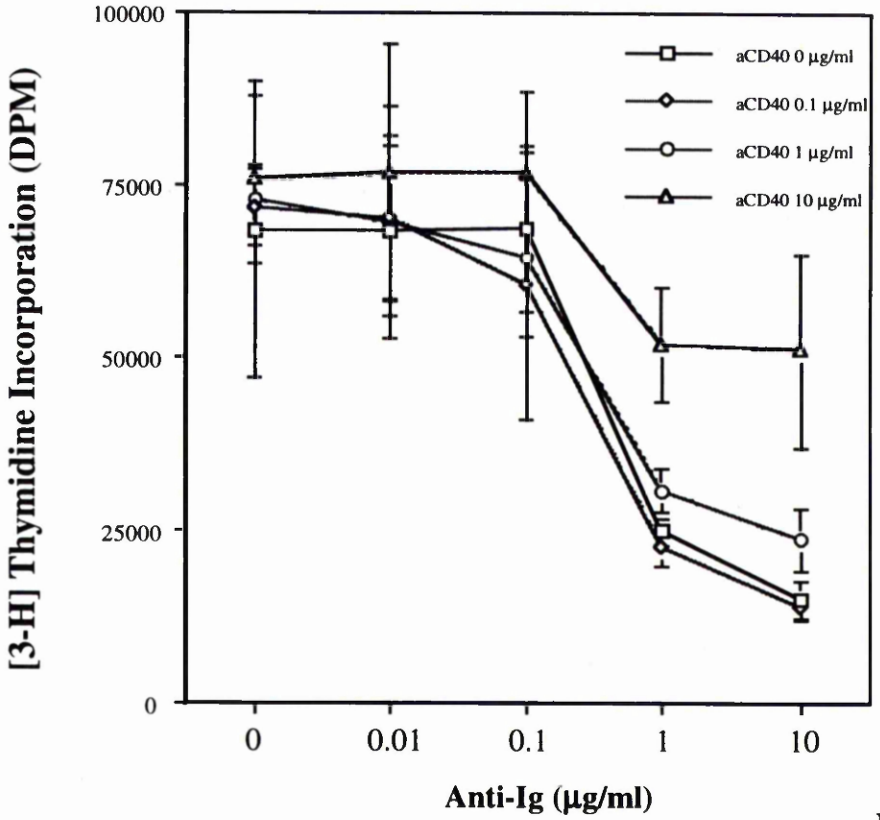
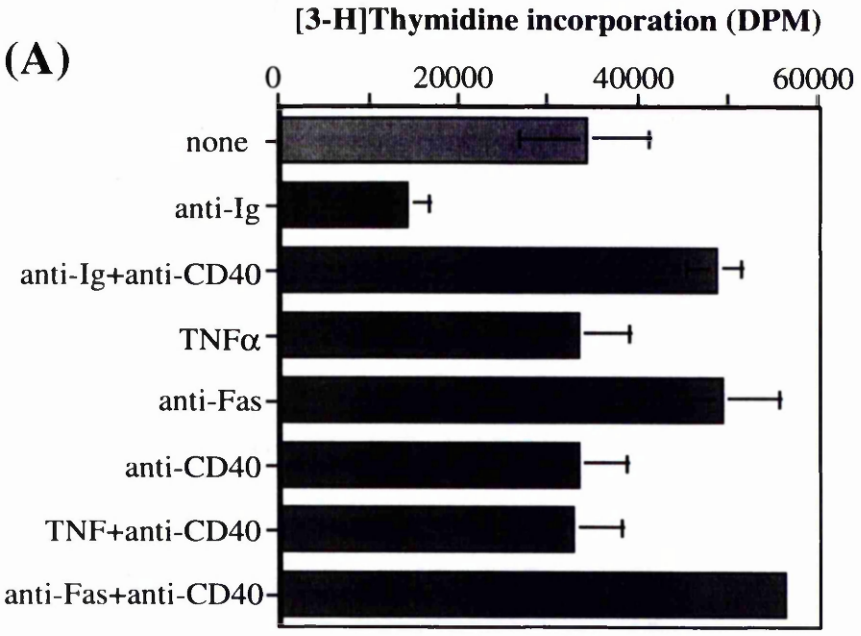

[3-H] Thymidine Incorporation (DPM)
100000
75000
50000
25000
0
aCD40 0 µg/ml
aCD40 0.1 µg/ml
aCD40 1 µg/ml
aCD40 10 µg/ml
0
0.01
0.1
1
10
Anti-Ig (µg/ml)

Figure 15

Figure 16. Neither Fas or TNF-α induce growth arrest in WEHI-231 cells.

A. WEHI-231 cells (10^4/well) were cultured in the presence or absence of anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml), anti-Fas (1 μg/ml) or TNF-α (1 ng/ml). Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values are the means ± SD of quadruplicate wells. **B.** WEHI-231 ($5x10^5$/ml) were cultured in the presence or absence of anti-Ig (1 μg/ml) or anti-Fas (1 μg/ml). Levels of apoptosis were determined by merocyanine binding and FACS analysis after 48 hours as described in Materials and Methods.


(A)
[3-H]Thymidine incorporation (DPM)
0
20000
40000
60000
none
anti-Ig
anti-Ig+anti-CD40
TNFα
anti-Fas
anti-CD40
TNF+anti-CD40
anti-Fas+anti-CD40

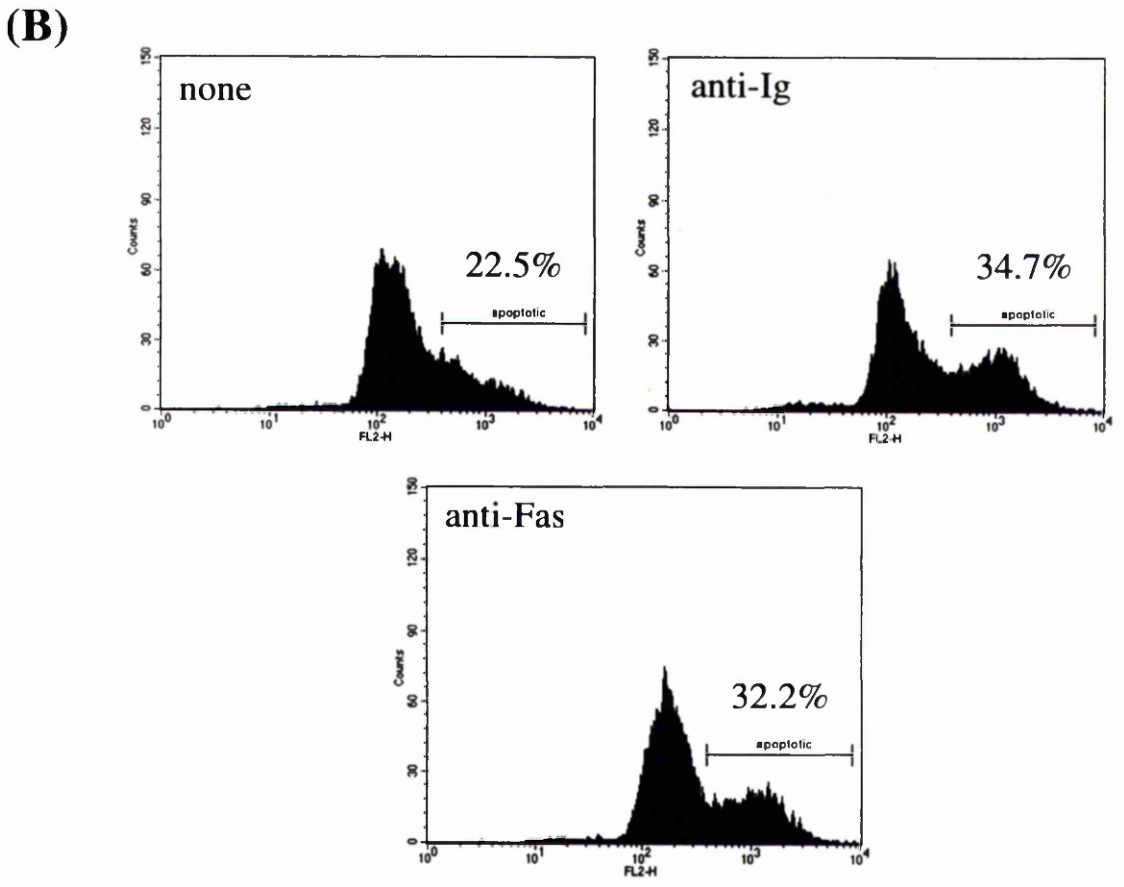

(B)
none
22.5%
apoptotic
anti-Ig
34.7%
apoptotic
anti-Fas
32.2%
apoptotic
Counts
FL2-H

Figure 16

Figure 17. Kinetics of anti-Ig mediated growth arrest and CD40-mediated rescue. **A**, WEHI-231 cells (10^4/well) were cultured for the indicated time periods in the presence of 10 μg/ml anti-Ig. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are means ± SD of quadruplicate wells. **B**, WEHI-231 cells (10^4/well) were incubated with anti-Ig (10μg/ml). anti-CD40 (10μg/ml) was added at the indicated time periods post anti-Ig treatment. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are means ± SD of quadruplicate wells.

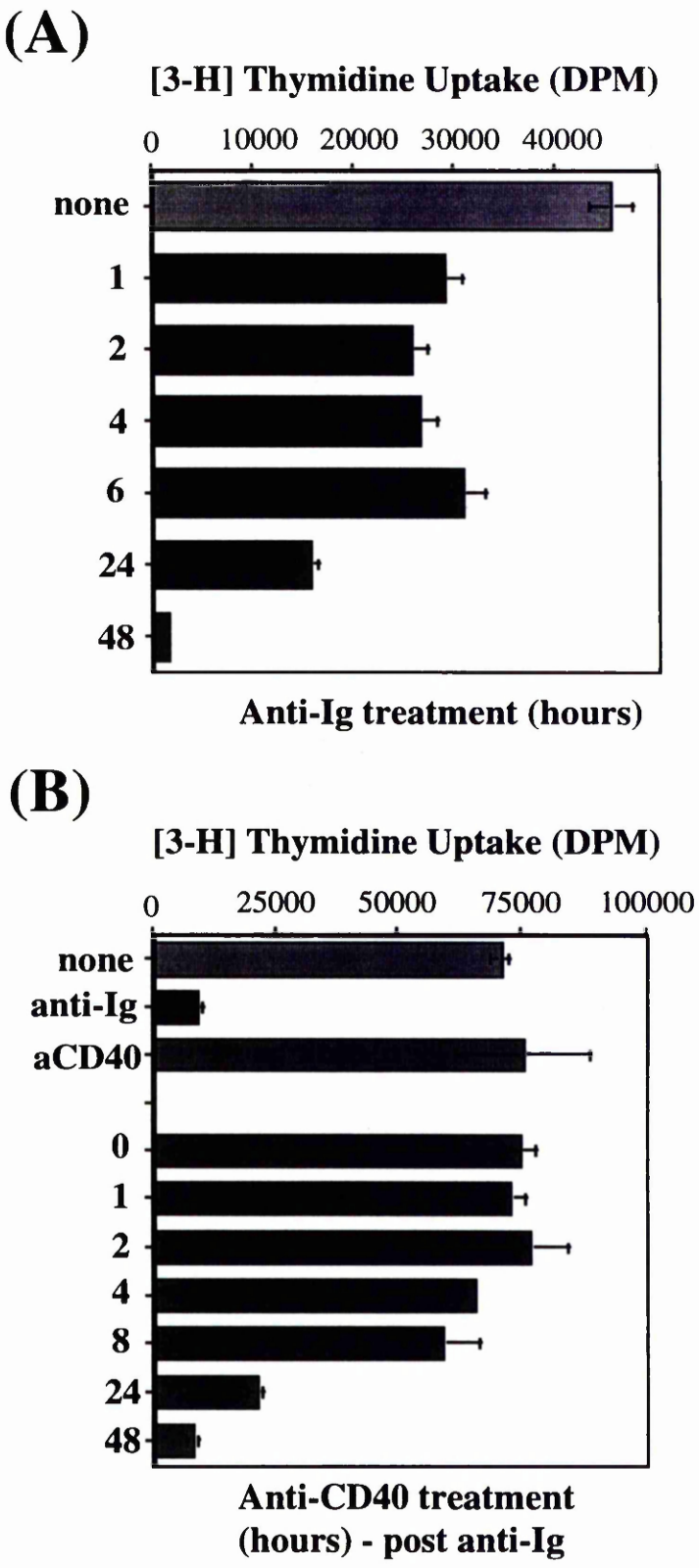

(A)
[3-H] Thymidine Uptake (DPM)
0
10000
20000
30000
40000
none
1
2
4
6
24
48
Anti-Ig treatment (hours)
(B)
[3-H] Thymidine Uptake (DPM)
0
25000
50000
75000
100000
none
anti-Ig
aCD40
0
1
2
4
8
24
48
Anti-CD40 treatment
(hours) - post anti-Ig

Figure 17

Figure 18. Anti-Ig treatment causes apoptosis in WEHI-231 cells which is reversed by co-treatment with anti-CD40 antibodies.

WEHI-231 ($5x10^5$/ml) were cultured in the presence or absence of anti-Ig at 10 μg/ml, anti-CD40 at 10 μg/ml or dexamethasone at 1 μM. Levels of apoptosis were determined by PI staining and FACS analysis after 48 hours as described in Materials and Methods.

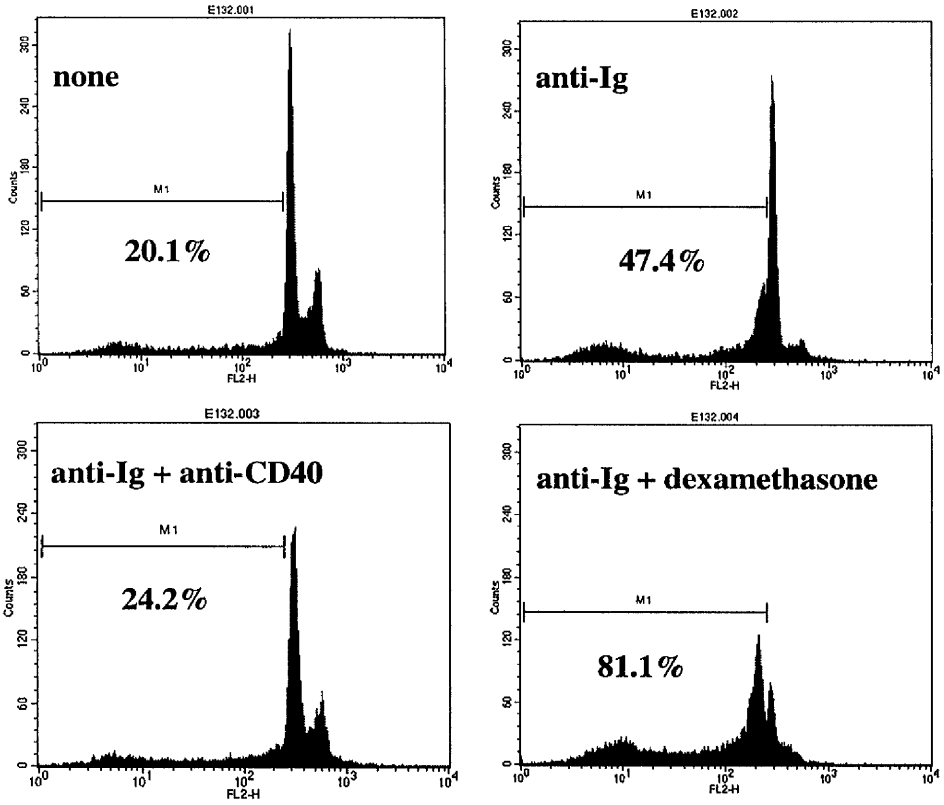

E132.001
none
M1
20.1%
Counts
FL2-H
E132.002
anti-Ig
M1
47.4%
Counts
FL2-H
E132.003
anti-Ig + anti-CD40
M1
24.2%
Counts
FL2-H
E132.004
anti-Ig + dexamethasone
M1
81.1%
Counts
FL2-H

Figure 18

Figure 19. BCR ligation suppresses cell division in WEHI-231 cells. WEHI-231 cells were loaded with the cell permeable dye CFSE (2.5 μM). Cells were then left untreated, or stimulated with combinations of anti-Ig and anti-CD40 (both 10 μg/ml) for 48 hours. Cells were then harvested, washed and analysed by FACS for the fluorescence intensity of CFSE, which gives an indication of the number cell divisions per population for each stimulation.

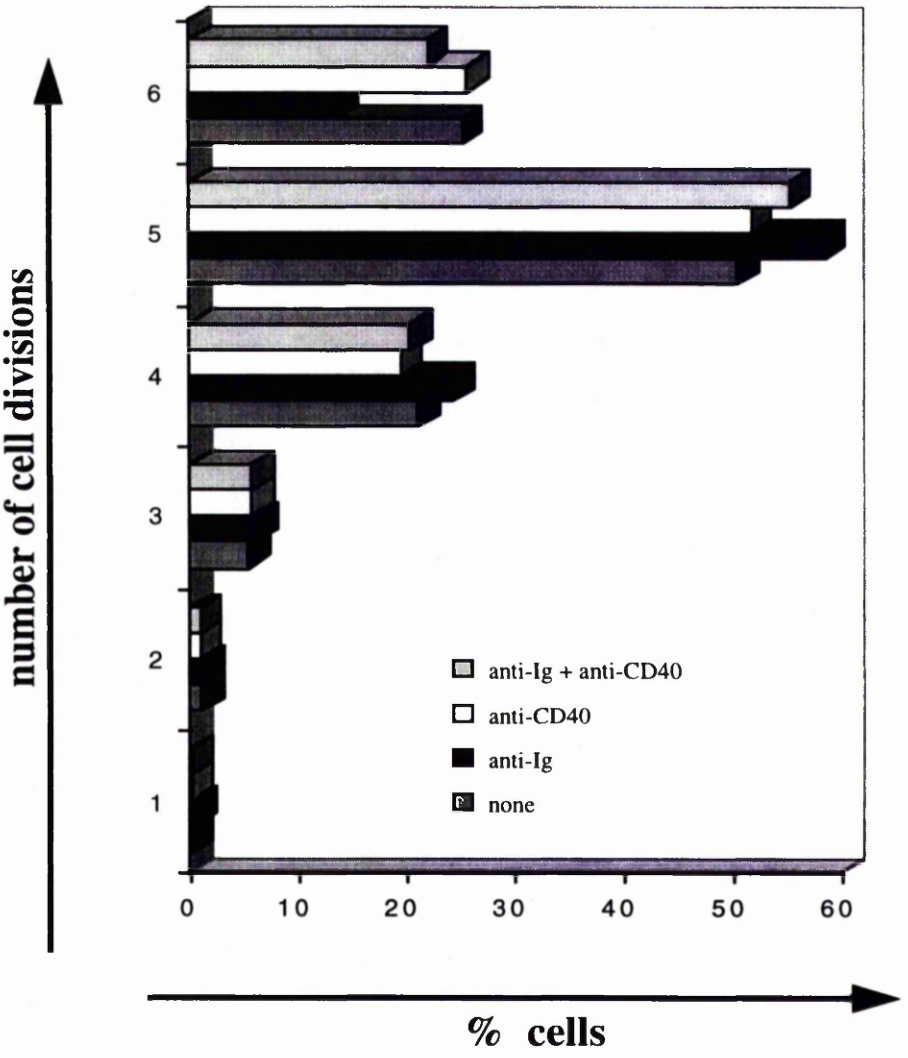

number of cell divisions
6
5
4
3
2
1
0
10
20
30
40
50
60
anti-Ig + anti-CD40
anti-CD40
anti-Ig
none
% cells

Figure 19

Figure 20. Morphological changes in WEHI-231 cells undergoing apoptosis or rescue from apoptosis via anti-CD40 co-treatment.
A. WEHI-231 cells ($5x10^5$/ml) were treated for 48 hours with medium only (control) (A), 10μg/ml anti-Ig (B), 10μg/ml anti-Ig and 1μM dexamethasone (C), or 10μg/ml of both anti-Ig and anti-CD40 . Magnification x200. **B.** WEHI-231 cells ($5x10^5$/ml) were treated for 48 hours with medium only (control), 10μg/ml anti-Ig or 10μg/ml of both anti-Ig and anti-CD40. Cells were then examined by FACS analysis for size (FSC) plotted against granularity (SSC).

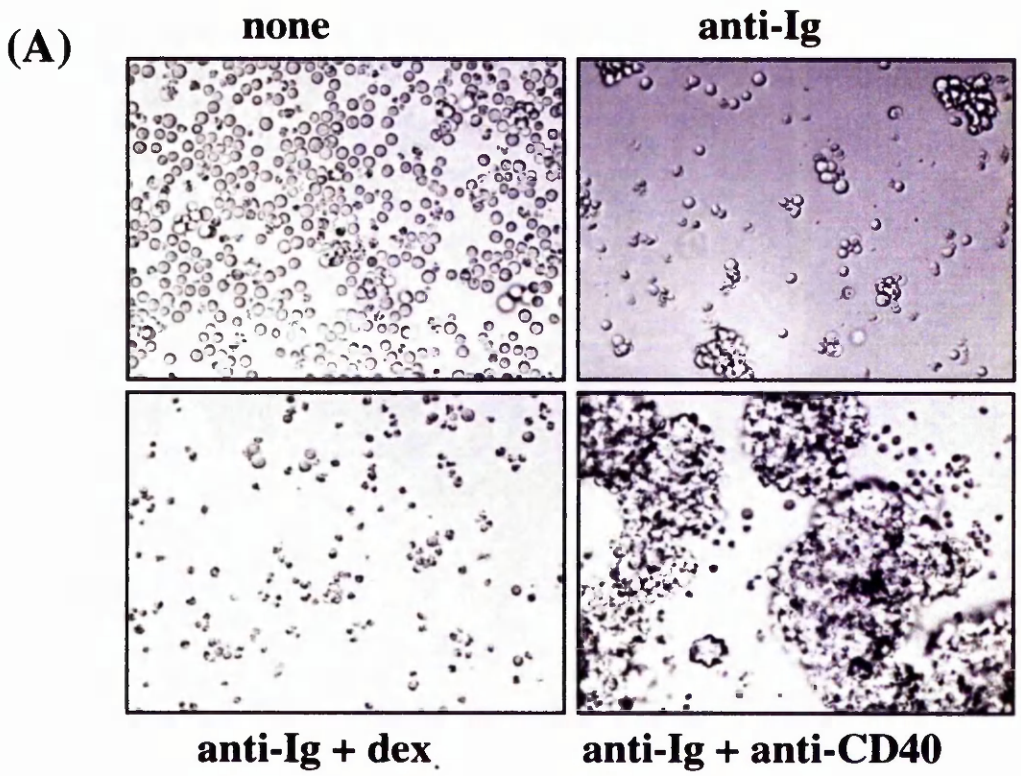

(A)
none
anti-Ig
anti-Ig + dex
anti-Ig + anti-CD40
(B)
none
16.8%
R2
73.7%
R1
SSC-H
FSC-H
anti-Ig
39.5%
R2
49.6%
R1
SSC-H
FSC-H
anti-Ig + anti-CD40
19.4%
R2
66.6%
R1
SSC-H
FSC-H

Figure 20

CHAPTER 4 - Differential Role for MAPKinases in WEHI-231 cell apoptosis/survival.

4.1 Overview of protein kinases.

Signal transduction cascades play important roles in the physiology of a wide range of cell types ranging from muscle cells to the cells of the immune system and can mediate signals leading to proliferation, differentiation or apoptosis. These signalling cascades are activated by the binding of a wide range of stimuli, including growth factors, hormones and cytokines to specific cell surface receptors. Upon surface receptor ligation, the signal is amplified by the utilisation of specific signalling cascades. These cascade mechanisms recruit a wide range of intracellular enzymes, each of which fulfil a specific role in the amplification of a particular physiological signal. Some of these enzymes are responsible for a wide range of post-translational modifications made to proteins which function to further amplify the signalling cascade.

Protein phosphorylation is perhaps the most well studied of the post-translational modifications made to proteins in signalling. The addition of a phosphate group to a specific amino acid is carried out by a family of enzymes called protein kinases. The alcoholic groups of serine and threonine as well as the phenolic group of tyrosine provide the major phosphorylation-sites for many of these kinases (91). Although many families of protein kinases have been discovered, protein kinases can be assigned to one of three groups, depending on the specificity of their amino acid substrates. Consequently, protein kinases can either be classified as serine/threonine (Ser/Thr) kinases, tyrosine (Tyr) kinases, or perhaps the most interesting, dual specificity kinases. Dual specificity kinases can phosphorylate both Ser/Thr as well as Tyr residues and are known to play central roles in the regulation of cell fate. Following the activation of a signalling cascade by cell surface receptor ligation, these three families of protein kinases are known to act in signalling modules to both communicate and amplify these signals.

4.2 MAPKinase family.

The MAPKinase family of protein kinases are activated under a wide range of physiological stimuli and have central roles in a wide range of cellular responses including proliferation and cell death. One fascinating aspect of MAPKinase signalling is the ability of these protein kinases to regulate different biological mechanisms within the same cell. Perhaps the clearest example of this phenomenon occurs in the PC12 neuronal cell line. These cells have been shown to differentiate into neurons or undergo proliferation depending on whether the ERK-MAPKinase family is activated by nerve

growth factor (NGF) or epidermal growth factor (EGF) respectively (92). The regulation of these differential responses has been suggested to be due to the activation of distinct sets of transcription factors. However, recent studies have also suggested the manner of MAPKinase activation in PC12 cells to have profound effects on the overall biological outcome of cell stimulation (42) in that transient Ras-dependent MAPKinase activation results in proliferation, whilst sustained Rap1-dependent MAPKinase activation induces differentiation.

Presently, there are three known MAPKinase families. These include the classical ERK-MAPKinase family (extracellular signal-regulated kinases), the c-Jun N-terminal kinases (JNK), also known as the stress-activated protein kinases (SAPKs) and finally, the p38 MAPKinases (93). The regulation and activation of these different MAPKinase families is due to distinct upstream MAPKinase kinases (MEKs), which lead to dual phosphorylation on both a tyrosine and threonine residue in a threonine-X-tyrosine activation motif. Each family of the MAPKinase group is activated by specific MEKs allowing independent activation and regulation following cell stimulation. ERK-MAPKinase, JNK and p38 MAPKinases are known to phosphorylate different sets of transcription factors (**figure 21**). These include the transcription factors Elk-1 and c-Myc activated by ERK-MAPKinase MAPKinase, c-Jun and ATF-2 by JNK, and ATF-2 and MAX by p38 MAPKinase (93). Previous work on U937 human monoblastic leukaemia cells (94) has suggested that the balance between the ERK-MAPKinase and the stress-activated protein kinases (SAPKs) was an important factor in determining cell fate suggesting that the pattern of transcription factor activation and hence new mRNA and protein production may play key roles in determining cell fate.

In a typical MAPKinase signalling module, the kinase-core consists of a minimum of three kinases (91) (95), an upstream Ser/Thr kinase, a middle dual specificity kinase and a downstream Ser/Thr kinase. Perhaps the most well known, and documented mammalian example of this consists of the Raf-MAPKinase cascade. This signalling cascade is common to many cell types and is activated by many cell surface receptors including the EGFR (**figure 22**). In order to initiate the signalling cascade, the effector molecule, Shc, is recruited to the cytoplasmic tail of the receptor molecule. The molecule Shc is then phosphorylated which allows it to interact with the adaptor molecule, Grb2, through its SH2 (tyrosine-binding) domains. This allows Grb2 to recruit the Ras guanine nucleotide exchange factor SOS (SH3 domain interaction) to the complex which coverts Ras from its GDP-bound inactive form, into its active GTP-bound form (96). Ras is then converted into its active, GTP-bound, state and facilitates the translocation of the Ser/Thr kinase Raf-1 to the plasma membrane, where it associates with and is activated by Ras. Active Raf-1, subsequently, activates the dual-specificity kinase MEK1 (MAPKinase-kinase 1) and MEK2 (MAPkinase-kinase 2) through the phosphorylation of $Ser^{217/218}$ and Ser^{221}

residues. MEK1 and MEK2 then proceed to activate ERK1 (p44) and ERK2 (p42) MAPK, respectively, on specific Thr^{202} and Tyr^{204} residues within a characteristic TPY motif. Activated ERK-MAPKinase has been shown to act on a wide range of downstream molecules, including cytoplasmic molecules such as $cPLA_2$ (53) and nuclear factors such as Elk-1 and c-Myc (97).

In addition to regulatory control by specific upstream MEKs, the MAPKinase family of enzymes are also under the control of a wide range of other signalling intermediates. This is particularly true for the ERK-MAPKinase family. Over the past 5-10 years a wealth of literature has arisen highlighting the importance of molecules such as PKC, PI(3)K, GTPase-activating proteins, adaptor proteins and phosphatases in the regulation of ERK-MAPKinase activation or inactivation.

4.3 Role of MAPKinases in lymphocyte signalling.

BCR-mediated activation of ERK-MAPKinase is well documented (93, 97, 98). These studies also show that the BCR is coupled to ERK-MAPKinase activation at different developmental stages of B lymphocyte development. Together, these studies suggest that ERK-MAPKinase may be involved in different cellular responses at distinct stages of B lymphocyte development such as cell proliferation or death. Studies using the chicken DT40 B cell line (reviewed in (36)) have also shown that BCR-mediated ERK-MAPKinase activation is under the control of a number of signalling pathways including those regulated by PKC or Shc. **Figure 23** (adapted from (36)) shows current models leading to ERK-MAPKinase activation following BCR-ligation.

In addition to ERK-MAPKinase, the role of the other members of the MAPKinase family following BCR-ligation have also been closely studied. Indeed, studies by Graves *et. al.* (99) have indicated that JNK and p38 MAPKinases, but not ERK-MAPKinase, are downstream targets in BCR-mediated cell death in the human B102 immature B cell line. Other groups have also shown the activation of both JNK and p38 MAPKinase in both mature splenic B lymphocytes and also in the immature WEHI-231 B cell line following BCR crosslinking. However, because BCR-ligation in these cell types brings about different cellular responses, proliferation and cell death respectively, it is difficult to draw conclusions concerning their precise roles in BCR signalling.

In addition to BCR ligation, the MAPKinase families have also been shown to be activated in B lymphocytes following CD40 ligation (93, 97, 100). Since it is well known that CD40 ligation can rescue immature B lymphocytes from BCR-mediated cell death, the MAPKinase family may play a key role in this phenomenon. In addition, papers by Berberich *et. al.* and Sutherland *et. al.* (93, 100) have suggested that BCR

and CD40 ligation lead to different patterns of MAPKinase family activation suggesting that the overall balance of MAPKinase activation could determine B lymphocyte fate.

Moreover, although it has been shown that both BCR and CD40 ligation both lead to ERK-MAPKinase activation, it has been suggested that these receptors utilise different up-stream signalling pathways in this process. Purkerson *et. al.* (97) showed that, both BCR- and CD40- resulted in MEK activation in splenic B lymphocytes. Pre-treatment of these cells with cholera toxin, an agent known to cause an increase in intracellular cAMP, was shown to abolish BCR-mediated ERK-MAPKinase activation, however, the same treatment had no effect on ERK activation following CD40 ligation. In addition, BCR-ligation, but not CD40-ligation, was shown to induce Shc phosphorylation and induce Raf-1 phosphorylation. This suggests that CD40-mediated activation of ERK-MAPKinase is PK-A insensitive and utilises a unidentified MEKK, rather than Raf-1, to regulate ERK-MAPKinase activity (**figure 24**). This study highlights the ability of different receptors to couple to MAPKinase pathways by the utilisation of distinct signalling pathways.

4.4 Aims of chapter.

This chapter aims to dissect the roles of the MAPKinases in the fate of WEHI-231 cells. In particular, it shall focus on:

1. the pattern of MAPKinase activation during BCR-induced growth arrest and apoptosis.
2. the signalling pathways involved in the coupling of the BCR to these MAPKinase pathways.
3. the role of the MAPKinases in CD40-mediated rescue in the WEHI-231 cell line and their kinetics of activation.

4.5 Results.

4.5.1 BCR-ligation of WEHI-231 cells induces ERK-MAPKinase activation.

Treatment of WEHI-231 immature B lymphocytes with anti-Ig has previously been shown to inhibit DNA synthesis and induce apoptosis (**figures 15A and 18**). In addition, previous work in our group has shown this may be due to the activation of an early $cPLA_2$ pathway leading to AA generation (53). Interestingly, activation of this negative $cPLA_2$ signal is known to require both calcium and MAPK activation. We therefore investigated whether BCR-ligation induced the activation of ERK-MAPKinase and whether this coupled the BCR to a PLA_2-dependent apoptotic pathway. In order to determine if the ERK family of MAPKs was activated under these conditions, western blot analysis of activated ERK-MAPKinase was carried out on whole cell lysates from cells treated with anti-Ig at a concentration of 10 μg/ml as this concentration was previously shown to induce maximum growth-arrest in WEHI-231 cells. We chose to utilise one of the commercially available antibodies specific to the dually-phosphorylated form (active form) of p44 and p42 ERK-MAPKinase as an indication of ERK-MAPKinase activation. Anti-Ig (10 μg/ml) treatment resulted in the activation of ERK as early as 1 minute, with maximal ERK activity after 30 minutes (**figure 25A**).

4.5.2 BCR ligation induces ERK-MAPKinase activation in a Ras-dependent manner only under conditions favourable to apoptosis.

Previous experiments (**figure 15A**) have shown that anti-Ig treatment induces growth-arrest in a dose-dependent manner in WEHI-231 cells. In addition, the experiment described above suggests that BCR-ligation induces the activation of an early ERK-MAPKinase signal. In order to confirm that ERK-MAPKinase activation was central to the induction of apoptosis, WEHI-231 cells were treated with increasing amounts of anti-Ig to examine under what conditions ERK-MAPKinase was activated (**figure 25B**).

In addition, studies were carried out to examine the induction of apoptosis in WEHI-231 cells treated with increasing concentrations of anti-Ig. **Figure 26** shows that anti-Ig concentrations of 10 μg/ml are required to induce apoptosis in WEHI-231 cells. These results indicate that ERK-MAPKinase activation correlates with the induction of apoptosis in WEHI-231 cells. Interestingly, these results also show that anti-Ig concentrations that induce growth-arrest need not necessarily lead to the

activation of ERK-MAPKinase. These results suggest that the induction of growth arrest and apoptosis by BCR-ligation may be regulated by two distinct mechanisms.

Further studies were carried out to characterise the mechanisms of ERK-MAPKinase activation under these conditions. In order to determine if the BCR-mediated activation of ERK-MAPKinase was Ras-dependent, studies were carried out to examine the pattern of Ras activation under similar experimental conditions. These studies utilised the availability of glutathione-coupled Ras-RBD substrates, which bind selectively to active-GTP-bound Ras and not inactive-GDP-bound Ras. Therefore, the levels of activated Ras after cell stimulation can be assayed by the selective pull-down of active-Ras from whole-cell lysates as indicated by **figure 25C**. Ras activation was shown to precede the kinetics of anti-Ig mediated ERK-MAPKinase activation, indicating that the BCR utilises a Ras-dependent mode of ERK-MAPKinase activation. These results suggest that a Ras/ERK-MAPKinase may be coupled to the BCR under conditions that lead to the induction of apoptosis.

4.5.3 Inhibition of BCR-mediated ERK-MAPKinase activation reduces BCR-mediated apoptosis.

In order to address whether ERK-MAPKinase activated by the BCR was coupled to apoptotic pathways, we utilised the ERK-MAPKinase inhibitor U0126 to examine whether inhibition of ERK-MAPKinase activity could abolish BCR-induced apoptosis. This inhibitor blocks ERK-MAPKinase activation by preventing the catalytic activity of its upstream MAPKinase-kinase (MEK). Initial experiments confirmed the ability of U0126, and another ERK-MAPKinase inhibitor, PD98059, to inhibit the activity of ERK-MAPKinase in WEHI-231 cells stimulated with anti-Ig (10 μg/ml) for 30 minutes (previously shown to strongly activate ERK-MAPKinase, **figure 25A**). As shown in **figure 27A** and **27B**, PD98059 and U0126 were effective in the inhibiting ERK phosphorylation in both a time (pre-treatment prior to BCR ligation) and dose-dependent manner. Identical studies using the p38 MAPKinase inhibitor, SB203580, indicated that inhibition of p38 MAPKinase had no affect on the activation of early ERK-MAPKinase signals induced following BCR-ligation (**figure 31C**). This was used as a specificity control for the inhibition of ERK-MAPKinase by PD98059 and U0126, but also indicates that p38 MAPKinase plays no role in the upstream regulation of ERK-MAPKinase activation in WEHI-231 cells.

We were therefore interested to examine whether inhibition of this early ERK-MAPKinase signal would prevent BCR-mediated apoptosis in WEHI-231 cells. WEHI-231 cells were pre-treated with different concentrations of U0126 for 1 or 2 hours prior to cell stimulation. Cells were then left unstimulated, or stimulated with anti-Ig or a combination of anti-Ig and anti-CD40. Cell-cycle analysis experiments

using propidium iodide incorporation was used as a measure of sub-diploid, or apoptotic cells. Unstimulated cells (**figure 28A**) were shown to have low levels of apoptotic cells which increased upon anti-Ig treatment (**figure 28B**) from 14.8% to 28.9%. Co-treatment of anti-Ig treated cells with anti-CD40 substantially reduced BCR-induced apoptosis (**figure 28C**) whilst also reducing cells in G_1 arrest and therefore driving cells into cell-cycle. Pre-treatment of WEHI-231 cells with different concentrations of U0126 caused no increases in either basal apoptosis or basal G1 arrest compared to untreated (control) cells (**figures 28 D-E**). However, pre-incubation with U0126 were shown to reduce anti-Ig mediated apoptosis (**figures 28 F-G**). Similar results were obtained with both 1 and 2 hour pre-incubations, therefore only the results for the 2 hour pre-incubations are shown. In addition, another MEK inhibitor, PD98059 was also shown to reproducibly block BCR-induced apoptosis, but not as effectively as U0126.

Interestingly, these results also show that anti-Ig induced growth-arrest at the G_1-phase of the cell-cycle appeared to be unaffected by pre-treatment of cells with either PD98059 or U0126 suggesting that BCR-ligation may induce the initiation of two quite distinct mechanisms, one apoptotic and one promoting growth-arrest whilst also suggesting that ERK-MAPKinase is necessary for cell growth.

These results confirm a negative regulatory role for ERK-MAPKinase following BCR ligation in WEHI-231 cells which may be linked to the induction of $cPLA_2$ activity and AA generation as previously described (53).

4.5.4 Anti-CD40 stimulation does not induce ERK-MAPKinase activation, but does alter BCR-mediated ERK-MAPKinase activity.

Previous experiments have shown the ability of anti-CD40 stimulation to prevent BCR-mediated apoptosis in WEHI-231 cells. We therefore examined not only whether anti-CD40 stimulation induced ERK-MAPKinase activation, but also if it modulated BCR-mediated ERK-MAPKinase activation. In contrast to anti-Ig stimulation, anti-CD40 (10 μg/ml), which does not induce growth arrest or apoptosis, was shown to be ineffective in inducing ERK-MAPKinase activation (**figure 29A**). This is in contrast to another study (101) who reported early ERK activation following anti-CD40 stimulation in the same cell line. However, in our study we used antibodies to detect dually-phosphorylated ERK, which is known to be required for full activation of this enzyme. The study by Kashiwada *et. al.* (101) detected increases in tyrosine phosphorylation only, and therefore may not reflect true activation of this enzyme.

However, anti-CD40 co-stimulation was shown to alter the kinetics of ERK-MAPKinase activation in anti-Ig treated WEHI-231 cells. **Figure 29B** shows that co-treatment with CD40, known to prevent BCR-induced growth-arrest and apoptosis,

altered the kinetics of anti-Ig mediated ERK activation leading to an early peak at 5 minutes, but strongly down-regulated ERK MAPKinase activation after this time period. Interestingly, Ras activity was also altered with anti-CD40 co-treatment (**figure 29C**) with similar kinetics to the activation of ERK-MAPKinase. This suggests that co-treatment with CD40 may rescue WEHI-231 cells from apoptosis by uncoupling the BCR from an early Ras/ERK-MAPKinase/cPLA$_2$ pathway. Indeed, recent data from our lab has shown that CD40 co-stimulation can reduce BCR-mediated generation of AA in a dose-dependent manner (**figure 30**).

4.5.5 BCR ligation does not activate JNK or p38 MAPKinase in WEHI-231 cells.

In addition to examining the role of ERK-MAPKinase in BCR -mediated apoptosis, both JNK and p38 MAPKinases were also examined. Previous studies (93, 102) have shown only weak activation of either p38 and JNK following BCR ligation. Both studies, however, examined enzyme activation with stimulation times lasting 10-15 minutes. We therefore decided to utilise phospho-specific antibodies to both p38 MAPKinase and also the p46- and p54-isoforms of JNK, but examine their activity following BCR-ligation over a 60 minute period.

In contrast to the above studies by Salmon *et. al.* and Sutherland *et. al* no activation of either p38 or JNK MAPKinase could be detected (**figures 31A and 31B**) following BCR-ligation. Therefore, because we were unable to detect p38 MAPKinase activation, we wanted to verify that p38 MAPKinase was not involved in BCR-induced apoptosis. Subsequently, WEHI-231 cells were pre-incubated with the p38 MAPKinase inhibitor SB203580 90 minutes prior to anti-Ig stimulation. No significant decrease in either BCR-induced apoptosis or growth-arrest was noted (**figures 32 G-I**), data consistent with other studies (102) indicating that p38 MAPKinase does not appear to play a role in BCR-mediated apoptosis of WEHI-231 cells. Pre-incubation with SB203580 was also shown to be ineffective in reversing anti-CD40-mediated rescue from BCR-induced growth arrest and apoptosis (**figures 32 J-L**). Cells only treated with 5 μM SB203580 did show increases in apoptotoic cell numbers (42.0%) relative to untreated cells (27.3%) (**figure 32F**). Vehicle studies for this concentration (data not shown) produced similar levels of apoptosis to that found in untreated cells (30.8% as opposed to 27.3%) suggesting that p38 MAPKinase activity may play a role in the basal proliferation and survival of WEHI-231 cells. Parallel studies performed in macrophages confirmed that SB203580 was active as these results showed that pre-incubation with SB203580 blocked LPS-stimulated p38 MAPKinase activation and production of IL-12 and NO (results not shown).

No specific inhibitors for the JNK (SAPK) MAPKinases are, at present, commercially available and therefore similar studies examining the role of JNK MAPKinase in BCR mediated apoptosis were not possible.

4.5.6 Anti-Ig treatment of WEHI-231 cells causes the long-term down-regulation of ERK-MAPKinase activity.

Apoptosis of WEHI-231 B cells resulting from ligation of the BCR cannot be detected until 24-48 hours post-treatment. In addition, results from **figure 17A** suggest that WEHI-231 cells require anti-Ig stimulation for periods greater than 8 hours to fully commit to growth-arrest. Thus, we decided to investigate the pattern of MAPKinase activation over a 48 hour time period. Interestingly, in untreated WEHI-231 cells, basal ERK-MAPKinase activity was observed, which appeared to follow a cyclic pattern over a 48 hour period (**figure 33A**). This may be linked to the suggested role of ERK MAPKinase in G_1 cell-cycle progression (21). In contrast to this, BCR-ligation was shown to initiate an early transient activation of ERK MAPKinase, but abolished ERK-MAPKinase activation after this time point (**figure 33B**).

Taken together, these results suggest that there may be a potential requirement for ERK-MAPKinase activity in the basal proliferation of WEHI-231 cells and that the removal of this signal, due to BCR-ligation, may be sufficient to induce growth arrest and/or apoptosis. In addition previous studies have also shown that BCR ligation initiates the activation of an early, ERK-MAPKinase dependent, apoptotic pathway (**figures 25A and 28**). These results suggest differential roles for ERK-MAPKinase in WEHI-231 signalling, with both proliferative and apoptotic properties.

4.5.7 Anti-CD40-induced rescue from growth arrest involves the re-establishment of ERK-MAPKinase activity.

CD40 has previously been reported to be a very weak activator of ERK-MAPKinase in WEHI-231 cells and a potent activator of ERK-MAPKinase in mature B lymphocytes (93, 101). Our initial results would agree with previous reports in WEHI-231 cells. However, these studies have only analysed ERK-MAPKinase activation within 1 hr of anti-CD40 stimulation. Due to the proliferative nature of anti-CD40 treatment in WEHI-231 cells we analysed ERK-MAPKinase activation in cells treated with anti-CD40 over the 48 hour time period required for rescue from anti-Ig induced apoptosis. As shown in **figure 33D**, anti-CD40 treatment appears to induce a late, yet sustained, cyclic activation of ERK-MAPKinase. Although the pattern of ERK-MAPKinase activity is similar to that of untreated proliferating cells, densitometric

analysis indicates that anti-CD40 stimulation enhances ERK-MAPKinase activation relative to untreated cells.

Although the ability of CD40 to rescue WEHI-231 cells from anti-Ig-mediated growth arrest is well known, the precise signalling pathways involved are not. Previously (**figure 33B**) we showed that anti-Ig treatment caused the long-term downregulation of ERK-MAPKinase activation. Interestingly, treatment of cells with anti-Ig in combination with anti-CD40 treatment did not show any down-regulation of ERK-MAPKinase activation (**figure 33C**). Indeed, it appears that anti-CD40 treatment can both re-establish and strengthen ERK-MAPKinase activation in WEHI-231 cells under conditions in which CD40 rescues mIg-mediated growth arrest and apoptosis. This finding highlights a potential mechanism by which CD40 mediates it rescue signal.

4.5.8 Activation of p38 and JNK MAPKinases in long-term WEHI-231-231 stimulations.

In addition to examining MAPKinase activation under growth-arrest/apoptotic conditions as well as during CD40-mediated rescue, the activation kinetics of both p38 MAPK and JNK MAPK were also examined. Interestingly, p38 MAPK was weakly activated (24-48 hours) during basal proliferation of WEHI-231 cells, but anti-Ig treatment did not appear to activate p38 MAPK over the whole time course (**figures 34A and 34B**). Indeed, anti-Ig appeared to downregulate basal p38 MAPKinase activation. p38 MAPKinase was however, activated in a bi-phasic manner following co-treatment with anti-CD40 (**figure 34C**). This suggests a possible role for p38 MAPK in the rescue of WEHI-231 cells from growth-arrest/apoptosis by CD40 ligation, and interestingly, its kinetics of activation preceded that of ERK-MAPKinase in similarly stimulated samples. However, results obtained in **figure 32** suggested that p38 MAPKinase does not play an essential role in CD40-mediated rescue. Therefore further studies have to done to address this issue. In contrast to p38 MAPKinase JNK appeared to be weakly activated by anti-Ig treatment (**figure 34D**). In contrast, no JNK activity could be detected in basal or anti-CD40 (either alone or in combination with anti-Ig)-treated cells suggesting, but as yet unconfirmed, that it may play a role in the induction of negative signalling pathways.

Additional studies were carried out to examine the activation of Rac and Cdc42, known upstream activators of JNK. These results indicated that neither of these molecules were activated in either basal; anti-Ig treated or anti-Ig and anti-CD40 treated cells (data not shown).

4.5.9 ERK activity contributes to the basal proliferation of WEHI-231 cells, but does not appear to be involved in CD40-mediated rescue.

Due to the prolonged, but cyclic, activation of ERK-MAPKinase in untreated cells and the ability of CD40 stimulation to restore ERK-MAPKinase activity in BCR stimulated WEHI-231 cells, additional studies were carried out using the MEK inhibitors PD98059 and U0126 to confirm a role of ERK-MAPKinase in proliferative WEHI-231 cell responses. Initial studies suggested that ERK inhibition (PD98059) could weakly block basal proliferation of WEHI-231 cells (approximately 30% reduction in thymidine incorporation at 5 μM, **figure 35A**). Vehicle studies confirmed this was not due to toxic effects of the PD98059 vehicle medium, suggesting a role for ERK-MAPKinase in the basal proliferation of WEHI-231 cells. However, pre-incubation with PD98059 was ineffective in reversing BCR-mediated growth-arrest (**figure 35A**). Interestingly, earlier studies (**figure 28**) confirmed that pre-incubation of anti-Ig treated cells with U0126, whilst effective in reversing BCR-mediated apoptosis, did not reverse BCR-mediated growth arrest, data consistent with the above results (**figure 35A**). This data is also consistent with other findings (**figure 33B**) which suggested that BCR-ligation down-regulated ERK-MAPKinase activity in WEHI-231 cells, confirming a positive role for ERK-MAPKinase in WEHI-231 cell proliferation.

To provide further evidence for a role of ERK-MAPKinase in WEHI-231 cell proliferation but not BCR-mediated growth arrest, a dose-response study was carried out comparing varying the concentrations of both anti-Ig and PD98059. **Figure 35B** shows that costimulation of non-growth arrest promoting concentrations of anti-Ig with PD98059 results in the induction of growth arrest in a dose-dependent manner. Interestingly, at higher anti-Ig concentrations, which themselves induce growth-arrest, the affect of co-stimulation with PD98059 was reduced. This suggests the ability of BCR-ligation to suppress ERK-MAPKinase activity has reached saturation at these higher anti-Ig concentrations. Further studies using U0126 suggested that this inhibitor could actually enhance anti-Ig mediated growth arrest (**figure 36B**). However, this is probably due a reduction in the levels of growth-arrest normally expected at higher (1-10 μg/ml) anti-Ig concentrations in this particular experiment. This suggests that BCR-mediated suppression of basal ERK-MAPKinase activity may also be reduced, allowing U0126 to enhance growth-arrest even at anti-Ig concentrations where growth-arrest is normally at saturated levels.

The role of ERK-MAPKinase in CD40-mediated rescue of WEHI-231 cells was also examined. Pre-incubation of cells with increasing doses of PD98059 were only marginally effective in blocking rescue from growth-arrest following co-treatment with

anti-CD40 (**figure 35A**). In addition to PD98059, the MEK inhibitor U0126 was applied in similar experiments. Like PD98059, U0126 was effective in inducing growth arrest in otherwise untreated cells (**figure 36A**). In addition, co-stimulation of anti-Ig and anti-CD40 treated cells with U0126 was capable of reversing CD40-mediated rescue from growth-arrest. Additional studies have shown that this reduction in DNA synthesis is not due to the inhibitor vehicle (results not shown) suggesting that ERK-MAPKinase inhibition is sufficient to block CD40-mediated rescue.

However, these results do confirm that ERK-MAPKinase plays an important role in WEHI-231 cell proliferation and, coupled to previous findings (**figure 33C**), suggests that CD40 stimulation plays an important role in restoring vital ERK-MAPKinase-dependent proliferative signals in WEHI-231 cells.

4.5.10 Prolonged ERK-MAPKinase inhibition can block basal proliferation and CD40-mediated rescue.

Previous results which utilised the MEK inhibitors, PD98059 and U0126, suggested that U0126 was more efficient in its ability to promote growth arrest in WEHI-231 cells. Therefore, we wanted to examine the efficacy of both MEK inhibitors in blocking ERK-MAPKinase activation especially over a 48 hour time period. Unstimulated WEHI-231 cells, which express a level of basal ERK-MAPKinase activity, were pre-incubated with either PD98059 (1 μM) or U0126 (1 μM) and cultured for an additional 1, 6 or 24 hours before they were harvested and lysed. Our results indicated that whilst U0126 was effective in blocking early, basal, ERK-MAPKinase signals, both inhibitors were ineffective in blocking basal ERK-MAPKinase activity in long-term cultures (>12 hours) (**results not shown**).

In order to re-evaluate the role of ERK-MAPKinase in basal proliferation and CD40-mediated rescue from growth arrest cells were treated with PD98059 (1 μM) and U0126 (1 μM) or a combination of both. However, in order to overcome the instability of these two inhibitors, further doses were given every four hours of the stimulation, up to a total of 32 hours. Following a stimulation time of 48 hours both inhibitors were effective in inducing growth arrest of basal (untreated) cells as well as enhancing growth arrest from cell treated with anti-Ig alone. Both inhibitors were, in addition, effective in preventing CD40-mediated rescue from growth arrest (**figure 37A**). Interestingly, a combination of the two inhibitors, compared to each individually, appeared to enhance growth arrest under all stimulation conditions (**figure 37B**), although this probably reflects the different mechanisms used by these two inhibitors to suppress ERK-MAPKinase activity. PD98059 inhibits ERK-MAPKinase activation by binding to MEK and preventing its phosphorylation by Raf, conversely, U0126 blocks

ERK-MAPKinase activation by directly inhibiting the catalytic activity of MEK. Our results are also similar to other studies which have shown that PD98059 was ineffective in the complete inhibition of ERK-MAPKinase, and the authors suggested this may be due to incomplete inhibition of MEK2, which possesses an IC_{50} 10-fold higher than MEK1 for PD98059 (103). Moreover, U0126 is equally effective on both MEK1 and MEK 2. In addition, vehicle studies indicate that, at the concentrations of inhibitors used, no adverse effect on basal cells was apparent. These results suggest a role for ERK in both the basal proliferation of WEHI-231 cells and in the rescue of WEHI-231 cells from anti-Ig mediated growth arrest by CD40, confirming earlier observations.

4.5.11 CD40-mediated activation of ERK protects WEHI-231 cells from anti-Ig induced growth arrest.

In order to determine if the results indicated in growth arrest studies (**figure 37B**) were a consequence of apoptosis, DNA content levels were determined by FACS analysis utilising cell-cycle analysis by propidium iodide incorporation (**table 1**). Unstimulated cells showed cell-cycle profiles consistent with this cell type. However, prolonged ERK-MAPKinase inhibitor treatment (PD98059 and U0126 at 1μM every 4 hours) appeared to drive these cells into apoptosis to levels similar to anti-Ig treatment suggesting that deprivation of cycling ERK-MAPKinase activity results in cell death. Interestingly, whilst cells treated with a combination of both PD98059 and U0126 and anti-Ig, appeared to be rescued from apoptosis, they were driven strongly into growth arrest at G_0/G_1 again confirming that inhibition of an early ERK-MAPKinase signal can prevent BCR-mediated apoptosis.

Previous studies (**figure 33D**) have shown that anti-CD40 treatment of WEHI-231 cells can enhance ERK-MAPKinase activation in these cells. In addition anti-CD40 treatment leads to [^{3}H]thymidine incorporation to levels comparable with unstimulated basal cells (**figures 37A and 37B**). Analysis of the DNA content suggests that CD40 treatment may in some way alter the kinetics of cell-cycle progression, with more cells appearing in G_2/M phase as compared to unstimulated cells (**table 1**). CD40 stimulation of WEHI-231 cells may therefore promote cell-cycle progression in WEHI-231 cells. These results suggest a potential role of ERK-MAPKinase in driving B lymphocyte proliferation following CD40 treatment. Indeed, prolonged ERK-MAPKinase inhibition of CD40-stimulated cells reduced the numbers of cells found in S and G_2/M phases of the cell cycle whilst increasing percentages found in G_1. Interestingly, CD40 co-stimulation was shown to rescue cells from apoptosis induced by the inhibition of ERK-MAPKinase. These cells were however driven into G_1 arrest (**table 1**) suggesting that CD40 stimulation generates ERK-MAPKinase independent rescue signals. Moreover, prolonged ERK-MAPKinase inhibition did not reverse

CD40-mediated rescue from BCR-induced apoptosis, although these cells were still shown to be in G_1 arrest (**table 1**). These results again suggest that CD40 delivers ERK-MAPKinase independent signals in the rescue of WEHI-231 cells from apoptosis, whilst ERK-MAPKinase is important for the proliferation of these cells.

We also examined the ability of prolonged ERK-MAPKinase inhibition to block cell division measured by CFSE labelling (**figure 38**). Our results indicated that prolonged ERK-MAPKinase inhibition resulted in a reduction in the number of unstimulated cells entering a sixth round of cell division, with increased cells undergoing four or five rounds of cell division before undergoing growth arrest. Interestingly, prolonged ERK-MAPKinase inhibition appeared to enhance BCR-induced growth arrest, with increasing numbers of cells undergoing only three or four rounds of cell division. Prolonged ERK-MAPKinase inhibition also reduced in the number of cell divisions in cells co-stimulated with anti-Ig and anti-CD40. These results confirm earlier observations that ERK-MAPKinase is important for the basal proliferation of WEHI-231 cells and represents a target of CD40 signalling in the rescue of WEHI-231 cells from BCR-induced growth arrest.

In summary, these results suggest that CD40 rescues cells from BCR-mediated growth-arrest by re-establishing an essential, ERK-MAPKinase-dependent, proliferative signal suppressed following BCR ligation, but also initiates an anti-apoptotic signalling cascade. In addition, these results suggest that whilst ERK-MAPKinase is essential for the proliferation and survival of basal cells, they are not essential for cell survival in the presence of rescue signals provided by CD40.

4.6 Discussion.

It is well documented that BCR-ligation on WEHI-231 cells is a potent activator of ERK-MAPKinase (93). Whilst this may be true in the short term (0-60 minutes), which our data confirms, we have also shown that long-term BCR-ligation, under conditions which induce growth-arrest also results in the down-regulation of basal ERK-MAPKinase activity important for cell proliferation.

Studies in this chapter suggest that activation of an early (0-30 minute) ERK-MAPKinase signal correlates with the induction of apoptosis in WEHI-231 cells. Previous studies within our group have shown that BCR ligation of WEHI-231 cells also leads to the activation of $cPLA_2$, which is MAPKinase dependent. Our results suggest that BCR -mediated ERK-MAPKinase activation, which appears to be Ras-dependent, may play a role in the induction of apoptosis in WEHI-231 cells by the activation of an apoptotic $cPLA_2$ pathway. Indeed, studies have shown that inhibition of BCR-mediated ERK-MAPKinase activation, which prevents apoptosis, by using the ERK-MAPKinase inhibitors PD98059 or U0126 also reduces BCR-mediated activation of $cPLA_2$ activity (**figure 39**).

A role for ERK-MAPKinase in the induction of apoptotic mechanisms has also been reported in T-lymphocytes. However, in contrast to our studies where ERK-MAPKinase appears to link the BCR to the generation of apoptotic second messengers, ERK-MAPKinase activity in T lymphocytes has been reported to induce the expression of FasL (103, 104), allowing these cells to then undergo Fas-mediated cells death.

Although our results showed that BCR-mediated apoptosis of WEHI-231 cells can be reversed with co-stimulation with ERK-MAPKinase inhibitors, BCR-mediated growth-arrest remained intact. In addition, our studies indicate that ERK-MAPKinase follows a cyclic pattern of activation (presumably in response to serum- or autocrine factors) which may correlate with the reported ability of ERK-MAPKinase to drive cells through G_1 to the S phase of the cell cycle (21). We have also shown that inhibition of ERK-MAPKinase activity leads to growth-arrest in WEHI-231 cells confirming a role for ERK-MAPKinase in the basal proliferation of this cell line. Interestingly, BCR-ligation was shown to abolish ERK-MAPKinase activation in WEHI-231 cells after approximately 2-4 hours. This suggests a possible mechanism for the induction of BCR-mediated growth arrest in WEHI-231 cells. The ability of anti-CD40 co-stimulation to rescue WEHI-231 cells from BCR-mediated growth arrest was also shown to mirror the re-establishment of basal ERK-MAPKinase activity suggesting that CD40 co-stimulation reverses the signal delivered by BCR-ligation switching off this proliferative signal.

Our studies therefore indicate that ERK-MAPKinase may induce differential physiological responses in WEHI-231 cells which is dependent on its time and possibly

mode of activation. It appears that early ERK-MAPKinase signals (0-60 minutes) may induce, or precede, a "death signal" whilst late ERK-MAPKinase activation by CD40 stimulation may be protective and either activate survival signals or down-regulate "death signals".

In an attempt to dissect the role of differential, early and late, ERK-MAPKinase activities further studies were carried out to block the induction of these signals. Prolonged ERK-MAPKinase inhibition was shown to:

1. block basal proliferation and induce apoptosis in these cells.
2. block BCR-mediated apoptosis but not relieve growth arrest and finally,
3. block CD40-mediated rescue of BCR-induced growth arrest but not reverse CD40-mediated rescue from BCR-apoptosis (suggesting CD40-mediated rescue from apoptosis is partially ERK-MAPKinase independent).

These results confirm a role for ERK-MAPKinase as an essential proliferative signal in WEHI-231 cells and removal of this signal induces growth arrest and apoptosis. This is similar to the responses of PC12 neuronal cells in which withdrawal of nerve-growth-factor (NGF) leads a reduction of ERK-MAPKinase activity and the induction of apoptosis (105). Additionally, inhibition of the early, BCR-stimulated ERK-MAPKinase signal has been shown to block apoptosis, yet these cells still remain at G_1 arrest. This is presumably due to the inhibition of long-term, proliferative ERK-MAPKinase activity in WEHI-231 cells which we have also shown to be downregulated by BCR-ligation.

CD40 stimulation of WEHI-231 cells has been shown to upregulate the anti-apoptotic protein Bcl-x_L at both transcriptional and translational levels (17) and this has shown to play a key role in the rescue of WEHI-231 cells from BCR-mediated apoptosis (83). Although the signalling mechanisms required for the increase in Bcl-x_L expression following BCR-ligation are not known it, would be interesting to address whether they are ERK-MAPKinase independent or dependent. Additionally, Bcl-x_L has also been shown to inhibit the activation of $cPLA_2$ in breast carcinoma cells (106). As $cPLA_2$ has previously been implicated in BCR-mediated apoptosis in WEHI-231 cells it would be of interest to examine whether CD40-mediated increases in Bcl-x_L expression resulted in changes in $cPLA_2$ activity. In addition to Bcl-x_L, other molecules have been implicated in CD40 mediated rescue from BCR-induced apoptosis. In particular, a recent study has highlighted the role of another member of the Bcl-2 family, the anti-apoptotic protein A1 (107). Overexpression of this protein was shown to render WEHI-231 cells resistant to BCR-mediated apoptosis and CD40 stimulation was also shown to increase A1 RNA expression. However, the role of A1 in non-transfected

WEHI-231 cells remains to be explored, and the molecular mechanisms leading to its expression and activation elucidated.

The results in this chapter describe a differential role for ERK-MAPKinase in mediating both apoptotic and proliferative responses in WEHI-231 cells. How these differential ERK signals regulate two distinct biological responses is still unclear, but may in some way be related to the ability of ERK to act in both cytosolic and nuclear compartments as suggested by other studies (101). Indeed we have preliminary results to suggest that CD40 stimulation enhances nuclear ERK-MAPKinase activity, whilst BCR-stimulation results in largely cytoplasmic ERK-MAPKinase activity, but also some nuclear ERK-MAPKinase activity. These results are similar to recent findings by Shirakata *et. al.* (108). Whether the enhanced nuclear activity of ERK-MAPKinase results from the translocation of activated, cytosolic ERK-MAPKinase to the nucleus remains to be determined. However, the role of nuclear ERK-MAPKinase activity induced by CD40-stimulation fits current models relating to increased transcription factor activation (for example NF-κB) and new protein synthesis.

In addition to ERK-MAPKinase our results suggest that JNK and p38 MAPKinase may play roles in BCR-mediated apoptosis and CD40-mediated rescue respectively. However, both these signals appear to be very weak, and subsequently are difficult to examine by the use of inhibitors. Examination of these enzymes by in vitro kinase assay should help these sensitivity problems and help determine the pattern of activation of these enzymes under proliferative or apoptotic responses.

Currently however, our data suggests that p38 MAPKinase appears to be activated by CD40 with kinetics preceding that of ERK-MAPKinase. Utilisation of the p38 MAPKinase inhibitor, SB230580 however, suggests that p38 MAPKinase plays no apparent role in CD40-mediated rescue from growth-arrest and apoptosis, but does suggest that it may play a role in the basal proliferation of WEHI-231 cells. Interestingly, western blot analysis also detected low levels of p38 MAPKinase activity in untreated (basal) WEHI-231 cells (**figure 34A**).

In summary, the results obtained in this chapter indicate that ERK-MAPKinase can play differential roles in the biology of WEHI-231 immature B lymphocytes. Our results indicate that BCR-ligation leads to the activation of an ERK-MAPKinase-dependent apoptotic signal which is induced within 30 minutes of BCR-ligation. However, our results also indicate that ERK-MAPKinase plays an essential role in the proliferation of WEHI-231 cells. Interestingly, BCR-ligation appears to suppress this prolonged ERK-MAPKinase signal during the induction of growth-arrest, whilst CD40, which restores BCR-mediated growth arrest also re-establishes this ERK-MAPKinase signal. In addition we have also shown that CD40 stimulation appears to rescue WEHI-231 cells from BCR-mediated apoptosis by uncoupling ERK-

MAPKinase from a $cPLA_2$ dependent apoptotic signal, however, the role for an ERK-MAPKinase independent anti-apoptotic signal cannot be ruled out. Therefore we can begin to generate a model for the regulation of ERK-MAPKinase and the induction of apoptosis, growth-arrest and proliferation of WEHI-231 cells (**figure 40**).

Figure 21. MAPKinase signalling cassettes utilise different upstream elements for their activation. Activation of the MAPKinase family of protein kinases results from the initiation of a signalling cascade. Each member of the MAPKinase family is activated by an upstream MEK (MAPK 2), which was itself activated by a MEKK (MAPK 3). These MEKKs and MEKs are specific for each family member of the MAPKinase family. However, extensive cross-talk mechanisms exist in MAPKinase mediated activation of transcription factors.

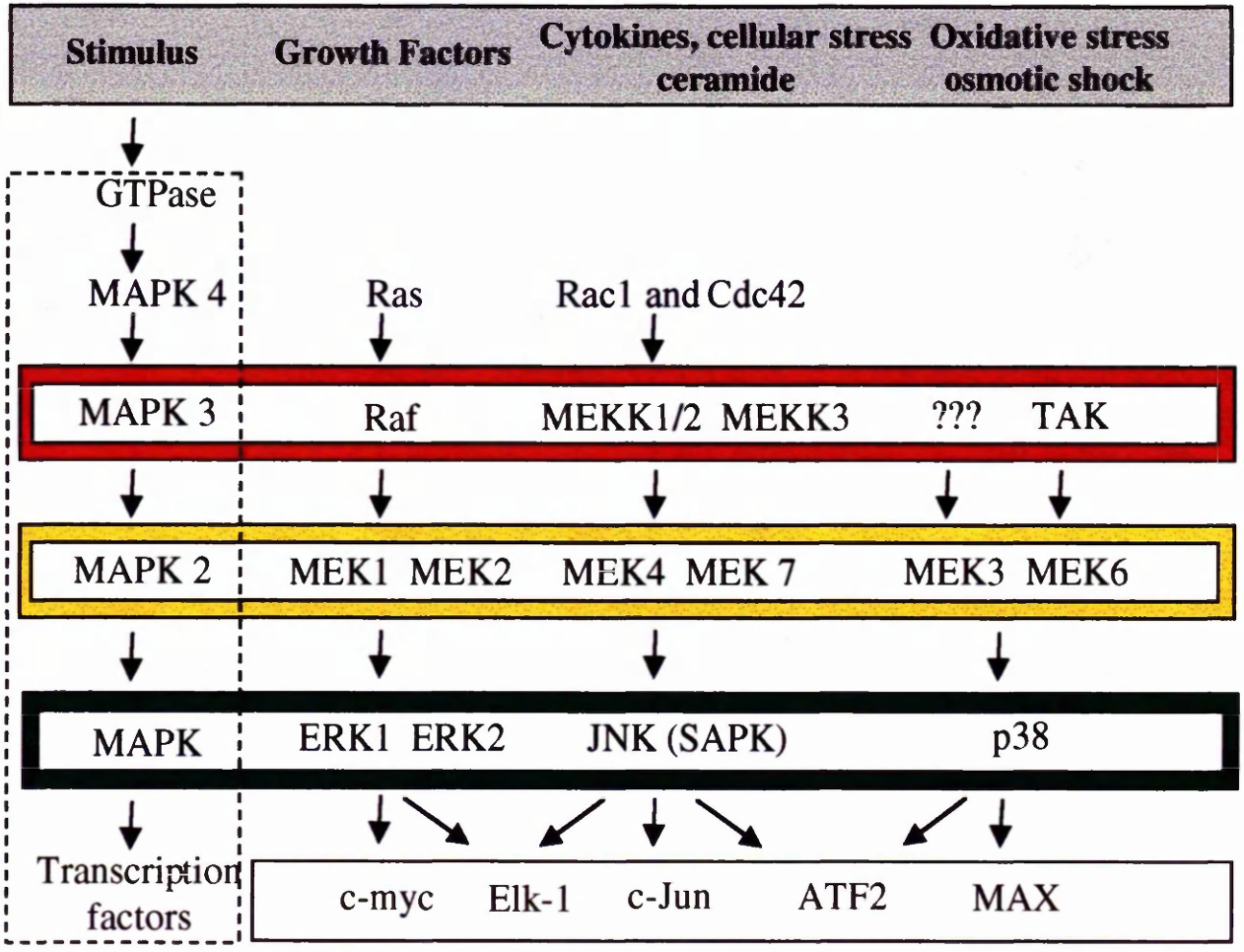

Stimulus
Growth Factors
Cytokines, cellular stress
ceramide
Oxidative stress
osmotic shock
GTPase
MAPK 4
Ras
Rac1 and Cdc42
MAPK 3
Raf
MEKK1/2
MEKK3
???
TAK
MAPK 2
MEK1
MEK2
MEK4
MEK 7
MEK3
MEK6
MAPK
ERK1
ERK2
JNK (SAPK)
p38
Transcription factors
c-myc
Elk-1
c-Jun
ATF2
MAX

Figure 21

Figure 22. EGFR-mediated activation of the Ras/Raf/MAPKinase signalling cascade. EGFR-ligation induces the rapid tyrosine phosphorylation of both Shc and BLNK/SLP-65 and an increase in the association of these molecules with the adaptor molecule Grb2 and Sos. Now Sos is recruited to the cell membrane, it directly activates Ras, which in-turn recruits and activates Raf-1 at the cell membrane. Raf-1 is then free to phosphorylate downstream MEKs which in turn phosphorylate their downstream substrates, the ERK-MAPKinases. Activated ERK-MAPKinase has both cytoplasmic and nuclear targets and its activity is tightly regulated post-activation by molecules such as phosphatases.

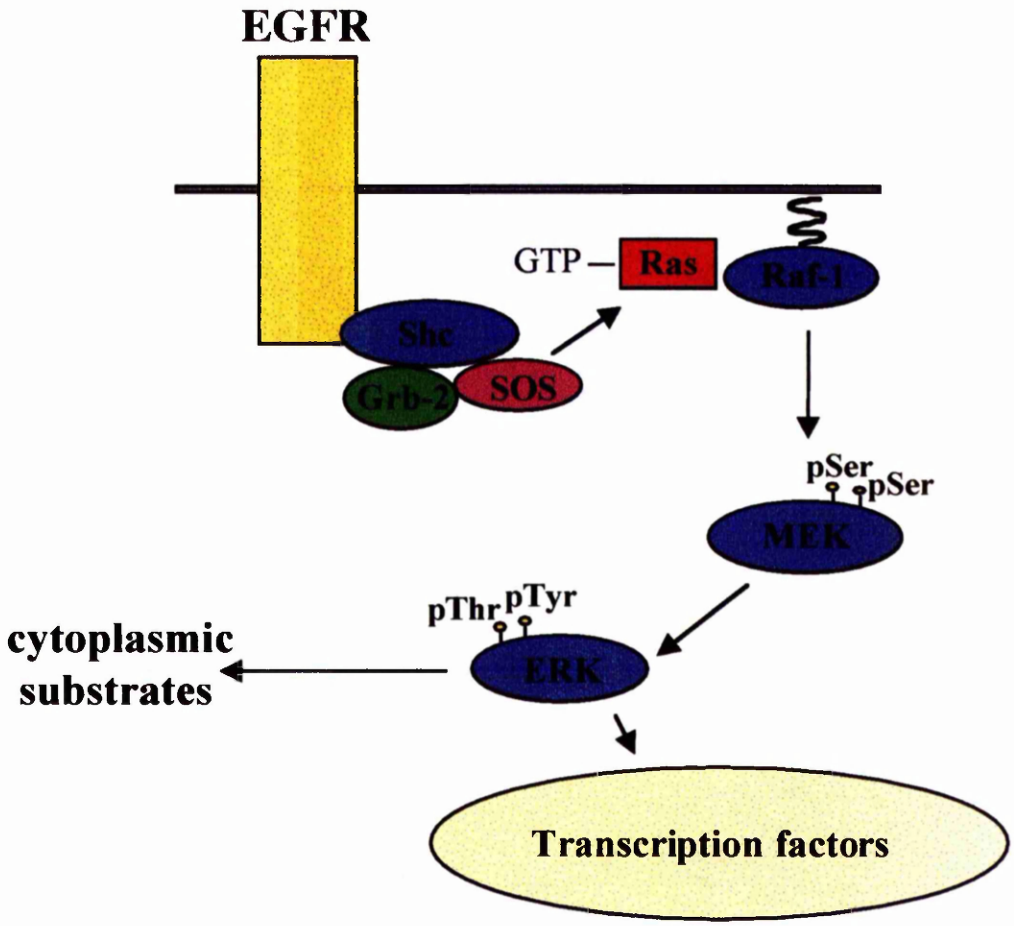

EGFR
GTP
Ras
Raf-1
Shc
Grb-2
SOS
pSer
pSer
MEK
pThr
pTyr
ERK
cytoplasmic
substrates
Transcription factors

figure 22

Figure 23. Summary diagram showing mechanisms leading to ERK-MAPKinase activation following BCR-ligation. BCR-ligation leads to the activation of ERK-MAPKinase by the activation of multiple signalling pathways. These include the classical Ras/Raf/MEK pathway regulated by the adaptor proteins Shc or BLNK, but also activation via DAG-sensitive PKC isoforms.

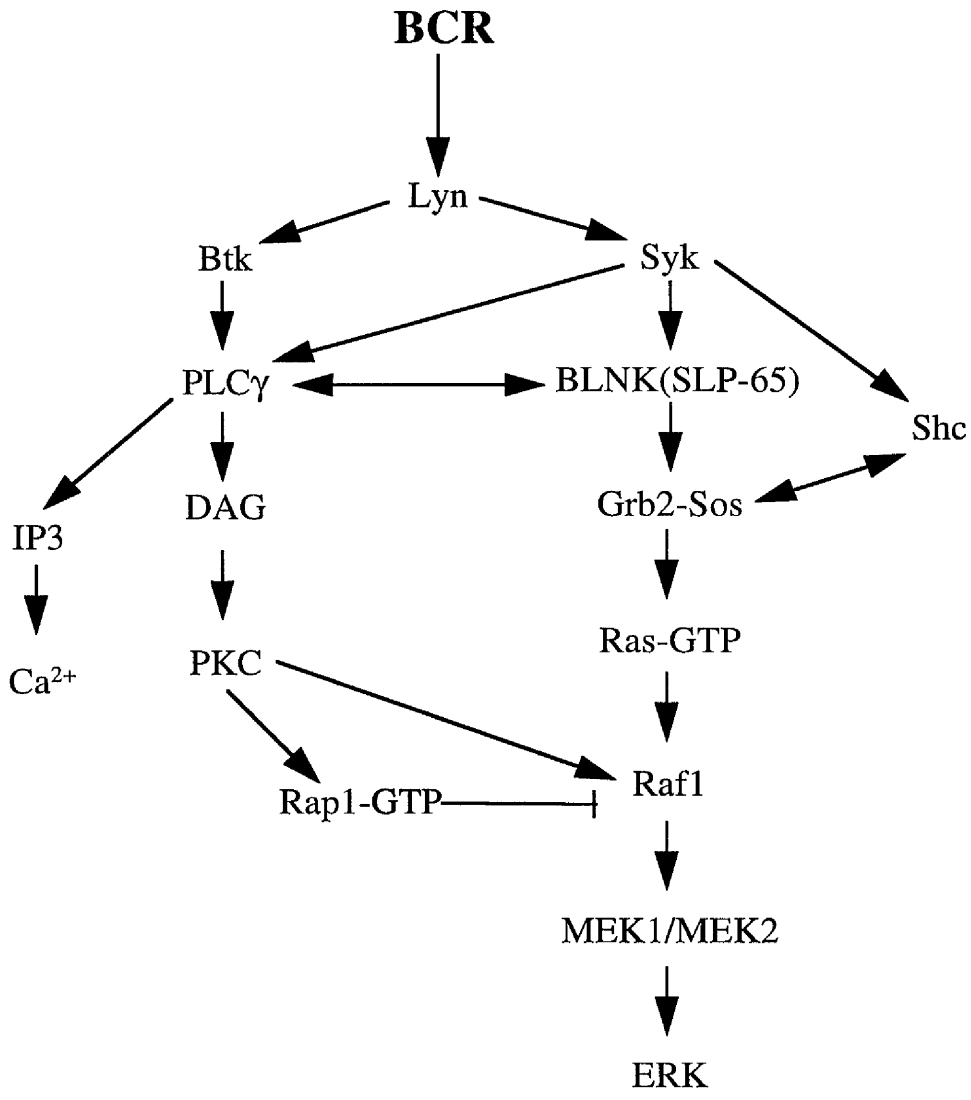

BCR
Lyn
Btk
Syk
PLCγ
BLNK(SLP-65)
Shc
IP3
DAG
Grb2-Sos
Ca2+
PKC
Ras-GTP
Rap1-GTP
Raf1
MEK1/MEK2
ERK

figure 23

Figure 24. Recent studies have shown that the BCR and CD40 utilise different pathways to activate ERK-MAPKinase which overlap at MEK1. Diagram adapted from Purkerson *et. al.*, (97).

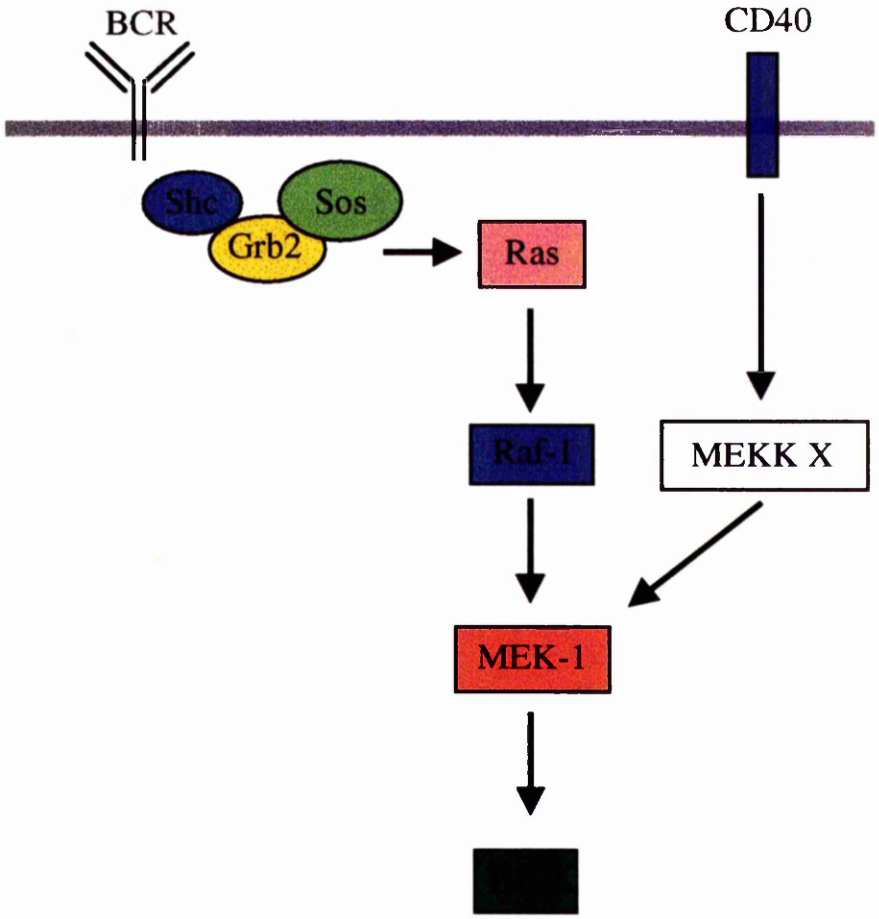
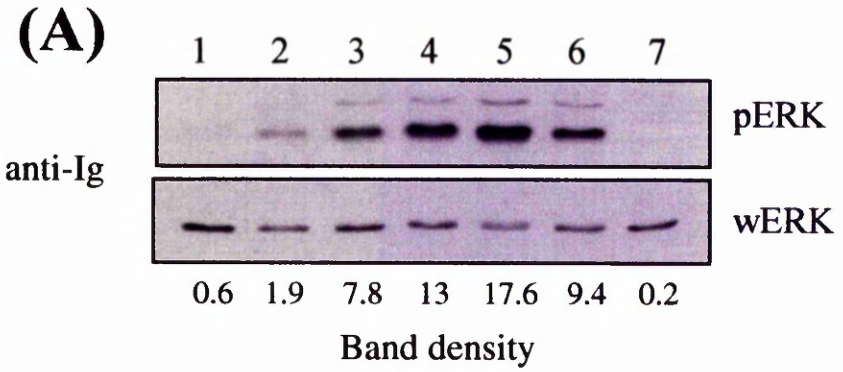
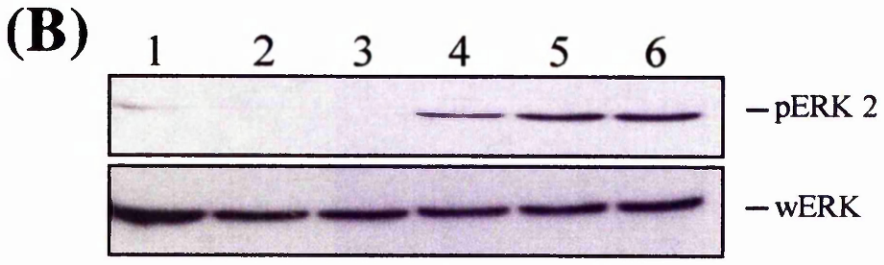


Figure 24

Figure 25. BCR-ligation activates an early but transient, Ras-dependent, ERK-MAPKinase signal. **A.** WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1,** cells left untreated for 0 minutes, **lane 2,** cells treated with anti-Ig (10 μg/ml) for 1 minute, **lane 3**, cells treated with anti-Ig (10 μg/ml) for 5 minutes, **lane 4**, cells treated with anti-Ig (10 μg/ml) for 10 minutes, **lane 5**, cells treated with anti-Ig (10 μg/ml) for 30 minutes, **lane 6**, cells treated with anti-Ig (10 μg/ml) for 60 minutes, **lane 7**, cells left untreated for 60 minutes. Levels of pERK/wERK expression were determined by Western blotting (15μg/lane). Densitometry shows relative band density of p42 ERK-MAPKinase measured on Gel-Pro Analysis software. **B.** WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1**, cells left untreated for 30 minutes, **lane 2**, cells treated with anti-Ig (0.1 μg/ml) for 30 minutes, **lane 3**, cells treated with anti-Ig (1 μg/ml) for 30 minutes, **lane 4**, cells treated with anti-Ig (5 μg/ml) for 30 minutes, **lane 5**, cells treated with anti-Ig (10 μg/ml) for 30 minutes, **lane 6**, cells treated with anti-Ig (25 μg/ml) for 30 minutes. Levels of pERK/wERK expression were determined by Western blotting (15μg/lane). **C.** WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1,** cells left untreated for 0 minutes, **lane 2,** cells treated with anti-Ig (10 μg/ml) for 1 minute, **lane 3**, cells treated with anti-Ig (10 μg/ml) for 5 minutes, **lane 4**, cells treated with anti-Ig (10 μg/ml) for 10 minutes, **lane 5**, cells treated with anti-Ig (10 μg/ml) for 30 minutes, **lane 6**, cells treated with anti-Ig (10 μg/ml) for 60 minutes, **lane 7**, cells left untreated for 60 minutes. GTP-bound Ras was then immunoprecipitated using Raf-1-RBD as bait. Ras activation was then determined by Western blotting using a anti-Ras antibody to equal levels of cell sample. Densitometry shows relative band density of Ras measured on Gel-Pro Analysis software.


(A)
1 2 3 4 5 6 7
anti-Ig
pERK
wERK
0.6 1.9 7.8 13 17.6 9.4 0.2
Band density


(B)
1 2 3 4 5 6
– pERK 2
– wERK

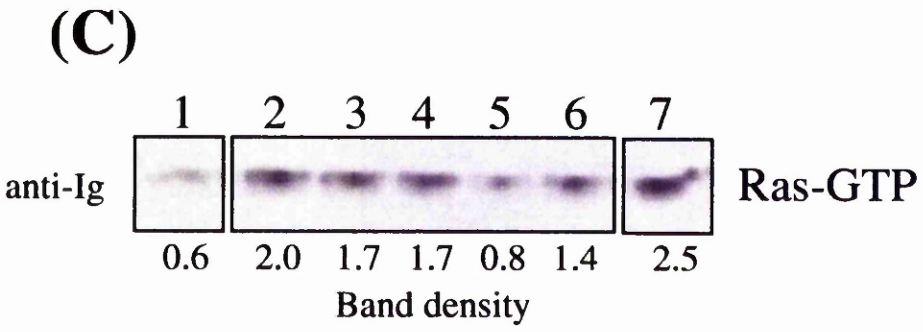

(C)
1 2 3 4 5 6 7
anti-Ig
Ras-GTP
0.6 2.0 1.7 1.7 0.8 1.4 2.5
Band density

Figure 25

Figure 26. Anti-Ig treatment induces a dose-dependent increase in WEHI-231 cell apoptosis.

WEHI-231 ($5x10^5$/ml) were cultured with increasing concentrations of anti-Ig (0, 0.1, 1, 10 and 50 μg/ml). Levels of apoptosis were determined by PI staining and FACS analysis after 48 hours as described in Materials and Methods.

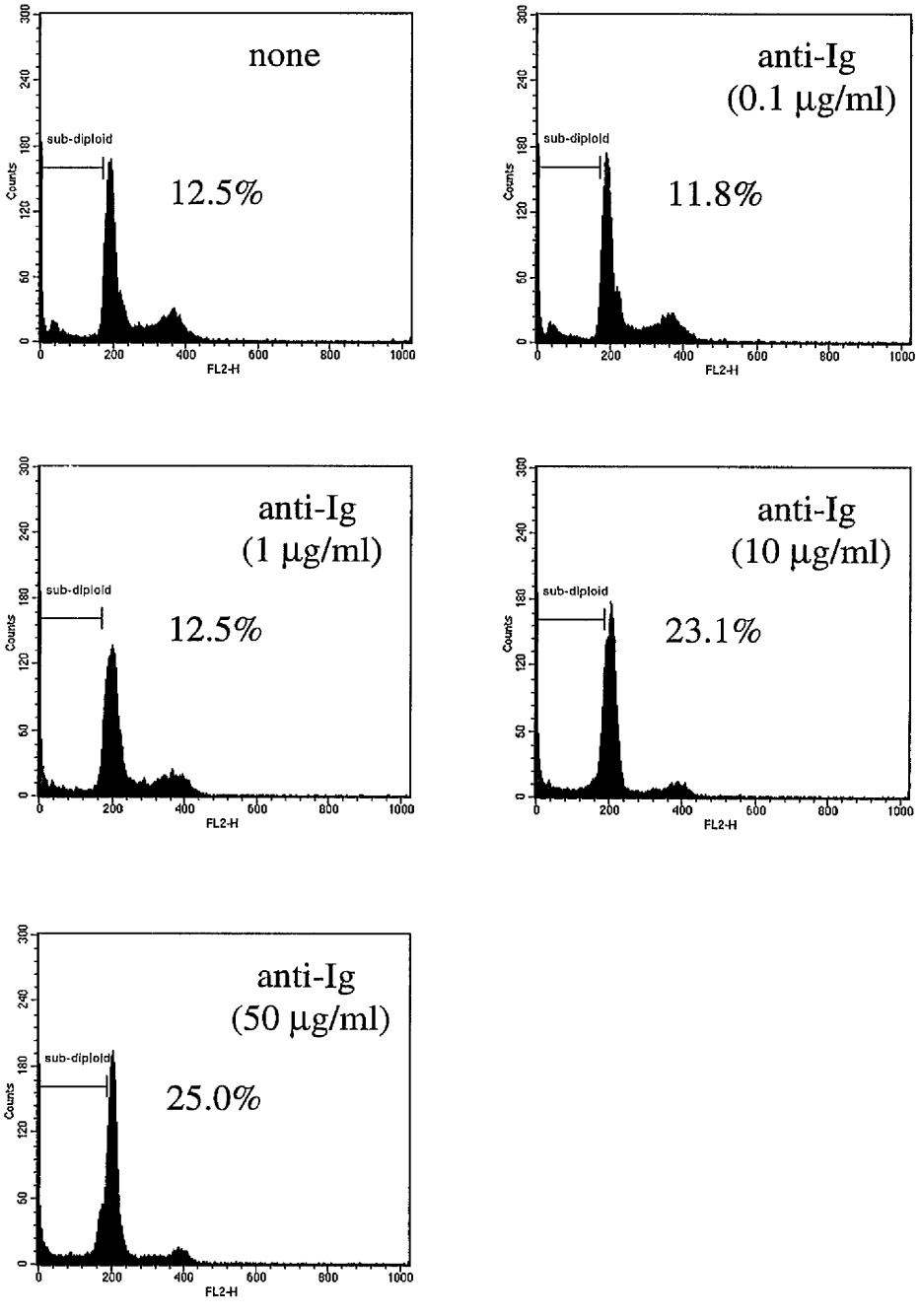

none
sub-diploid
12.5%
anti-Ig
(0.1 µg/ml)
sub-diploid
11.8%
anti-Ig
(1 µg/ml)
sub-diploid
12.5%
anti-Ig
(10 µg/ml)
sub-diploid
23.1%
anti-Ig
(50 µg/ml)
sub-diploid
25.0%
Counts
FL2-H

Figure 26

Figure 27. PD98059 and U0126 both inhibit anti-Ig induced ERK-MAPKinase activation in a time and dose-dependent manner. **A.**WEHI-231 cells (5 x 10^7/ml) were stimulated as follows: **lane 1**, cells left untreated for 0 minutes, **lane 2**, cells left untreated for 210 minutes, **lane 3**, cells treated with anti-Ig (10 μg/ml) for 30 minutes, **lane 4**, cells treated simultaneously with 0.1 μM PD98059 and anti-Ig (10 μg/ml) for 30 minutes, **lane 5**, cells treated simultaneously with 1 μM PD98059 and anti-Ig (10 μg/ml) for 30 minutes, **lane 6**, cells treated simultaneously with 5 μM PD98059 and anti-Ig (10 μg/ml) for 30 minutes , **lane 7**, cells were pre-incubated with PD98059 (0.1 μM) for 60 minutes and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 8**, cells were pre-incubated with PD98059 (1 μM) for 60 minutes and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 9**, cells were pre-incubated with PD98059 (5 μM) for 60 minutes and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 10**, cells were pre-incubated with PD98059 (0.1 μM) for 3 hours and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 11**, cells were pre-incubated with PD98059 (1 μM) for 3 hours and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 12**, cells were pre-incubated with PD98059 (5 μM) for 3 hours and then stimulated with anti-Ig (10 μg/ml) for 30 minutes. Levels of pERK/wERK expression were determined by Western blotting (15 μg/lane). **B.** WEHI-231 cells (5 x 10^7/ml) were stimulated as follows: **lane 1**, cells left untreated for 3 hours, **lane 2**, cells treated with anti-Ig (10 μg/ml) for 30 minutes, **lane 3**, cells pre-incubated with U0126 (0.1 μM) for 3 hours and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 4**, cells pre-incubated with U0126 (5 μM) for 3 hours and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 5**, cells pre-incubated with U0126 (20 μM) for 3 hours and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 6**, cells pre-incubated with U0126 (50 μM) for 3 hours and then stimulated with anti-Ig (10 μg/ml) for 30 minutes. Levels of pERK/wERK expression were determined by Western blotting (20 μg/lane).

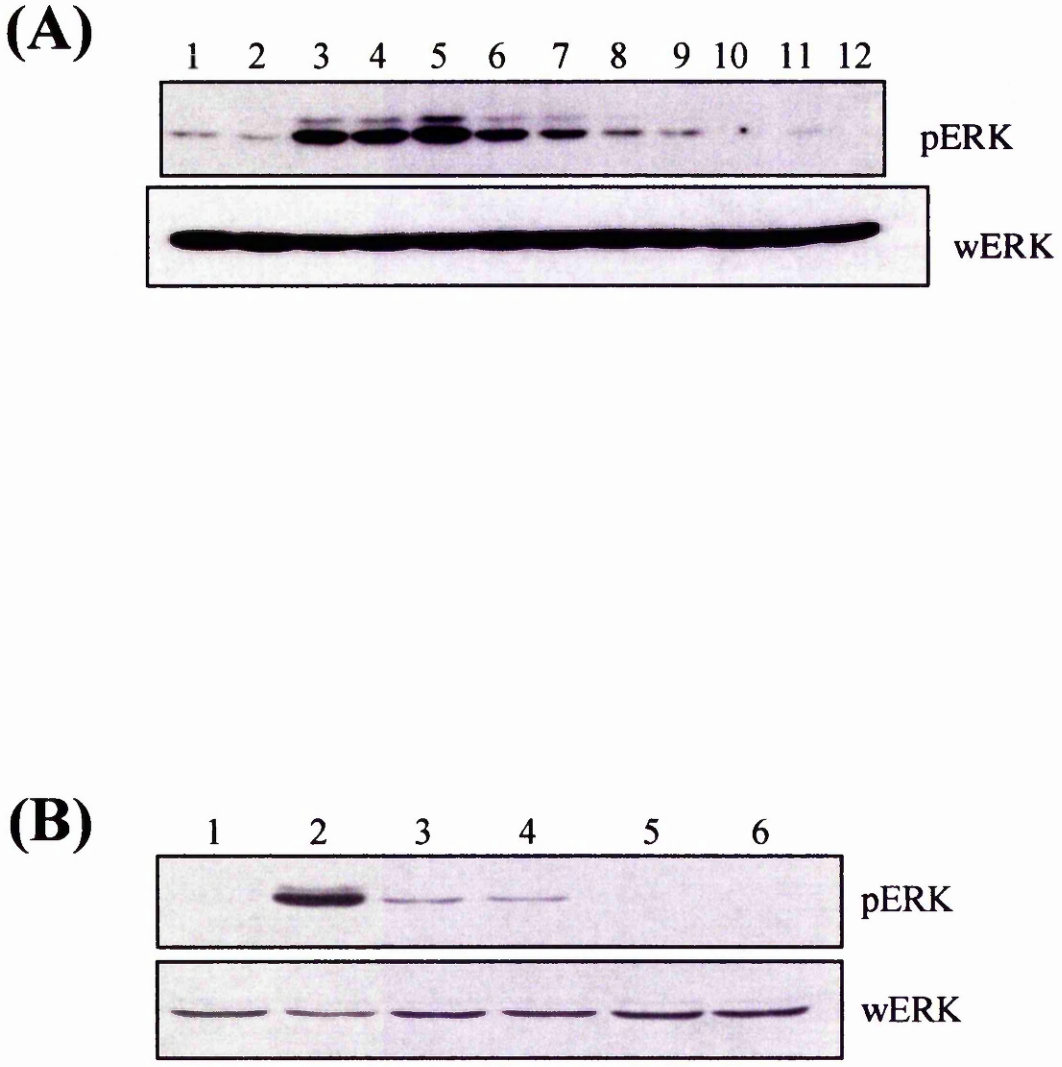


Figure 27

Figure 28. Inhibition of an early BCR-induced ERK-MAPKinase signal reduces apoptosis in WEHI-231 cells. WEHI-231 cells (5 x 10^5/ml) were pre-incubated with U0126 for two hours. Cells were then treated with the indicated stimuli (anti-Ig and anti-CD40 both at 10 μg/ml) for 48 hours. Levels of apoptosis were determined by PI staining and FACS analysis after 48 hours as described in Materials and Methods.

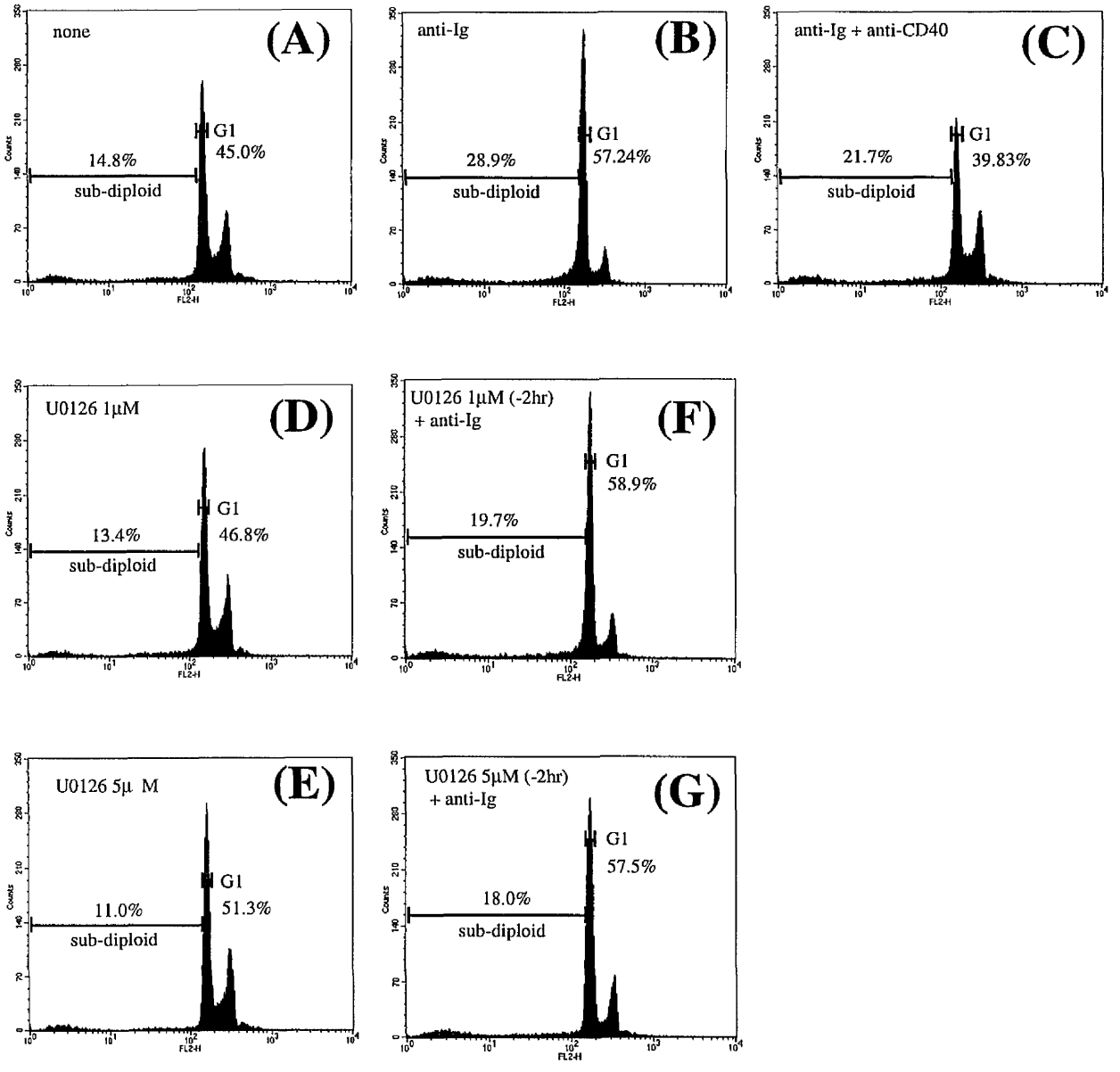

none
(A)
G1
45.0%
14.8%
sub-diploid
anti-Ig
(B)
G1
57.24%
28.9%
sub-diploid
anti-Ig + anti-CD40
(C)
G1
39.83%
21.7%
sub-diploid
U0126 1μM
(D)
G1
46.8%
13.4%
sub-diploid
U0126 1μM (-2hr)
+ anti-Ig
(F)
G1
58.9%
19.7%
sub-diploid
U0126 5μ M
(E)
G1
51.3%
11.0%
sub-diploid
U0126 5μM (-2hr)
+ anti-Ig
(G)
G1
57.5%
18.0%
sub-diploid

Figure 28

Figure 29. CD40 ligation does not activate ERK-MAPKinase in WEHI-231 cells. **A.** WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1,** cells left untreated for 0 minutes, **lane 2,** cells treated with anti-CD40 (10 μg/ml) for 1 minute, **lane 3**, cells treated with anti-CD40 (10 μg/ml) for 5 minutes, **lane 4**, cells treated with anti-CD40 (10 μg/ml) for 10 minutes, **lane 5**, cells treated with anti-CD40 (10 μg/ml) for 30 minutes, **lane 6**, cells treated with anti-CD40 (10 μg/ml) for 60 minutes, **lane 7**, cells left untreated for 60 minutes. Levels of pERK/wERK expression were determined by Western blotting (15μg/lane). **B.** WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1,** cells left untreated for 0 minutes, **lane 2,** cells treated with anti-Ig plus anti-CD40 (both 10 μg/ml) for 1 minute, **lane 3**, cells treated with anti-Ig plus anti-CD40 (both 10 μg/ml) for 5 minutes, **lane 4**, cells treated with anti-Ig plus anti-CD40 (both 10 μg/ml) for 10 minutes, **lane 5**, cells treated with anti-Ig plus anti-CD40 (both 10 μg/ml) for 30 minutes, **lane 6**, cells treated with anti-Ig plus anti-CD40 (10 μg/ml) for 60 minutes, **lane 7**, cells left untreated for 60 minutes. Levels of pERK/wERK expression were determined by Western blotting (15μg/lane). Densitometry shows p42 ERK-MAPKinase band density measured on Gel-Pro Analysis software. **C.** WEHI-231 cells ($1x10^8$/ml) WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1,** cells left untreated for 0 minutes, **lane 2,** cells treated with anti-Ig plus anti-CD40 (both 10 μg/ml) for 1 minute, **lane 3**, cells treated with anti-Ig plus anti-CD40 (both 10 μg/ml) for 5 minutes, **lane 4**, cells treated with anti-Ig plus anti-CD40 (both 10 μg/ml) for 10 minutes, **lane 5**, cells treated with anti-Ig plus anti-CD40 (both 10 μg/ml) for 30 minutes, **lane 6**, cells treated with anti-Ig plus anti-CD40 (10 μg/ml) for 60 minutes, **lane 7**, cells left untreated for 60 minutes. GTP-bound Ras was then immunoprecipitated using Raf-1-RBD as bait. Ras activation was then determined by Western blotting using a anti-Ras antibody to equal levels of cell sample. Densitometry shows band density of Ras measured on Gel-Pro Analysis software.

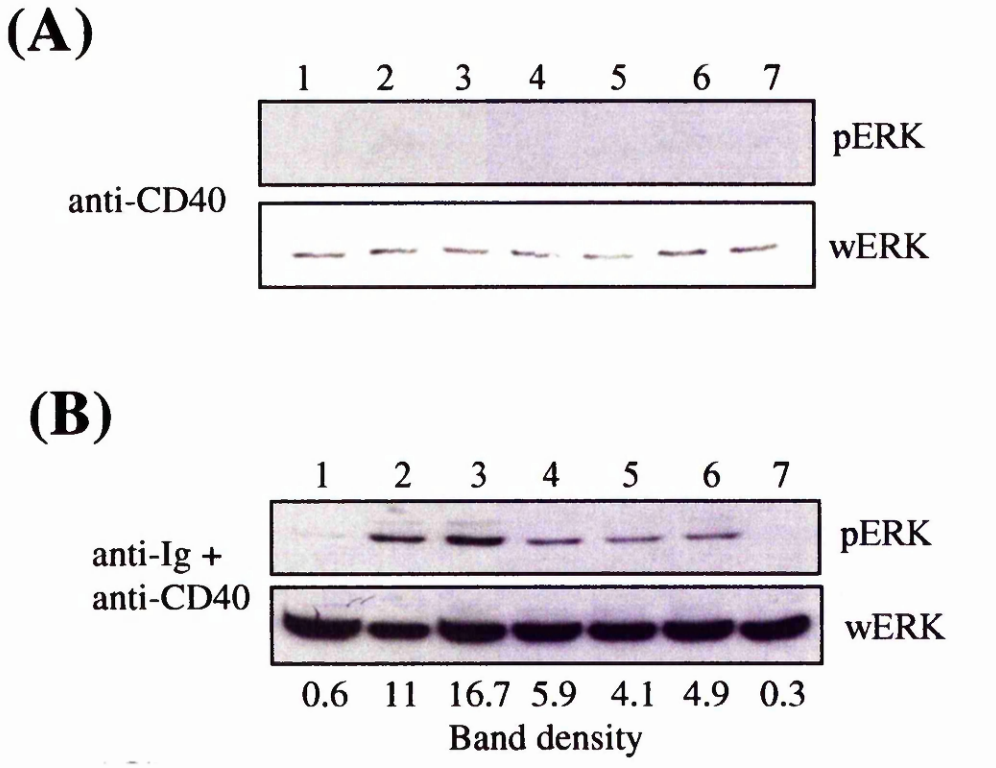

(A)
1 2 3 4 5 6 7
anti-CD40
pERK
wERK
(B)
1 2 3 4 5 6 7
anti-Ig +
anti-CD40
pERK
wERK
0.6 11 16.7 5.9 4.1 4.9 0.3
Band density
(C)
1 2 3 4 5 6 7
Ras-GTP
0.6 2.0 2.6 1.8 0.2 0.4 2.5
Band density

Figure 29

Figure 30. CD40 co-stimulation reduces BCR-mediated AA generation. Increasing concentrations (0.1-10 μg/ml as indicated) of anti-CD40 inhibit anti-Ig (1 μg/ml) stimulated PLA_2 activation as determined by the decreased generation of anti-Ig-stimulated [^{3}H]arachidonic acid production observed in WEHI-231 cells (30 minute stimulation).

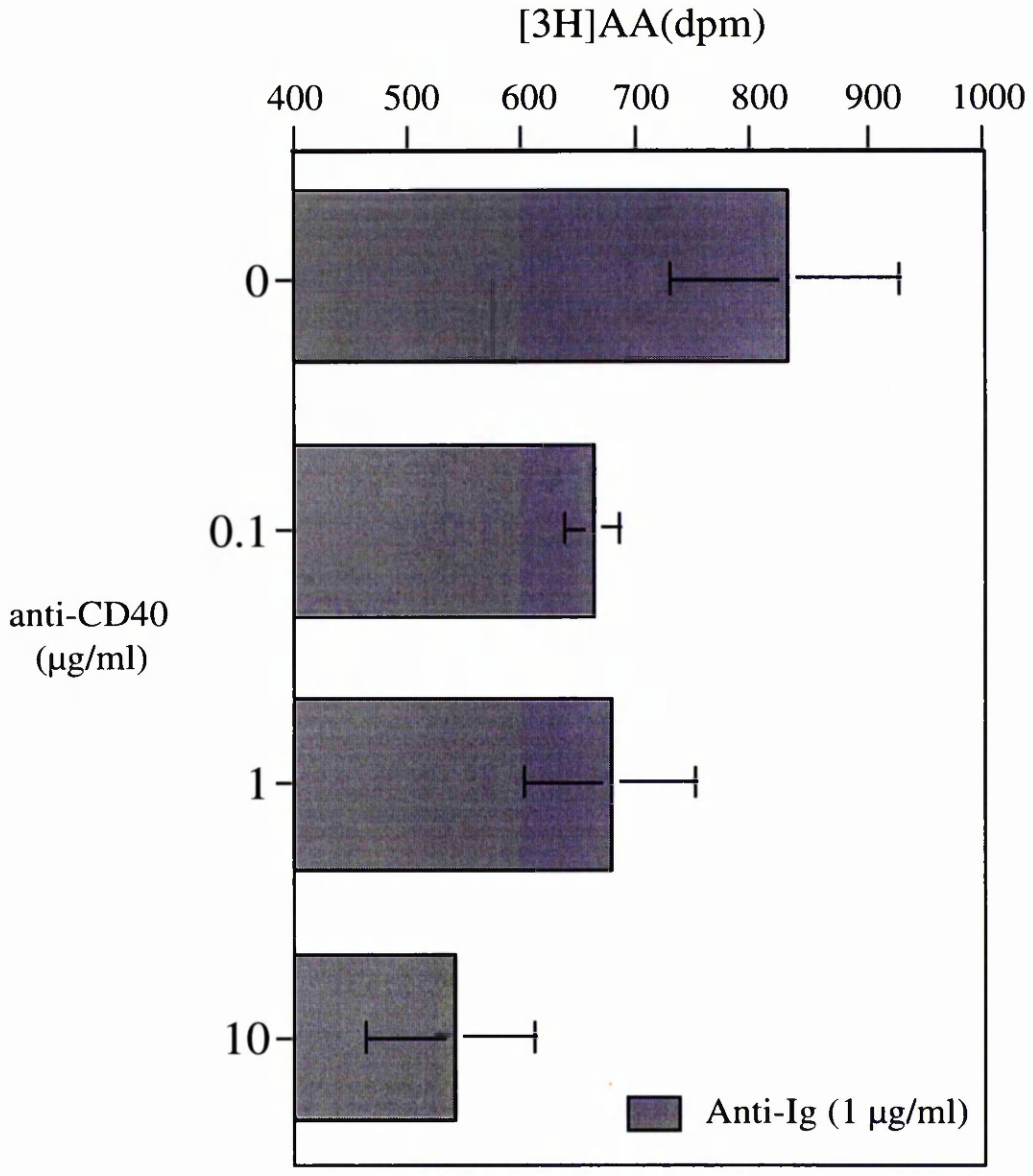

[3H]AA(dpm)
400
500
600
700
800
900
1000
0
0.1
1
10
anti-CD40
(μg/ml)
Anti-Ig (1 μg/ml)

Figure 30

Figure 31. BCR-ligation does not activate either p38-MAPKinase or JNK. **A.** WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1,** cells left untreated for 0 minutes, **lane 2,** cells treated with anti-Ig (10 μg/ml) for 1 minute, **lane 3**, cells treated with anti-Ig (10 μg/ml) for 5 minutes, **lane 4**, cells treated with anti-Ig (10 μg/ml) for 10 minutes, **lane 5**, cells treated with anti-Ig (10 μg/ml) for 30 minutes, **lane 6**, cells treated with anti-Ig (10 μg/ml) for 60 minutes, **lane 7**, cells left untreated for 60 minutes. Levels of pp38/wp38 expression were determined by Western blotting (15μg/lane). **B.** WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1,** cells left untreated for 0 minutes, **lane 2,** cells treated with anti-Ig (10 μg/ml) for 1 minute, **lane 3**, cells treated with anti-Ig (10 μg/ml) for 5 minutes, **lane 4**, cells treated with anti-Ig (10 μg/ml) for 10 minutes, **lane 5**, cells treated with anti-Ig (10 μg/ml) for 30 minutes, **lane 6**, cells treated with anti-Ig (10 μg/ml) for 60 minutes, **lane 7**, cells left untreated for 60 minutes. Levels of pp38/wp38 expression were determined by Western blotting (15μg/lane). **C.** WEHI-231 cells (5×10^7/ml) were stimulated as follows, **lane 1**, cells left untreated for 30 minutes, **lane 2**, cells left untreated for 60 minutes and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 3**, cells were pre-incubated with SB230580 (1 μM) for 60 minutes and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 4**, cells were pre-incubated with SB230580 (5 μM) for 60 minutes and then stimulated with anti-Ig (10 μg/ml) for 30 minutes pre-incubated with or without SB230580 (1 and 5μM) for 1 hour. Levels of pERK/wERK expression were determined by Western blotting (15 μg/lane).

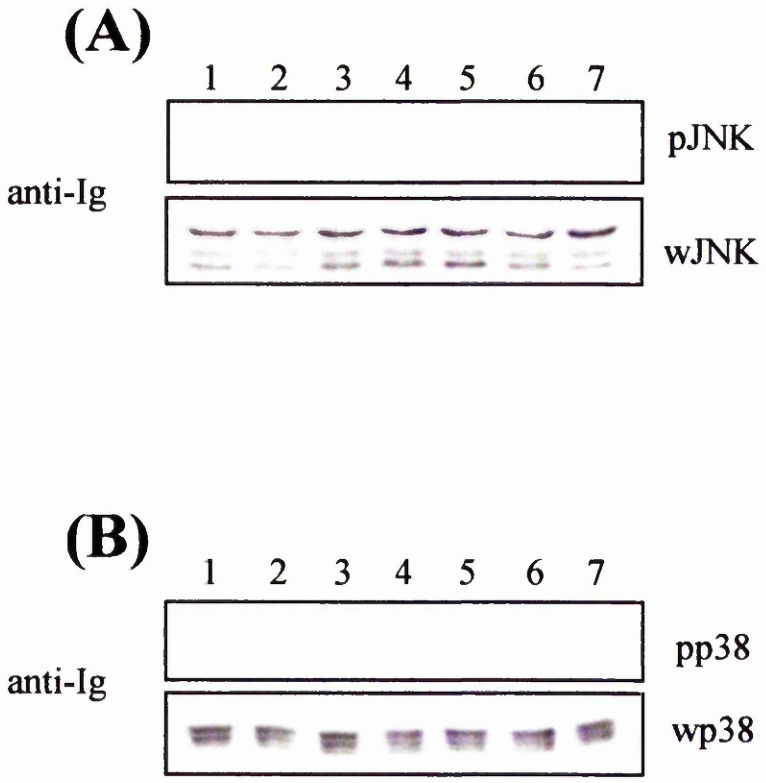

(A)
1 2 3 4 5 6 7
anti-Ig
pJNK
wJNK
(B)
1 2 3 4 5 6 7
anti-Ig
pp38
wp38
(C)
1 2 3 4
pERK
wERK
0.2 3.5 4.0 3.9

Figure 31

Figure 32. p38-MAPKinase is not essential for BCR-induced apoptosis. WEHI-231 cells (5 x 10^5/ml) were stimulated as indicated (anti-Ig and anti-CD40 both at 10μg/ml). Cells treated with SB203580 were pre-incubated in the presence of the indicated concentration of SB203580 for 90 minutes prior to further cell stimulation (if indicated). Levels of apoptosis were determined by PI staining and FACS analysis after 48 hours as described in Materials and Methods.

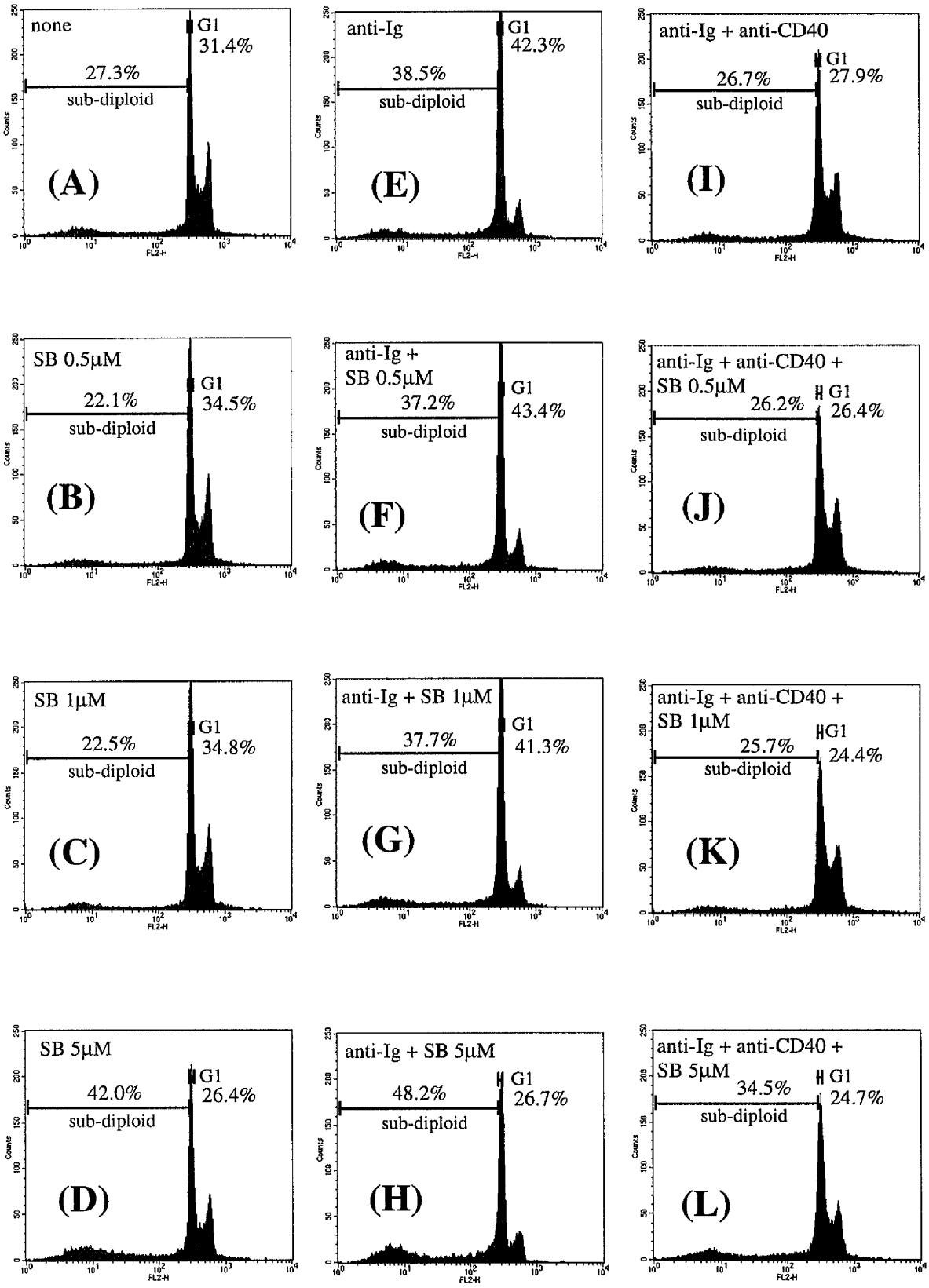

none
G1
31.4%
27.3%
sub-diploid
(A)
anti-Ig
G1
42.3%
38.5%
sub-diploid
(E)
anti-Ig + anti-CD40
G1
27.9%
26.7%
sub-diploid
(I)
SB 0.5μM
G1
34.5%
22.1%
sub-diploid
(B)
anti-Ig +
SB 0.5μM
G1
43.4%
37.2%
sub-diploid
(F)
anti-Ig + anti-CD40 +
SB 0.5μM
G1
26.4%
26.2%
sub-diploid
(J)
SB 1μM
G1
34.8%
22.5%
sub-diploid
(C)
anti-Ig + SB 1μM
G1
41.3%
37.7%
sub-diploid
(G)
anti-Ig + anti-CD40 +
SB 1μM
G1
24.4%
25.7%
sub-diploid
(K)
SB 5μM
G1
26.4%
42.0%
sub-diploid
(D)
anti-Ig + SB 5μM
G1
26.7%
48.2%
sub-diploid
(H)
anti-Ig + anti-CD40 +
SB 5μM
G1
24.7%
34.5%
sub-diploid
(L)
Counts
FL2-H

figure 32

Figure 33. Anti-Ig treatment causes the long-term down-regulation of pERK expression which can be re-established by co-stimulation with anti-CD40. A, WEHI-231 cells (5 x 10^5/ml) were stimulated as follows: **lane 1**, cells left untreated for 0 hours, **lane 2**, cells left untreated for 1 hour, **lane 3**, cells left untreated for 2 hours, **lane 4**, cells left untreated for 4 hours, **lane 5**, cells left untreated for 8 hours, **lane 6**, cells left untreated for 24 hours, **lane 7**, cells left untreated for 48 hours. Levels of pERK/wERK expression were determined by Western blotting (30 μg/lane). Densitometric analysis shows fold increase of p42 ERK-MAPKinase relative to lane 1. B, WEHI-231 cells (5 x 10^5/ml) were stimulated as follows, **lane 1**, cells left untreated for 0 hours, **lane 2**, cells treated with anti-Ig (1 μg/ml) for 1 hour, **lane 3**, cells treated with anti-Ig (1 μg/ml) for 2 hours, **lane 4**, cells treated with anti-Ig (1 μg/ml) for 4 hours, **lane 5**, cells treated with anti-Ig (1 μg/ml) for 8 hours, **lane 6**, cells treated with anti-Ig (1 μg/ml) for 24 hours, **lane 7**, cells treated with anti-Ig (1 μg/ml) for 48 hours. Levels of pERK/wERK expression were determined by Western blotting (30 μg/lane). Densitometric analysis shows fold increase of p42 ERK-MAPKinase relative to lane 1. C, WEHI-231 cells (5 x 10^5/ml) were stimulated as follows, **lane 1**, cells left untreated for 0 hours, **lane 2**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 1 hour, **lane 3**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 2 hours, **lane 4**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 4 hours, **lane 5**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 8 hours, **lane 6**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 24 hours, **lane 7**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 48 hours. Levels of pERK/wERK expression were determined by Western blotting (30 μg/lane). D, WEHI-231 cells (5 x 10^5/ml) were stimulated as follows, **lane 1**, cells left untreated for 0 hours, **lane 2**, cells treated with anti-CD40 (10 μg/ml) for 1 hour, **lane 3**, cells treated with anti-CD40 (10 μg/ml) for 2 hours, **lane 4**, cells treated with anti-CD40 (10 μg/ml) for 4 hours, **lane 5**, cells treated with anti-CD40 (10 μg/ml) for 8 hours, **lane 6**, cells treated with anti-CD40 (10 μg/ml) for 24 hours, **lane 7**, cells treated with anti-CD40 (10 μg/ml) for 48 hours. Levels of pERK/wERK expression were determined by Western blotting (30 μg/lane). Densitometric analysis shows fold increase of p42 ERK-MAPKinase relative to lane 1.

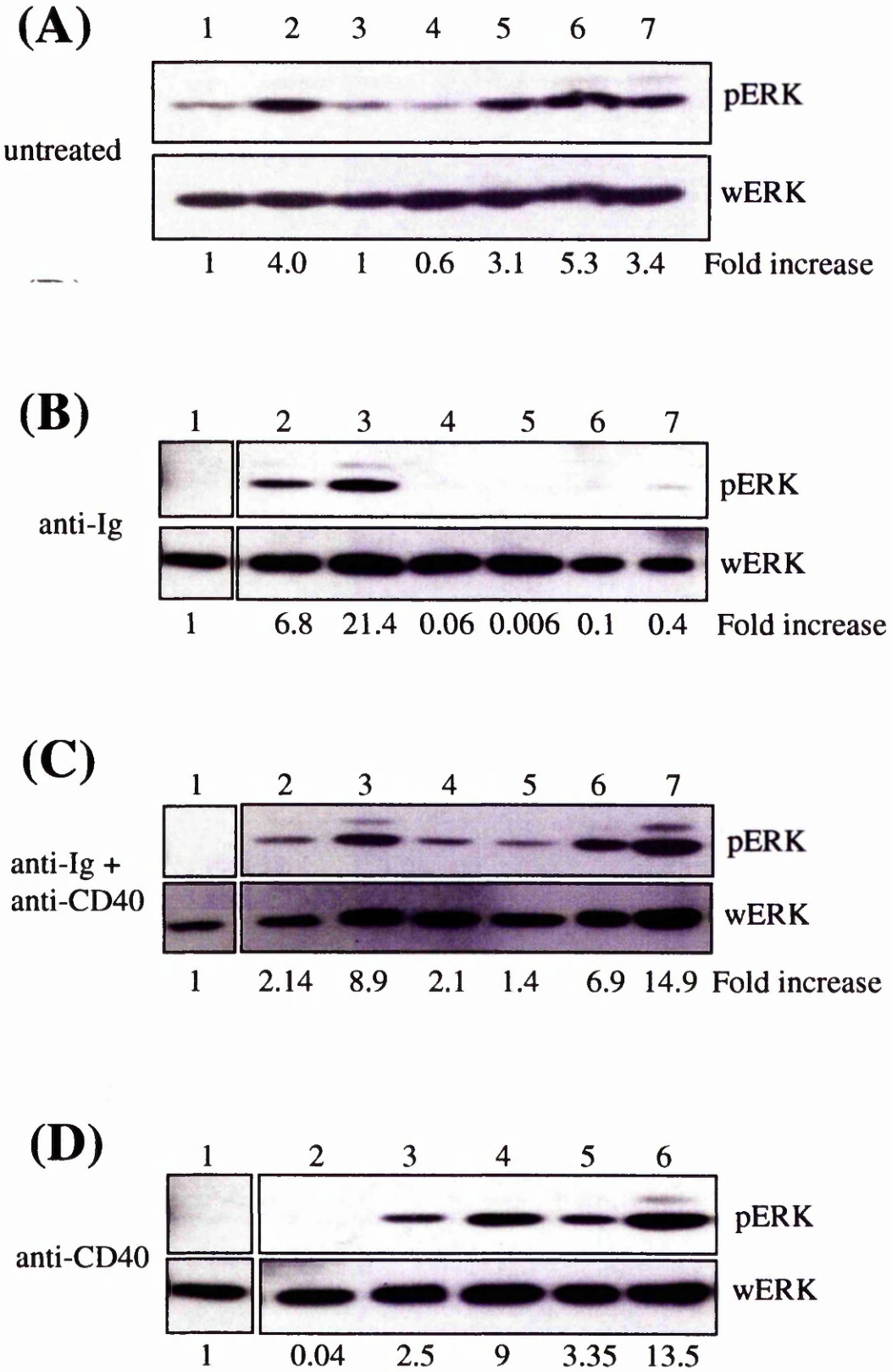

(A)
1 2 3 4 5 6 7
untreated
pERK
wERK
1 4.0 1 0.6 3.1 5.3 3.4 Fold increase
(B)
1 2 3 4 5 6 7
anti-Ig
pERK
wERK
1 6.8 21.4 0.06 0.006 0.1 0.4 Fold increase
(C)
1 2 3 4 5 6 7
anti-Ig +
anti-CD40
pERK
wERK
1 2.14 8.9 2.1 1.4 6.9 14.9 Fold increase
(D)
1 2 3 4 5 6
anti-CD40
pERK
wERK
1 0.04 2.5 9 3.35 13.5

Figure 33

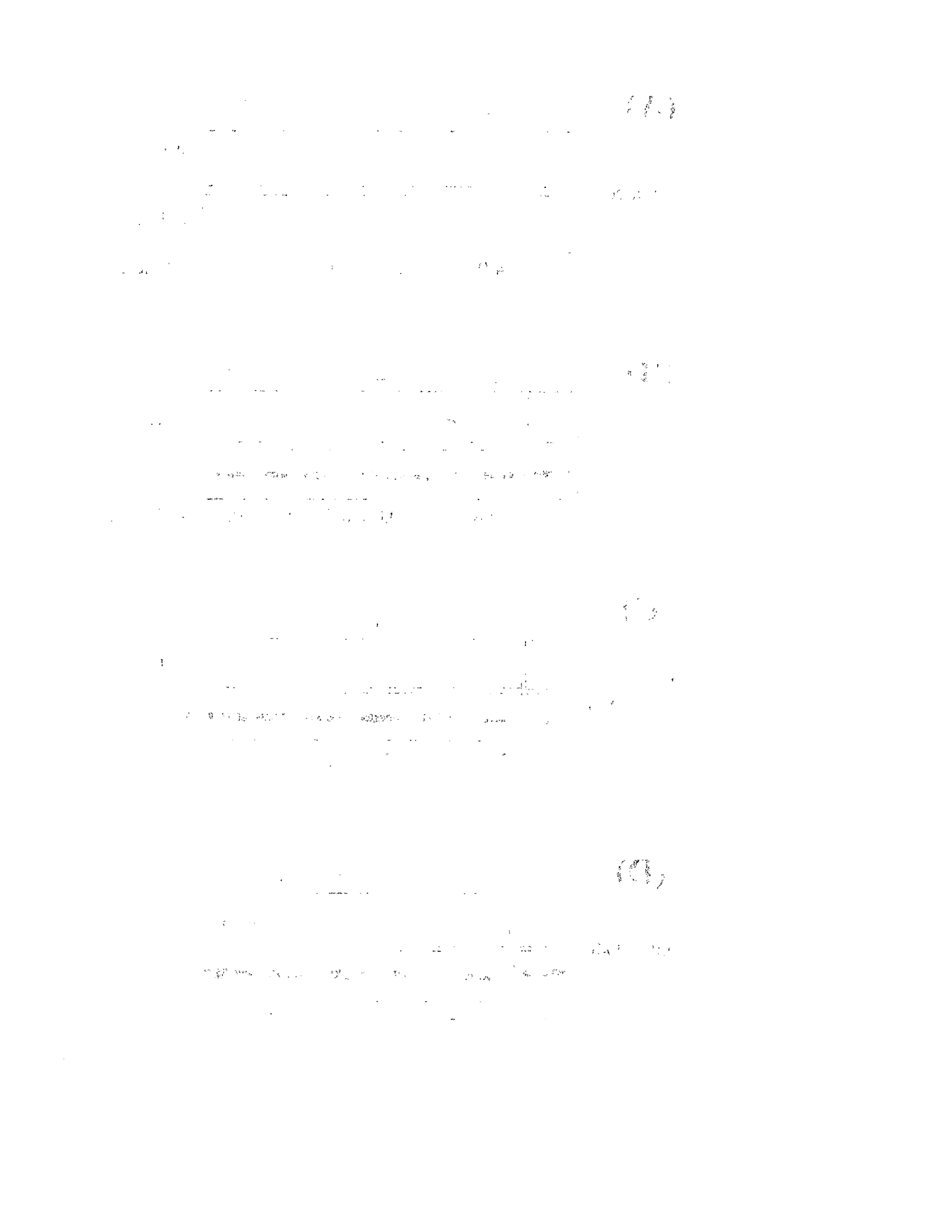


Figure 34. Activation of p38-MAPKinase or JNK in WEHI-231 cells. A, WEHI-231 cells (5 x 10^5/ml) were stimulated as follows: **lane 1**, cells left untreated for 0 hours, **lane 2**, cells left untreated for 1 hour, **lane 3**, cells left untreated for 2 hours, **lane 4**, cells left untreated for 4 hours, **lane 5**, cells left untreated for 8 hours, **lane 6**, cells left untreated for 24 hours, **lane 7**, cells left untreated for 48 hours, **lane 8**, hypoxic rat fibroblast positive control. Levels of pp38/wp38-MAPKinase expression were determined by Western blotting (30 μg/lane). B, WEHI-231 cells (5 x 10^5/ml) were stimulated as follows, **lane 1**, cells left untreated for 0 hours, **lane 2**, cells treated with anti-Ig (1 μg/ml) for 1 hour, **lane 3**, cells treated with anti-Ig (1 μg/ml) for 2 hours, **lane 4**, cells treated with anti-Ig (1 μg/ml) for 4 hours, **lane 5**, cells treated with anti-Ig (1 μg/ml) for 8 hours, **lane 6**, cells treated with anti-Ig (1 μg/ml) for 24 hours, **lane 7**, cells treated with anti-Ig (1 μg/ml) for 48 hours. Levels of p-p38/w-p38-MAPKinase expression were determined by Western blotting (30 μg/lane). C, WEHI-231 cells (5 x 10^5/ml) were stimulated as follows, **lane 1**, cells left untreated for 0 hours, **lane 2**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 1 hour, **lane 3**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 2 hours, **lane 4**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 4 hours, **lane 5**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 8 hours, **lane 6**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 24 hours, **lane 7**, cells treated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 48 hours. Levels of pp38/w38 MAPKinase expression were determined by Western blotting (30 μg/lane). D, WEHI-231 cells (5 x 10^5/ml) were stimulated as follows, **lane 1**, cells left untreated for 0 hours, **lane 2**, cells treated with anti-Ig (1 μg/ml) for 1 hour, **lane 3**, cells treated with anti-Ig (1 μg/ml) for 2 hours, **lane 4**, cells treated with anti-Ig (1 μg/ml) for 4 hours, **lane 5**, cells treated with anti-Ig (1 μg/ml) for 8 hours, **lane 6**, cells treated with anti-Ig (1 μg/ml) for 24 hours, **lane 7**, cells treated with anti-Ig (1 μg/ml) for 48 hours. Levels of pJNK/wJNK expression were determined by Western blotting (30 μg/lane).

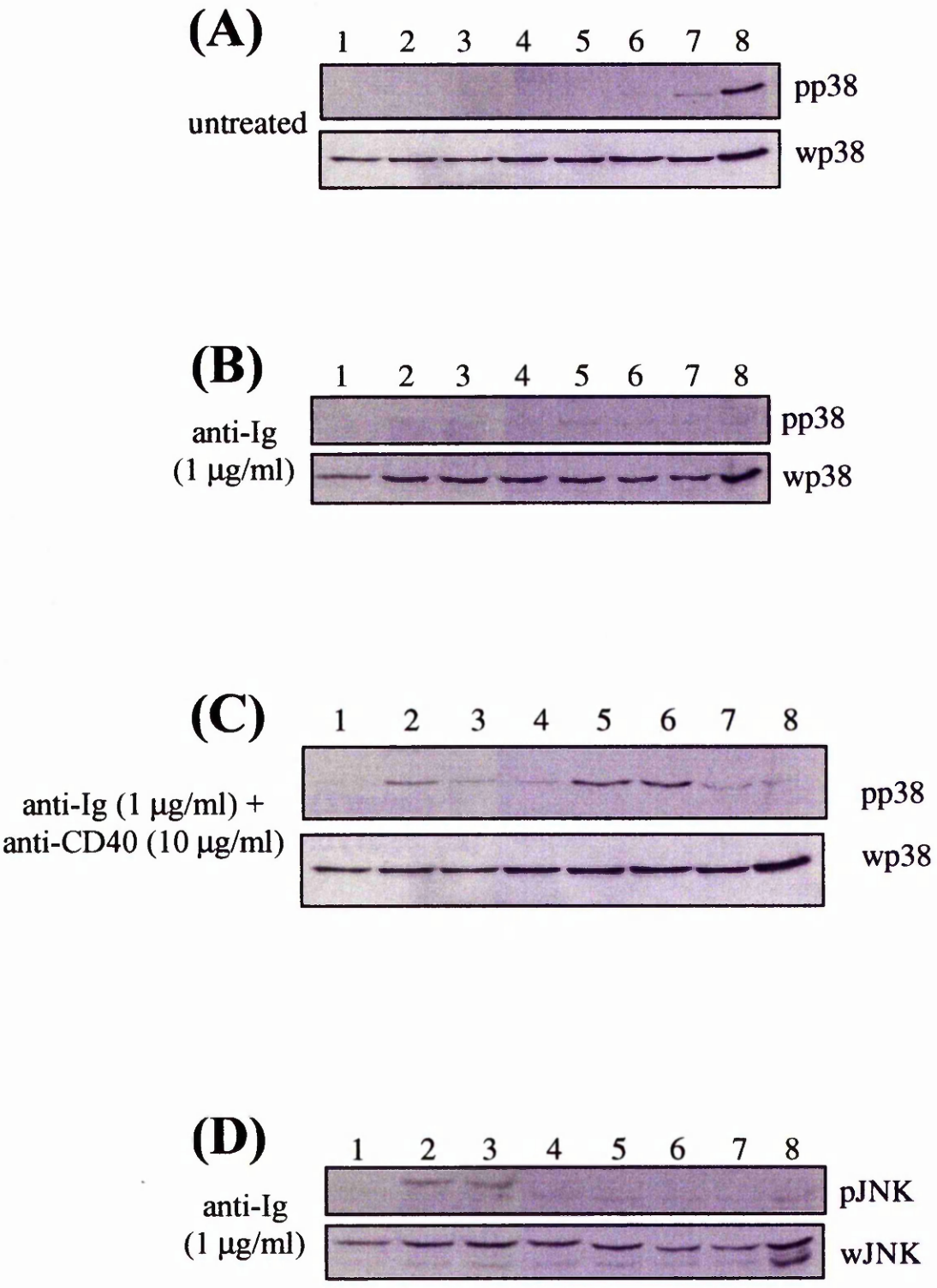
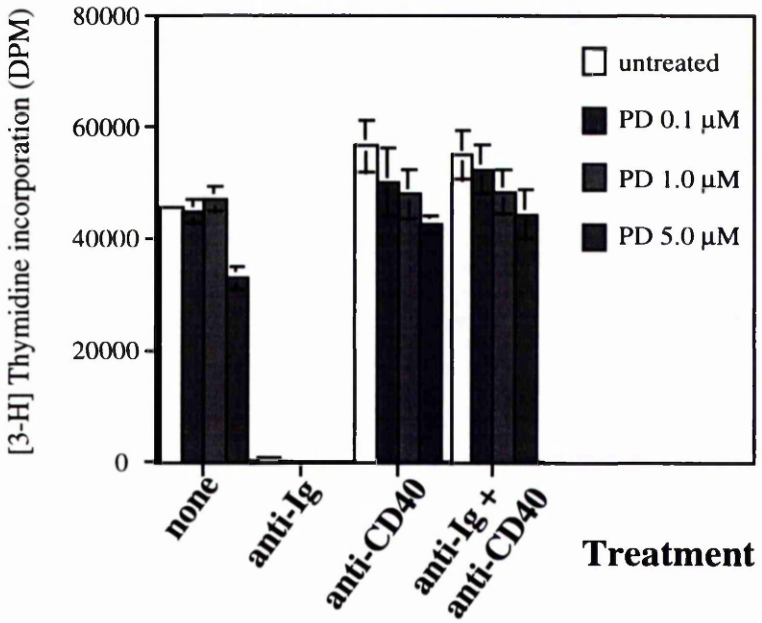

(A)
1 2 3 4 5 6 7 8
untreated
pp38
wp38
(B)
1 2 3 4 5 6 7 8
anti-Ig
(1 µg/ml)
pp38
wp38
(C)
1 2 3 4 5 6 7 8
anti-Ig (1 µg/ml) +
anti-CD40 (10 µg/ml)
pp38
wp38
(D)
1 2 3 4 5 6 7 8
anti-Ig
(1 µg/ml)
pJNK
wJNK

Figure 34

Figure 35. ERK activity is not involved in CD40-mediated rescue from growth arrest. A. WEHI-231 cells (10^4 cells/well) were pre-incubated for 3 hours in PD98059 (0.1-5 μM) prior to stimulation with anti-Ig (10 μg/ml); anti-CD40 (10 μg/ml) or a combination of both. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells. **B.** WEHI-231 cells (10^4 cells/well) were pre-incubated for 3 hours in PD98059 (0.1-5 μM) prior to stimulation with anti-Ig (0.001-10 μg/ml) or otherwise left untreated. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells.

(A)


[3-H] Thymidine incorporation (DPM)
80000
60000
40000
20000
0
untreated
PD 0.1 µM
PD 1.0 µM
PD 5.0 µM
none
anti-Ig
anti-CD40
anti-Ig + anti-CD40
Treatment

(B)

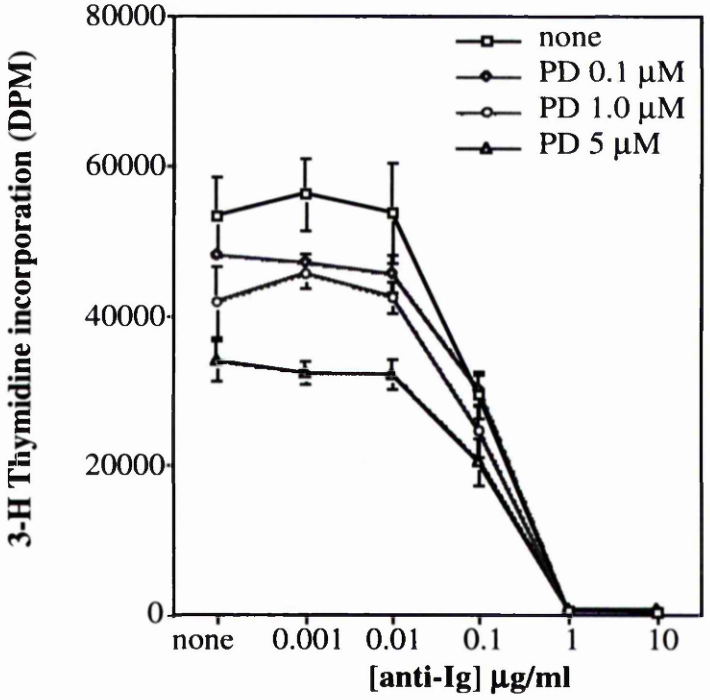

3-H Thymidine incorporation (DPM)
80000
60000
40000
20000
0
none
PD 0.1 µM
PD 1.0 µM
PD 5 µM
none
0.001
0.01
0.1
1
10
[anti-Ig] µg/ml

Figure 35

Figure 36. U0126 enhances basal and anti-Ig-mediated growth-arrest. **A.** WEHI-231 cells (10^4 cells/well) were left untreated or treated with 10μg/ml anti-Ig or 10μg/ml anti-Ig and anti-CD40. Were indicated, cells were pre-incubated for 3 hours in U0126 (1, 5, or 20μM) prior to stimulation with anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml) or left otherwise untreated. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells. **B.** WEHI-231 cells (10^4 cells/well) were pre-incubated for 3 hours in U0126(0.05-10 μM) prior to stimulation with anti-Ig (0.001-10 μg/ml) or otherwise left untreated. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells.

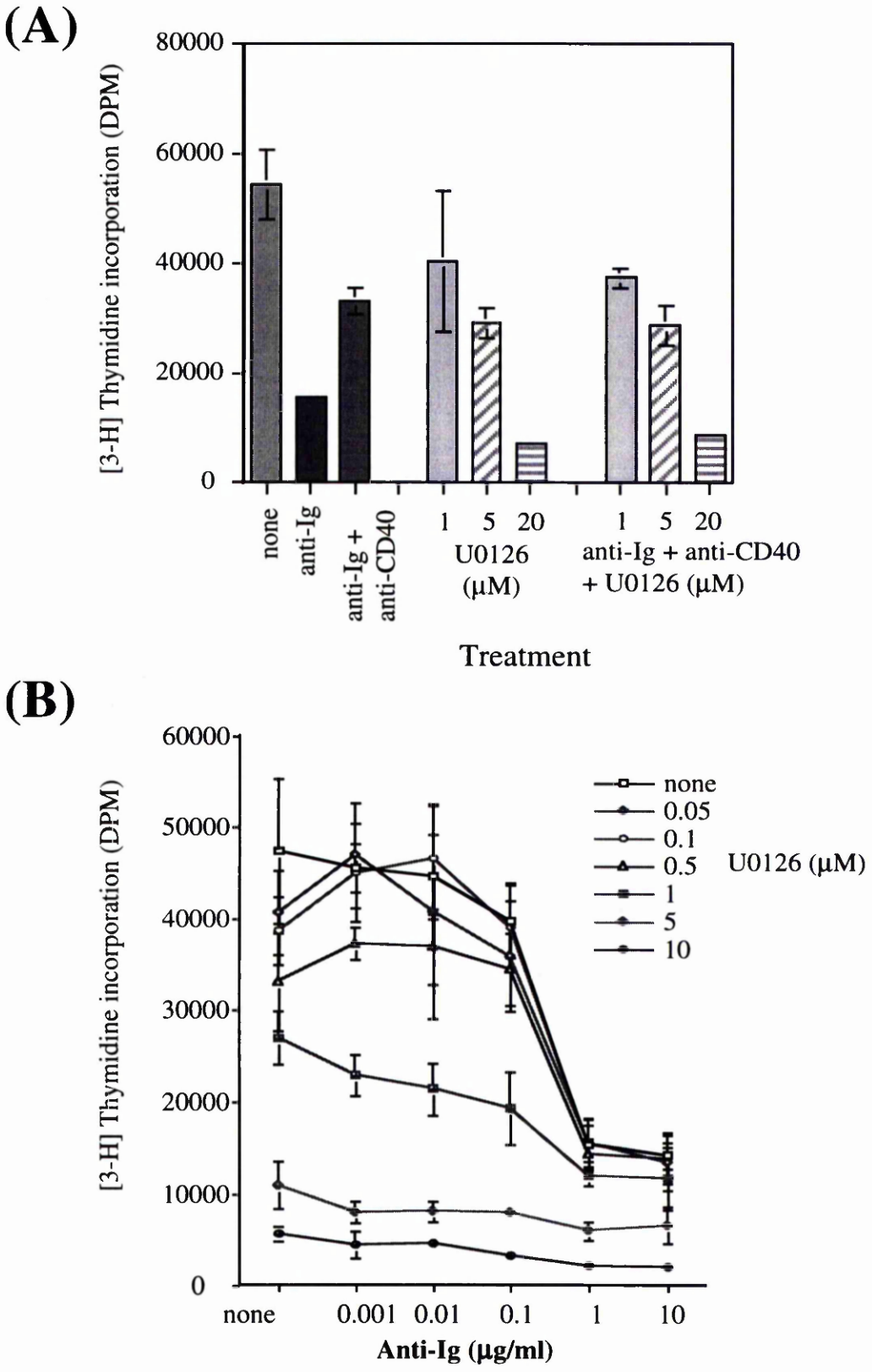
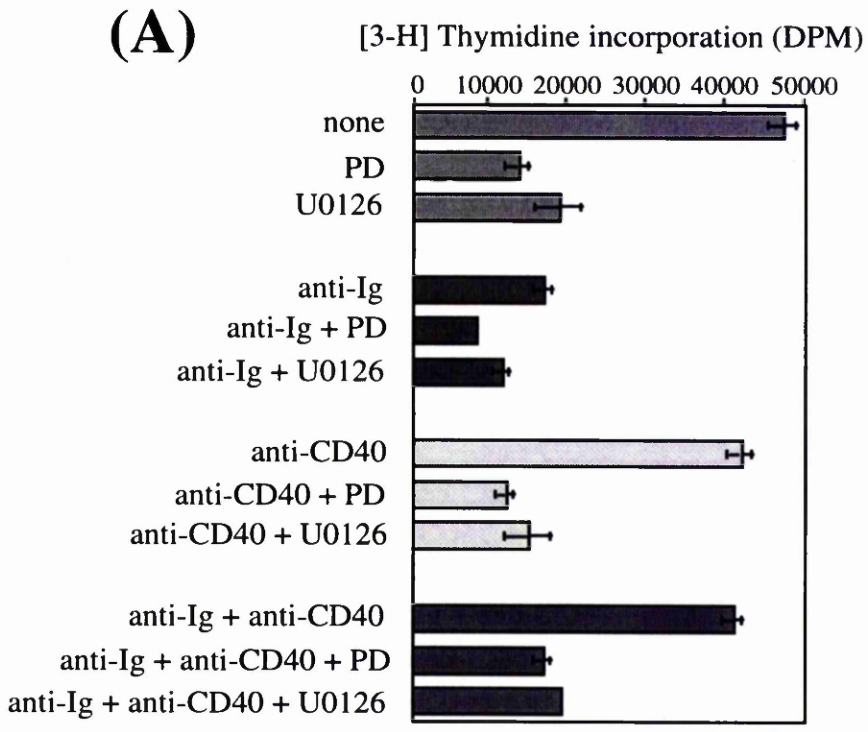

(A)
80000
60000
40000
20000
0
[3-H] Thymidine incorporation (DPM)
none
anti-Ig
anti-Ig + anti-CD40
1 5 20
U0126 (μM)
1 5 20
anti-Ig + anti-CD40 + U0126 (μM)
Treatment
(B)
60000
50000
40000
30000
20000
10000
0
[3-H] Thymidine incorporation (DPM)
none
0.05
0.1
0.5
1
5
10
U0126 (μM)
none
0.001
0.01
0.1
1
10
Anti-Ig (μg/ml)

Figure 36

Figure 37. Sustained ERK inhibition prevents normal cell proliferation BUT also inhibits CD40-mediated rescue from anti-Ig induced growth arrest. A. WEHI-231 cells (10^4 cells/well) were pre-incubated for 3 hours with PD98059 or U0126 (both 1μM). Cells were then treated as indicated with 10μg/ml anti-Ig or 10μg/ml anti-CD40 or a combination of both. Cells treated with PD98059 and U0124 were treated with an additional dose (1 μM) every 4 hours for a total of 28 hours post-pre-incubation. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells. **B.** WEHI-231 cells (10^4 cells/well) were pre-incubated for 3 hours with PD98059 and U0126 (both 1 μM). Cells were then treated as indicated with 10μg/ml anti-Ig or 10μg/ml anti-CD40 or a combination of both. Cells treated with PD98059 and U0124 were treated with an additional dose (1 μM) every 4 hours for a total of 28 hours post-pre-incubation. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells.


(A)
[3-H] Thymidine incorporation (DPM)
0
10000
20000
30000
40000
50000
none
PD
U0126
anti-Ig
anti-Ig + PD
anti-Ig + U0126
anti-CD40
anti-CD40 + PD
anti-CD40 + U0126
anti-Ig + anti-CD40
anti-Ig + anti-CD40 + PD
anti-Ig + anti-CD40 + U0126

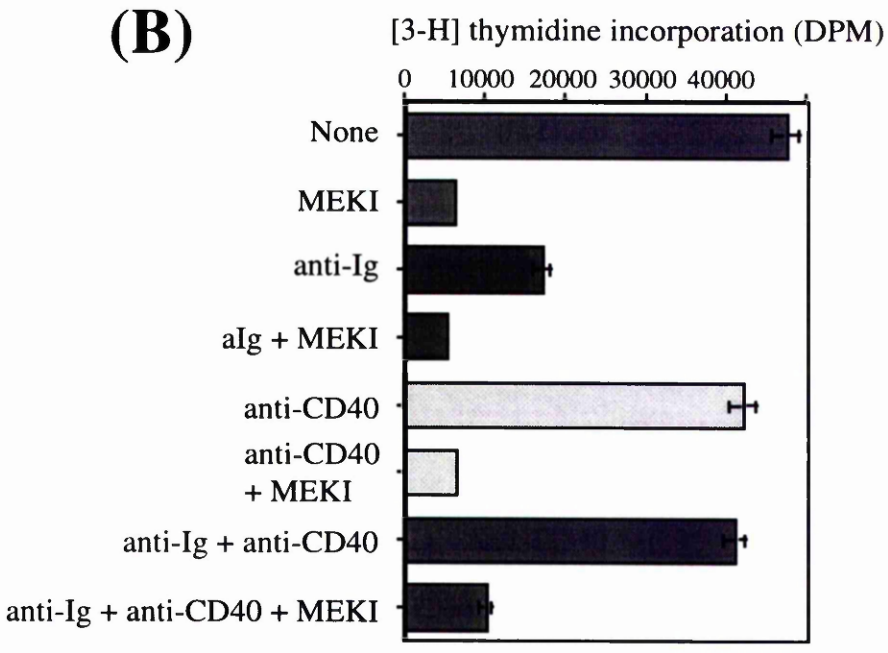

(B)
[3-H] thymidine incorporation (DPM)
0
10000
20000
30000
40000
None
MEKI
anti-Ig
aIg + MEKI
anti-CD40
anti-CD40
+ MEKI
anti-Ig + anti-CD40
anti-Ig + anti-CD40 + MEKI

Figure 37

TABLE 1. ERK inhibition drives cells into apoptosis which is reversed by anti-CD40 treatment. WEHI-231 cells (5 x 10^5/ml) were pre-incubated with PD98059 (1 μM) and U0126 (1 μM) or a combination of the two for 3 hours. Cells were then stimulated as indicated with 10 μg/ml anti-Ig or 10 μg/ml anti-CD40 or a combination of both. Cells originally treated with PD98059 or U0126 (or both) were treated with additional doses (1 μM) every 4 hours for a total of 28 hours post-pre-incubation. Cells were then harvested after a total of 48 hours and DNA content analysed by PI staining and FACS analysis as described in Materials and Methods.

Condition(s)	Apoptotic	G1	S	G2
untreated	7.1	48.7	29.1	15.7
untreated + PD/U0126	81.8	15.9	2.9	0.2
aIg	85.1	11.8	3.4	0.2
aIg + PD/U0126	3.5	66.2	14.5	16.0
aCD40	4.6	46.5	25.8	21.5
aCD40 + PD/U0126	5.4	60.4	19.8	14.5
I/C	9.8	52.4	23.3	14.5
I/C + PD/U0126	6.6	58.5	20.7	14.1

Table 1

Figure 38. Inhibition of ERK-MAPKinase suppresses cell division in WEHI-231 cells. WEHI-231 cells were loaded with the cell permeable dye CFSE (2.5 μM). Cells were then left untreated, or stimulated with combinations of anti-Ig and anti-CD40 (both 10 μg/ml) for 48 hours, both in the presence and absence of PD98059 and U0126 (1 μM) which were added every 4 hours over a 32 hour time period. Cells were then harvested, washed and analysed by FACS for the fluorescence intensity of CFSE, which gives an indication of the number cell divisions per population for each stimulation.

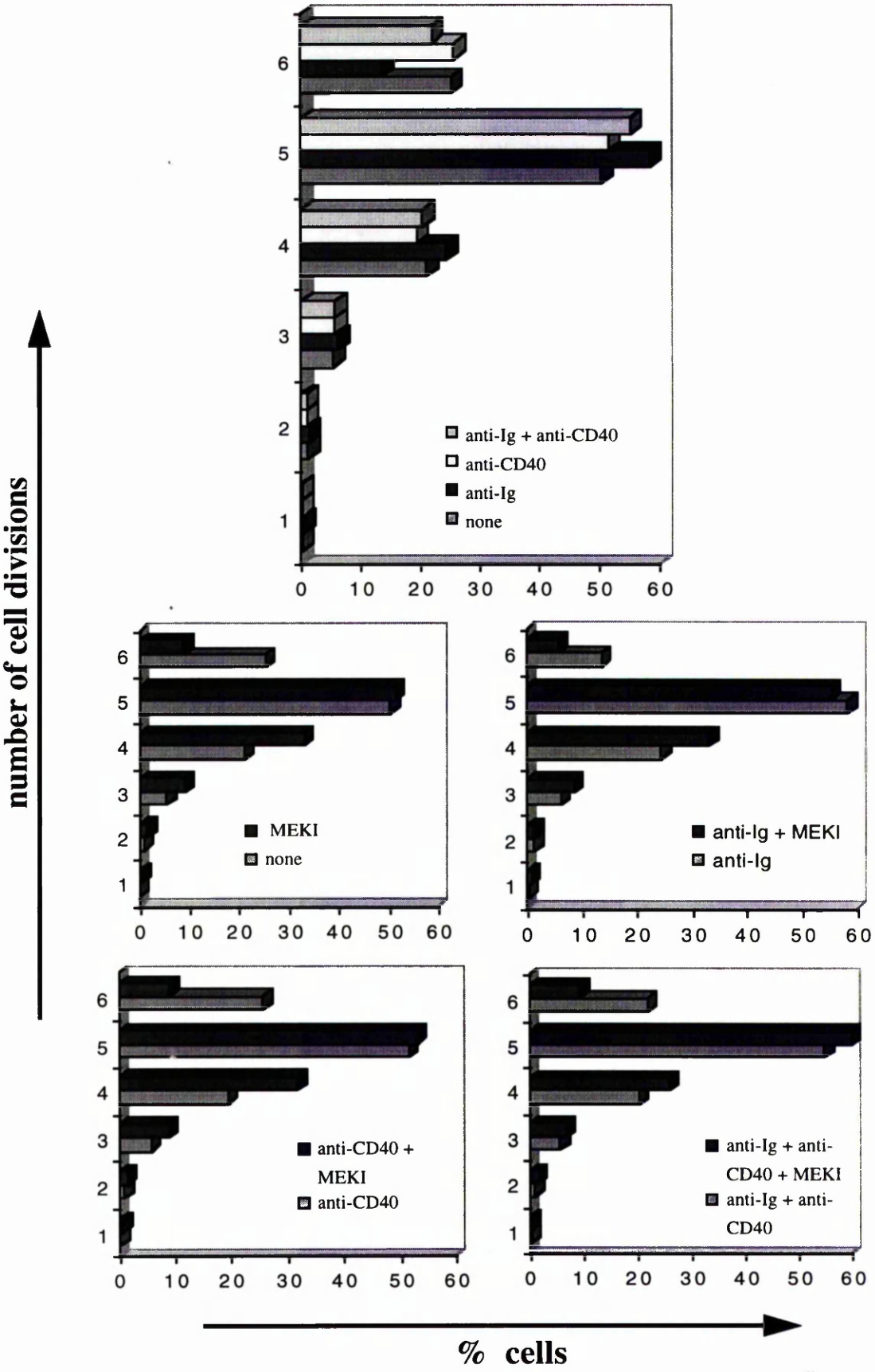

number of cell divisions
% cells
anti-Ig + anti-CD40
anti-CD40
anti-Ig
none
MEKI
none
anti-Ig + MEKI
anti-Ig
anti-CD40 + MEKI
anti-CD40
anti-Ig + anti-CD40 + MEKI
anti-Ig + anti-CD40

figure 38

Figure 39. PD98059 and U0126 reduce BCR-mediated $cPLA_2$ activity in WEHI-231 cells. WEHI-231 cells (5 x 10^6 total) were left untreated or pre-incubated with either PD98059 (10 μM) or U0126 (10 μM) or a combination of both for 60 minutes. Cells were then stimulated with anti-Ig (10 μg/ml) for 30 minutes before being analysed for $cPLA_2$ activity by colourimetric analysis as described in Materials and Methods.

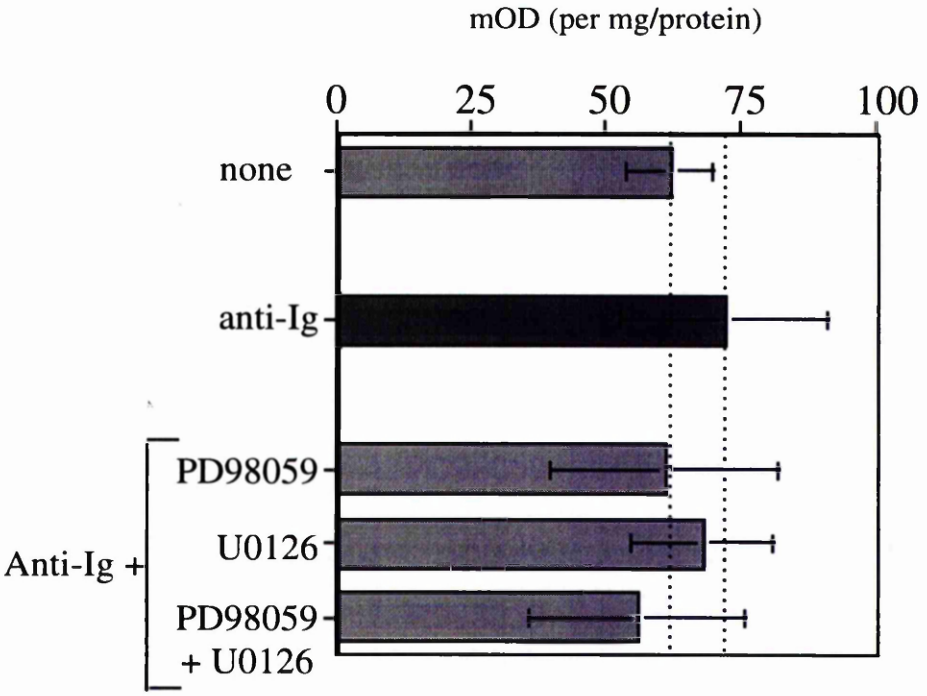

mOD (per mg/protein)
0
25
50
75
100
none
anti-Ig
Anti-Ig +
PD98059
U0126
PD98059
+ U0126

Figure 39

Figure 40. Model for the role of ERK-MAPKinase in WEHI-231 apoptosis, growth-arrest and proliferation. BCR ligation initiates the activation of an early, transient ERK-MAPKinase signal positively coupled to apoptotic signals including $cPLA_2$ activity. BCR ligation also suppresses basal ERK-MAPKinase activity which is required for WEHI-231 cell proliferation. CD40 co-stimulation rescues WEHI-231 cells from BCR-induced apoptosis and growth arrest by uncoupling the BCR from activation of the early, apoptotic ERK-MAPKinase signal, and re-establishes the long-term basal ERK-MAPKinase activity. CD40 co-stimulation also initiates the activation of other anti-apoptotic signals including those mediated by Bcl-x_L.

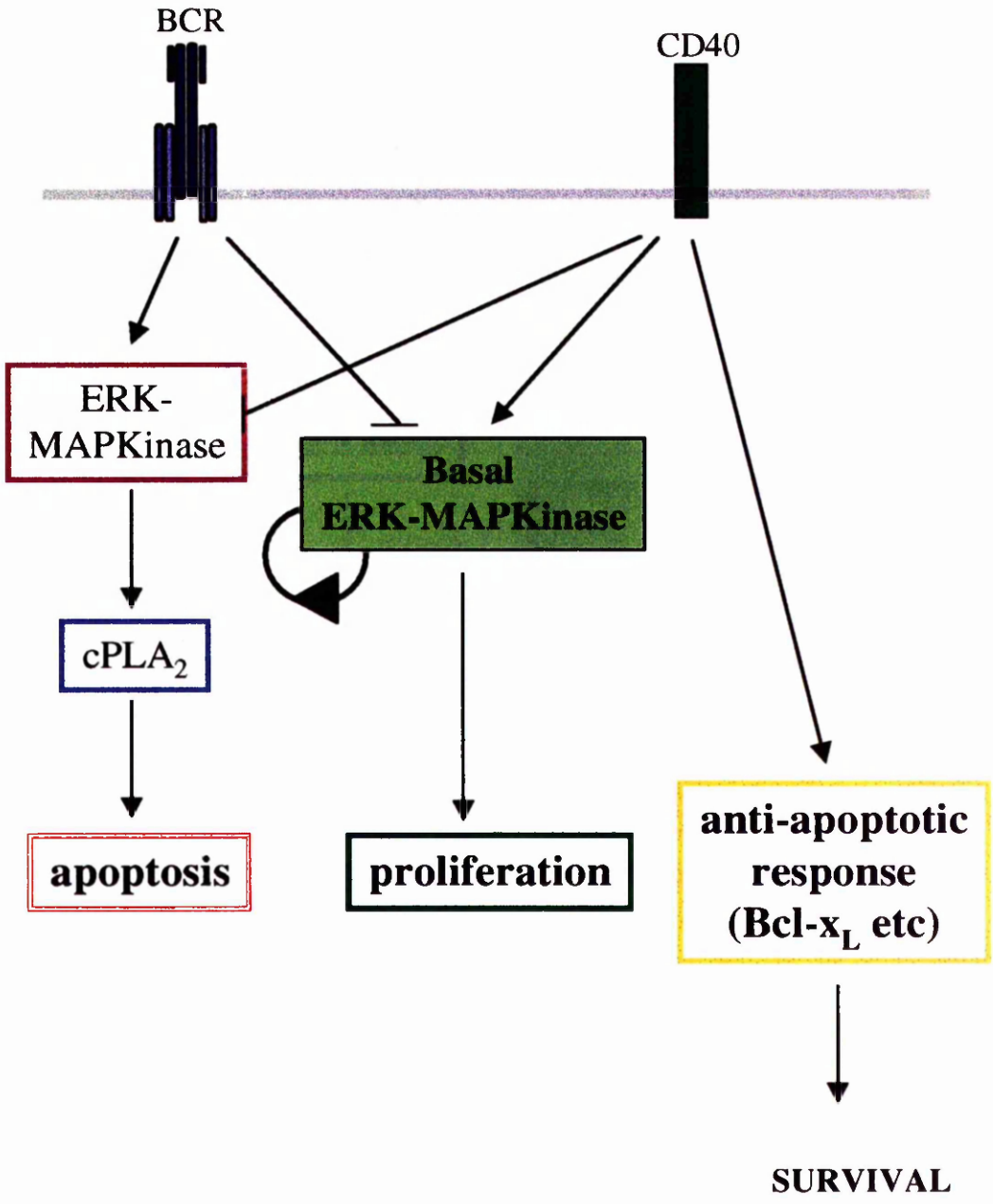

BCR
CD40
ERK-
MAPKinase
Basal
ERK-MAPKinase
cPLA2
apoptosis
proliferation
anti-apoptotic
response
(Bcl-xL etc)
SURVIVAL

Figure 40

CHAPTER 5 - Downstream mediators of BCR and CD40 signalling.

5.0 Introduction.

The studies outlined in the previous chapter have described differential biological roles for ERK-MAPKinase in WEHI-231 cells. We have shown that BCR-ligation not only leads to the activation of an early, transient, apoptotic signal, but also suppresses basal ERK-MAPKinase signals required for WEHI-231 cell proliferation. Moreover, CD40 treatment rescues these cells from growth-arrest and apoptosis by re-establishing basal ERK-MAPKinase activity and downregulating early BCR-coupled ERK-MAPKinase signals respectively. Both routes of ERK-MAPKinase activation appear to be Ras-dependent. Thus, how do signals transduced through ERK-MAPKinase lead to such different physiological responses. One possibility may be due to the differential activation of downstream intermediates following BCR or CD40 ligation. Previous studies within our lab have proposed a role for cytosolic PLA_2 as a key mediator of the apoptotic responses in WEHI-231 B cells (53).

5.1 PLA_2 family enzymes.

Membrane phospholipids provide an important source of lipid second messengers following receptor aggregation and activation. A family of phospholipases hydrolyse membrane phospholipids in a specific manner to yield a wide range of lipid products responsible for different physiological responses. The phospholipase A_2 (PLA_2) family of enzymes hydrolyse fatty acid from the sn-2 position of phospholipids generating a range of free fatty acids including arachidonic acid (AA) and lysophospholipids. In recent years, modern molecular biology cloning techniques has allowed this enzyme family to be characterised into distinct sub-sets according to different structural and functional properties. These include the secretory PLA_2 ($sPLA_2$), Ca^{2+}-independent PLA_2 ($iPLA_2$) and cytosolic PLA_2 ($cPLA_2$) families of PLA_2 enzymes.

$sPLA_2$s are low molecular weight (~14-kDa) enzymes that require millimolar concentrations of Ca^{2+} to exert their full enzymatic activity (109) and are abundantly expressed in the mammalian pancreas where they function as digestive enzymes for dietary phospholipids.

On the other hand, $iPLA_2$ has been shown to be Ca^{2+}-independent and exists in both 26-kDa lysosomal and a 86-kDa cytosolic forms and evidence exists for their role in phospholipid remodelling of the cell plasma membrane (109).

$cPLA_2$ is a 85-kDa protein, which shares no homology with other PLA_2s and preferentially hydrolyses sn-2 arachidonic acid from membrane phospholipids (110).

However, both sPLA2 and $iPLA_2$, which are non-specific for fatty acid species at the sn-2 position of phospholipid substrates, are also known to promote AA generation. Nevertheless it is $cPLA_2$ that plays the major role in the generation of AA from membrane phospholipids and is tightly regulated by post-receptor signalling events and its activity has been implicated in a range of biological responses, including inflammation (111).

5.2 Regulation of $cPLA_2$ activity.

5.2.1 Regulation of $cPLA_2$ activity by calcium.

Unlike $sPLA_2$ which requires millimolar levels of Ca^{2+} for enzymatic activity, $cPLA_2$ only requires submicromolar concentrations of Ca^{2+} (109). Ca^{2+} has been shown to be essential for the translocation and binding of $cPLA_2$ to the lipid membrane bringing the enzyme into close proximity with its chosen substrate. This is due to a N-terminal calcium-dependent phospholipid binding domain that has been shown to share close homology to the C2 domains of the conventional (Ca^{2+} dependent) isoforms of PKC (110). *In vitro* experiments using phospholipid vesicles as substrate have shown that Ca^{2+} concentrations required to promote $cPLA_2$ catalytic activity also correlate with Ca^{2+} concentrations required for membrane binding of $cPLA_2$ (110). These reports are consistent with the hypothesis that $cPLA_2$ is held in a cytoplasmic state at low Ca^{2+} concentrations and that receptor mediated calcium influxes promote its translocation to the lipid membrane. Additional studies by Hirabayashi et. al., 1999 (112), have since addressed how these Ca^{2+} signals regulate Ca^{2+} translocation. They reported that selective inhibition of platelet-activating factor receptor mediated Ca^{2+} influxes using the Ca^{2+} chelator EGTA were effective in preventing AA release (and therefore $cPLA_2$ activity) after critical time periods. This suggests that the duration of $[Ca^{2+}]_i$ elevation plays a key role in $cPLA_2$ translocation and subsequent activation.

5.2.2 Regulation of $cPLA_2$ activity by protein kinases.

In addition to a N-terminal calcium-dependent phospholipid binding domain, $cPLA_2$ contains a MAPKinase-phosphorylation site at Ser^{505} (113). Phosphorylation of $cPLA_2$ at this site by ERK-MAPKinase induces a gel shift of $cPLA_2$ as seen by SDS-PAGE and this has been used as a indicator of $cPLA_2$ activation. Phosphorylation of Ser^{505} is also essential for the *in vivo* activation of $cPLA_2$ as overexpression of mutant $cPLA_2$ (S505A) in Chinese hamster ovary cells failed to generate AA release seen in wild-type cells under similar conditions (110). Other protein kinases have been

implicated in regulating $cPLA_2$ activity. PKC, presumably via PKC-mediated activation of MAPKinase, and PKA, have both been shown to phosphorylate $cPLA_2$ *in vitro*. No evidence exists for PKC mediated phosphorylation of $cPLA_2$ *in vivo* however. One additional study also suggests that $sPLA_2$ may play an important role in $cPLA_2$ activation by directly activating ERK-MAPKinase (113).

The phosphorylation and activation of $cPLA_2$ is therefore both calcium- and MAPKinase-dependent. However, a number of studies have suggested that the order in which these activation signals are delivered is vital for proper enzymatic activation of $cPLA_2$. Subsequently, it has been proposed that phosphorylation of $cPLA_2$ by MAPKinases must precede the influx of calcium, which is required for the translocation of this enzyme from the cytoplasm to the cell membrane (110, 114). This data suggests that $cPLA_2$ cannot be phosphorylated after it has translocated to the cell membrane.

5.3 Downstream Effectors of $cPLA_2$ Signalling.

AA is a well known lipid second messenger, but also plays a vital role as the major substrate for a number of lipid pathways including the lipoxygenase and cyclo-oxygenase enzyme systems. AA has also been shown to regulate a wide range of intracellular signalling molecules including PLCγ, $p21^{ras}$GAP and certain PKC isoforms. In addition, TNFα mediated AA generation has also known to be a co-mitogen in the Swiss 3T3 cell line (115). However, it is its role as a mediator of TNFα-mediated cell death that has received much of the effort of investigators examining AA in signalling mechanisms. TNF signalling has been shown to activate a neutral SMase which in turn hydrolyses membrane sphingomyelin to ceramide. Ceramide has been shown to activate a 97-kDa kinase termed ceramide-activated protein kinase (CAPK). This kinase is capable of phosphorylating and activating Raf-1 on Thr^{269}, thus linking ceramide to the activation of ERK-MAPKinase (116, 117). This has been shown to result in $cPLA_2$ activation and AA generation (111). However, it has also been reported that AA can induce the activation of SMase with resultant generation of ceramide. This suggests that ceramide production can also lie downstream of cPLA2 activity.

In addition to TNFα signalling, AA is also known to play a role in the induction of apoptosis signalling via other cell surface receptors. These include Fas-mediated apoptosis. Indeed, a recent study by Atsumi *et. al.* (118) presented data to suggest that Fas-mediated apoptosis in U937 cells was mediated by the generation of AA from $iPLA_2$ activity. Interestingly, these studies also showed that $cPLA_2$ activity was downregulated in these cells, which was shown to be due to the cleavage of the $cPLA_2$ protein by caspase-3. This is in agreement with other findings which have suggested a

role for $iPLA_2$ in the induction of late PLA_2 activity during apoptosis, which was also shown to be secondary to caspase activity (26).

Additional factors which modulate the downstream effectors of $cPLA_2$ activity include the localisation of $cPLA_2$. The induction of apoptosis by TNFα-mediated activation of $cPLA_2$ has previously been shown to interfere with the electron flow in the mitochondria (119) suggesting a direct link between the induction of apoptosis and the mitochondria. Recent work in our lab has shown that $cPLA_2$ specifically translocates to the mitochondria after BCR-ligation. This results in the destabilisation of the mitochondrial membrane potential resulting death by apoptosis. Studies have also shown that this can be mimicked by exogenous treatment with AA, confirming a role for $cPLA_2$ in this process.

It is therefore clear that the location of AA generation by $cPLA_2$ activity is a major determinant in the subsequent downstream effectors activated by this lipid second messenger.

5.4 Metabolism of Arachidonic Acid

5.4.1 The Lipoxygenase Pathway.

The metabolites of lipoxygenase action have been implicated in TNF responses such as TNF-induced cytotoxicity, induction of the transcription factor c-fos and the mitochondrial superoxide radical scavenging enzyme manganous superoxide dismutase. They are produced by the enzymes 5-, 12- and 15-lipoxygenase which lead to the oxidation of AA, generating compounds such as HPETES, HETES, DHET, EET and leukotrienes. These compounds can subsequently be broken down into oxygen free radicals which are known to induce cell cytotoxicity (111).

5.4.2 The Cyclooxygenase Pathway.

Cyclooxygenase (COX) or prostaglandin H_2 synthase was first purified 25 years ago and subsequently cloned some 12 years later in the late 1980s. COX is the key enzyme in the synthesis of prostaglandins (PGs) from AA and catalyses the first two stages of primary prostanoid production (**figure 41**). Pharmacologically, COX is an important target of anti-inflammatory drugs. As early as the 1970s it was reported that non-steroidal anti-inflammatory drugs (NSAIDs) inhibited PG formation and that *in vitro* inhibition of PG formation correlated with their anti-inflammatory properties *in vivo* (120). Currently, two isoforms of COX are known to exist. They reflect both constituitively expressed, and inducible isoforms of COX and have been termed COX-1

and COX-2 respectively. COX-2 is induced by a wide range of pro-inflammatory cytokines and microbial agents. However, its role as a purely pro-inflammatory enzyme is still unclear. Although selective COX-2 inhibitors have been shown to reduce murine air pouch and foot pad inflammation, selective inhibition of COX-2 has also been shown to delay healing of gastric ulcers in mice and exacerbate induced colitis in rat models (121). PG synthesis has also been proposed to play a role in the development of colorectal cancers with elevated levels of PGE_2 and COX-2 both reported in human colorectal adenocarcinomas (122). Subsequent overexpression of COX-2 in intestinal epithelial cells has been shown to result in decreases in the number of cells undergoing apoptosis and is associated with rises in the anti-apoptotic protein Bcl-2. Studies have therefore suggested that COX-2 leads to decreases in apoptosis by regulating Bcl-2 expression via PGE_2 production whilst also inducing the activation of mitogenic signals such as ERK-MAPKinase. This leads onto studies suggesting that COX-2, but not COX-1, can be located in perinuclear regions. Indeed PGJ_2 derivatives have been shown to act as potent ligand for the nuclear peroxisome proliferator-activated receptor-γ (PPAR-γ) which acts as a transcription factor for genomic DNA (123).

Both COX isoforms share similar structural and catalytic activities, with both isoforms approximately 600 amino acids in length and sharing 63% sequence homology. They do however differ greatly at the gene level, reflecting their expression characteristics. Subsequently, COX-1 is known to be widely expresses in most mammalian organs, including the stomach and kidneys, whilst COX-2 is expressed constituitively in only a few organs including the brain (123). COX-1 originates from a 22-kb gene in humans, whist the inducible COX-2 originates from a smaller 8.3-kb immediate early gene (123). Despite structural similarities, COX-1 and COX-2 can be distinguished by both substrate and inhibitor selectivity. For example, COX-2 will accept a wider range of fatty acids as substrates than COX-1 whilst aspirin-mediated acetylation of COX-1 will prevent its oxidisation of AA, although COX-2 is unaffected (123).

The intracellular regulation of COX-2 expression and subsequent PGE_2 formation has been addressed in a range of cell types. Studies by Barry *et. al.* (1999) (124) provided evidence for the upregulation of COX-2 expression in platelets. Their studies highlighted that COX-2 expression was dependent on the activation of a membrane-linked signalling cascade, rather than the activation of nuclear receptors regulating gene expression. This signalling pathways was shown to be dependent on PKC-mediated activation of ERK-MAPKinase in a PI(3)K sensitive manner. Additional studies, using human lung fibroblasts (125), have shown that CD40 ligation also leads to dramatic increases in PGE_2 levels which were accompanied by CD40-

mediated increases in both COX-2 protein and mRNA levels. A role for CD40 in regulating PGE_2 levels is not restricted to lung fibroblasts. Interestingly, PGE_2, previously thought to act as an inhibitor of the immune response, has recently been shown to act in synergy with a number of cell surface receptors to enhance B lymphocyte proliferation. Studies by Garrone *et. al.* (1994) provided evidence to suggest that exogenous PGE_2 treatment could greatly enhance DNA synthesis of anti-CD40-activated B cells, as well as proliferative responses from IL-4 and IL-10 treated cells. In addition, these authors also reported the ability of PGE_2 to down-regulate IL-4-induced isotype switching, suggesting both positive and negative roles for PGE2 in B lymphocyte development. Reports have also suggested that PGE_2 prevents BCR-mediated DNA synthesis in human B cells, suggesting that PGE_2-mediated proliferative effects are very much receptor-specific and may indeed also reflect differential responses by different cell types. The role of PGE_2 as a negative regulator of growth may reflect the ability of this prostanoid to stimulate receptor-mediated cAMP generation via the activation of the adenylate cyclase pathway (126).

5.5 Role of $cPLA_2$ and AA in B lymphocyte development.

A recent study by Gilbert *et. al.* (53) highlighted a key role for $cPLA_2$ activation and AA generation in the maturation of T and B lymphocytes. This study addressed the role of $cPLA_2$ activation in the transduction of proliferative signals via T and B lymphocyte antigen receptors. Briefly, they provide data to suggest that $cPLA_2$ is expressed and activated in immature B lymphocytes and thymoctyes following antigen receptor ligation only under conditions leading to growth-arrest and/or apoptosis. Ligation of antigen receptors in mature B and T lymphocytes, which undergo proliferation under such circumstances, was not found to be coupled to $cPLA_2$ activation. Indeed, the expression of $cPLA_2$ protein was shown to be limited to developmental stages of B and T lymphocyte maturation where antigen receptor ligation leads to apoptotic signals. In particular, this study suggests that AA generation in immature B lymphocytes plays a key role in the deletion of these cells. Recent work within our lab (Katz *et. al.*, Journal of Immunology, in press), as previously mentioned, has indicated a role for mitochondiral $cPLA_2$ activity in the initiation of apoptosis in WEHI-231 cells following BCR-ligation. In addition, these results also show that exogenous treatment with AA can mimic the effects of BCR-ligation, corroborating a role for this lipid second messenger in mediating anti-proliferative responses in WEHI-231 cells.

5.6 Aims and objectives of this investigation.

The objectives of this investigation were to investigate downstream signalling events from ERK-MAPKinase and $cPLA_2$ and in particular:

1. to investigate the role of AA on ERK-MAPKinase activity in WEHI-231 cells.
2. to examine the biological role played by PGE_2 in WEHI-231 cells.
3. to address the role of CD40 in modulating AA-mediated responses.

5.7 Results.

5.7.1 Exogenous AA promotes WEHI-231 growth-arrest.

Studies by Gilbert et. al. (53) have shown that generation of AA may play a key role in mediating apoptotic responses in immature B lymphocytes. Indeed, AA generation only occurred when using anti-Ig concentrations favourable to growth-arrest/apoptosis, and anti-CD40 stimulation, which rescues WEHI-231 cells from anti-Ig-mediated growth-arrest does not induce AA generation. Indeed, we have previously shown that anti-CD40 co-stimulation is effective in reversing BCR-induced AA generation (**figure 30**). Additionally, we have also shown that U0126 inhibits BCR-induced apoptosis by inhibiting BCR-coupled ERK-MAPKinase activation, and presumably AA generation. In order to examine the downstream events of AA generation, WEHI-231 cells were treated with exogenous AA to mimic $cPLA_2$ activation via the BCR.

Exogenous treatment of WEHI-231 cells with AA (**figure 42A**) reduced DNA synthesis by approximately 36% whereas anti-Ig treatment, which has previously been shown to promote growth-arrest in WEHI-231 cells (**figure 15A**), reduced basal proliferation by as much as 60%. In addition, we have also shown that the ability of AA to induce growth arrest is concentration dependent (results not shown - concentrations used here after produce maximal growth arrest). The differences in the induction of growth arrest observed during anti-Ig or AA stimulation may also reflect differential effects of AA which is added to cells exogenously compared to AA generated intracellularly following BCR ligation. One interesting observation from these studies was the inability of CD40 ligation to rescue from AA-mediated growth arrest (**figure 42A**), suggesting that CD40-mediated rescue from BCR-induced growth-arrest occurs upstream from AA. These results are consistent with previous findings (**figure 28**) which showed the inability of the ERK-MAPKinase inhibitors PD98059 or U0126 to reverse BCR-induced growth arrest, yet successfully inhibit BCR-induced apoptosis.

Previous studies in this report had revealed the ability of BCR ligation to abolish a basal ERK-MAPKinase signal essential for WEHI-231 cell proliferation (**figures 33A and 42B**). As exogenous AA was shown to also induce growth arrest, albeit less than BCR-ligation, it was examined whether it exerted this effect by also down-regulating this ERK-MAPKinase signal. AA treatment was shown to induce a substantial increase in ERK-MAPKinase activation 2 hours post-AA treatment (**figure 42C**). This was also observed in cells treated with anti-Ig alone. However, in stark contrast to anti-Ig treated cells, ERK-MAPKinase activity was still present even after 4-8 hours post-treatment. Instead, ERK-MAPKinase activity was slowly down-regulated

by AA, but was abolished 24-48 hours post-AA treatment. Therefore, the inability of AA to completely abolish long-term ERK-MAPKinase signals, as seen in anti-Ig treated cells, may account for its weaker induction of growth-arrest. Interestingly, although previous studies have indicated the inability of anti-CD40 co-simulation to reverse AA-induced growth arrest, **figure 42C** confirms the ability of CD40 co-stimulation to reverse the late down-regulation of ERK-MAPKinase activity induced by AA treatment. This suggests that another block in the cell cycle apparatus, induced by AA treatment, is still active, and is not reversed by anti-CD40 co-stimulation.

5.7.2 AA also induces apoptosis in WEHI-231 cells.

Previous studies within our group had reported the ability of AA to induce DNA fragmentation and hence apoptosis in WEHI-231 cells (**figure 43A, Sandra Seatter, unpublished observation**) confirming an important role for $cPLA_2$ activation following apoptotic stimuli. These studies also indicate that exogenous AA treatment may bypass ERK-MAPKinase-$cPLA_2$ activation, as observed following BCR ligation, and exerts its apoptotic mechanism by activation of downstream apoptotic effectors. Interestingly, unlike BCR-ligation, AA treatment did not induce activation of an early ERK-MAPKinase signal (**figure 43B**), but did appear to enhance ERK-MAPKinase activity approximately 2 hours post-stimulation (**figure 42C**). Additionally, studies in our group suggest AA stimulation may also activate a positive feedback mechanism promoting further AA generation (**figure 44, Caroline Lord, unpublished observation**). Together with the results obtained in figure 42C, these studies would appear to suggest that AA stimulation promotes the activation of a positive feedback mechanism resulting in further AA generation possibly through the activation of a ERK-MAPKinase/$cPLA_2$ dependent pathway.

In addition, and similar to results obtained from cells treated with anti-Ig, AA did not induce activation of either p38 MAPKinase or JNK over a similar time course (**figure 43B**) although a role for these MAPKinases in the induction of AA-induced apoptosis cannot be discounted and further studies must be carried out.

5.7.3 CD40 co-treatment reverses AA-induced apoptosis.

Having previously shown a important role for CD40 co-treatment in reversing BCR-mediated apoptosis and growth-arrest we investigated whether AA-induced apoptosis could also be reversed by CD40 treatment. Cells were treated with a range of AA concentrations with or without 10 μg/ml anti-CD40 co-stimulation for a 48 hour period and analysed by FACS as previously described to determine cells undergoing apoptosis or in G_1 arrest. Results obtained (**figure 45**) confirmed previous

observations with BCR ligation driving cells into both apoptosis and growth-arrest. In addition, anti-CD40 treatment substantially blocked apoptosis as well as reducing cells in G_1 arrest to below basal (untreated levels) indicating cells were now progressing through cell-cycle. Cells treated with increasing concentrations of AA (5-50 μM) yielded a number of interesting observations. Cells treated with 5 μM AA produced apoptotic/G_1 arrest profiles similar to untreated cells. Cells treated with 25 μM AA, compared to untreated cells, whilst seeing no induction of apoptosis, experienced a decrease in G_1 arrest indicating that these cells were engaged in cell-cycle progression. In contrast, cells treated with 50 μM, whilst also seeing a reduction in G_1 arrest, were also shown to undergo apoptosis. These results indicate that whilst low AA concentrations promote cell-cycle progression higher concentrations of AA induce apoptosis in addition to cell-cycle progression in WEHI-231 cells. Indeed, examination of the cell cycle profiles of these cells confirmed earlier observations showing that whilst AA induces apoptosis at higher concentrations (50 μM), the number of cells in G_1 arrest compared to untreated cells was substantially decreased and a increase in cells in the S phase was also observed (**table 2**).

This may result from the non-synchronised nature of WEHI-231 cell populations, suggesting that AA treatment may induce differential biological responses depending on the stage of cell cycle when the stimulation was encountered, but is also suggestive of cells having to progress through the cell cycle before they die by apoptosis. Interestingly, co-treatment with anti-CD40 antibodies was shown to substantially reduce AA-mediated apoptosis at these higher concentrations, indicating that anti-CD40 treatment exerts one of its anti-apoptotic roles in WEHI-231 cells downstream of AA generation, possibly through the regulation of the anti-apoptotic protein, Bcl-x_L. In addition, cells in G_1 arrest were reduced, compared to 50 μM AA alone, indicating CD40 treatment has an additional mitogenic signal alongside its anti-apoptotic role, results which would confirm earlier findings suggesting that CD40 co-stimulation could enhance ERK-MAPKinase activity in AA treated cells (**figure 42C**).

The ability of anti-CD40 co-stimulation to reverse AA-induced apoptosis suggests that CD40 acts downstream of AA to reverse the induction of pro-apoptotic mechanisms, or may actually result from the metabolism of AA into an non-apoptotic intermediate. Studies within our lab, whilst not ruling out a role for the former, have provided some data to confirm the latter hypothesis. We have shown that CD40 co-stimulation appears to be ineffective in reversing apoptosis mediated by $AACOCF_3$, a AA derivative that cannot be further metabolised. Although these results are preliminary and require further study, they do suggest that CD40 co-stimulation may result in the conversion of AA into non-apoptotic intermediates.

Another interesting finding was the apparent ability of the ERK-MAPKinase inhibitor U0126 to reduce AA-mediated apoptosis, albeit very weakly. This again suggests that AA stimulation may actually induce a positive feedback mechanism leading to the induction of ERK-MAPKinase and $cPLA_2$ activities and hence generation of AA.

Taken together, these results provide evidence for the role of AA as a negative regulator of WEHI-231 cell fate, but also suggests that AA may play other quite distinct roles in determining cell proliferation.

5.7.4 Exogenous PGE_2 treatment enhances BCR-mediated growth-arrest in WEHI-231 cells.

Previous reports by other groups have suggested that COX-2 can decrease apoptosis by regulating Bcl-2 expression via PGE_2 production (122) whilst also inducing the activation of mitogenic signals such as ERK-MAPKinase. In addition, CD40 ligation has also been shown to enhance COX-2 expression in certain cell types (125). Taken together, these results suggest that CD40-mediated rescue from BCR-induced apoptosis, in addition to up-regulating Bcl-x_L expression and reversing BCR-mediated decreases in mitochondrial membrane potential, may also drive the production of PGs from AA generated following BCR ligation. Thus CD40 could mediate rescue signals by converting the negative regulator AA to a positive regulator, PGE_2 of WEHI-231 cell fate.

In order to determine whether PGE_2 plays a role in regulating the fate of WEHI-231 cells, exogenous PGE_2 was added to the culture of anti-Ig stimulated cells or cells left otherwise untreated. Therefore, if PGE_2 plays an important role in CD40-mediated rescue from growth-arrest, could PGE_2 treatment reverse anti-Ig-mediated growth-arrest. Results indicated that instead of a positive role in WEHI-231 cells fate, exogenous PGE_2 treatment was actually inhibitory in nature (**figure 46A**). PGE_2 treatment was shown to cause a dose-dependent increase in growth-arrest at higher concentrations, whilst also acting in synergy with anti-Ig to enhance growth-arrest.

Having shown that exogenous PGE_2 treatment may inhibit cell proliferation in WEHI-231 cells, we examined the effect of exogenous PGE_2 treatment in mature splenic B lymphocytes which are known to proliferate following BCR-ligation (**figure 46B**). PGE_2 treatment had no affect on the resting state of mature splenic B lymphocytes, however, cell activation and hence DNA synthesis activated by BCR-ligation was markedly reduced by co-culture with PGE_2 in a dose-dependent manner. These results provide further evidence to suggest that exogenous PGE_2 treatment plays a negative role in B lymphocyte function.

The above studies suggest that exogenous PGE_2 treatment activates anti-proliferative signals in B lymphocytes. Interestingly, the EP2 receptor, one of three receptors for PGE_2, is known to induce increase in intracellular cAMP (127), a negative regulator of B lymphocyte proliferation. It is therefore tempting to speculate that the negative effects observed in the above studies may be mediated by the binding of PGE_2 to EP2, although the expression of this receptor on these cells has not been examined.

5.7.5 Intracellular production of PGE_2 is suppressed by BCR-ligation.

Although the above results suggest PGE_2 treatment leads to the induction of anti-proliferative mechanisms in both immature and mature B lymphocytes, this may result from the activation of specific receptors on the cell surface by PGE_2 and therefore may not truly mimic the biological effect of intracellular PGE_2. Therefore we examined the ability of BCR-ligation with or without anti-CD40 co-stimulation to modulate intracellular PGE_2 levels (**figure 47**). Results obtained suggested that PGE_2 levels in basal proliferating cells varied substantially with time, suggesting that PGE_2 production was closely regulated during the cell-cycle. Perhaps more interesting was the ability of anti-Ig treatment to significantly reduce PGE_2 levels over an 8-48 hour period relative to untreated cells, suggesting that BCR-ligation suppresses COX activity. Coupled to the ability of CD40 co-stimulation to restore anti-Ig-mediated decreases in PGE_2 levels to almost basal (untreated) levels, these results suggest that receptor-mediated PGE_2 production predominately occurs during proliferative responses. Although we were unable to control for cell death in this study, which may account for the reduction in PGE_2 production by anti-Ig treated cells, it should be noted that the suppression of PGE_2 production by anti-Ig, compared to untreated cells or those co-stimulated with anti-CD40, was most noticeable between 8-24 hours. Previous studies have shown that this time period precedes that of cell death, suggesting that decreases in intracellular PGE_2 levels are the result of decreased production and not cell death. In addition, cell death by 24 h is of the order of 10-15% above basal levels, a value considerably less than % reduction in PGE_2 observed here.

The results described in this chapter provide evidence to suggest both a positive and negative role for PGE_2 in lymphocyte proliferation depending on whether it is intra- or extracellular. It was therefore important to investigate whether PGE_2 was actually secreted by WEHI-231 cells following cell stimulation. Cells were left untreated, or treated with anti-Ig, anti-CD40 or both anti-Ig and anti-CD40 for 1, 8, 24 or 48 hours. At these time points a sample of cultured supernatant was removed and analysed for PGE_2 levels. Results indicated that no PGE_2 was present, or secreted into the culture media by WEHI-231 cells following any of the above treatments (**results not shown**). These results suggest that PGE_2 plays no extracellular role in WEHI-231 cell

physiology. Nevertheless, the results indicate that exogenous PGE_2, possibly though receptor mediated activation of adenylate cyclase, can negatively regulate B lymphocyte proliferation.

5.7.6 Indomethacin inhibits basal and CD40-stimulated proliferation, but does not affect CD40-mediated rescue from BCR-induced growth-arrest.

Our studies suggest a possible role for the intracellular production of PGE_2 as a mechanism for CD40-mediated rescue from BCR ligation growth arrest. In addition, CD40 treatment has been shown to upregulate both the message and protein expression of COX-2 in human lung fibroblasts (125). We therefore wanted to address whether inhibition of both COX-1 and COX-2 using the chemical inhibitor indomethacin would block CD40-mediated rescue (**figure 48**). Treatment of WEHI-231 cells with indomethacin was shown to induce a marginal decrease in cell proliferation compared to untreated cells (vehicle studies confirmed no toxic effects from inhibitor vehicle). Co-treatment with indomethacin was marginally effective in reducing anti-CD40 but not anti-IL-4 mediated increases in proliferation. In addition, indomethacin was ineffective in preventing anti-CD40-mediated rescue from BCR-induced growth arrest. Although indomethacin treatment did not prevent CD40-mediated rescue in these cells, this may reflect the availability of AA as a substrate for COX-1 and COX-2 and the inability of indomethacin to effectively block all COX activities possibly due to the instability of this inhibitor over long-term cultures as we have previously seen with the ERK-MAPKinase inhibitors PD98059 and U0126. It would therefore be favourable to repeat these experiments with prolonged treatment with indomethacin and perhaps try other, more specific COX inhibitors.

5.8 Discussion.

Although the activation of $cPLA_2$ in the WEHI-231 cell line has been shown to play an important role in the induction of apoptotic signals following BCR-ligation and also as a key mediator in maturational state-dependent BCR responses, the role of key downstream effectors is still unclear. Arachidonic acid is the major product of $cPLA_2$ activity and is produced following BCR, but not CD40 ligation in WEHI-231 cells confirming its role as a negative mediator. How AA mediates its biological effects are unclear. Recent work within our group (Katz *et. al.*, Journal of Immunology, in press) have highlighted the role of the BCR in mediating both the activation and translocation of $cPLA_2$ to the mitochondria, where it has been shown to induce disruption of the mitochondrial membrane potential, which is known to be pro-apoptotic. AA is also the major source for the production of prostaglandins, lipid mediators with a wide range of physiological roles. We therefore wanted to examine the role of AA in WEHI-231 cell responses and in particular, the role of the PGs under similar conditions.

Initial studies examining DNA fragmentation confirmed a role for AA in the induction of apoptosis in WEHI-231 cells. However, the use of FACS analysis to examine cell-cycle profiles by propidium iodide binding highlighted that AA may also play a role in cell proliferation in addition to apoptosis. These results could be interpreted in a number of ways. Firstly, although we know the BCR is coupled to AA generation under conditions of growth-arrest and apoptosis, the treatment of WEHI-231 cells with exogenous AA is very different to that produced intracellularly by way of signalling cascades. This may explain why high concentrations of AA are required to induce apoptosis in these cells. Secondly, with respect to the reduction of cells stuck in G_1 arrest, internalised AA may indeed be broken down by other intracellular mechanisms such as cyclooxygenase activity into growth-promoting lipid metabolites such as PGE_2, this may also explain why the AA analogue, $AACOCF_3$, which is resistant to enzymatic breakdown is so damaging the cells. Thirdly, the ability of high AA concentrations to induce both apoptosis and cell-cycle progression may reflect the non-synchronised nature of WEHI-231 cells. Consequently, cells already in G_1 arrest at time of AA treatment may be pre-disposed to undergo apoptosis, whilst other cells enter cell-cycle progression without blockages. However, it may also reflect the need for AA stimulated cells to progress through the cell cycle before committing to apoptosis.

Previous studies measuring cell proliferation by means of [^{3}H]thymidine incorporation indicated the ability of AA to induce growth-arrest in WEHI-231 cells, which unlike BCR-ligation, could not be reversed by co-treatment with anti-CD40. However, FACS profiles using propidium iodide suggested that CD40 treatment was capable of rescuing AA-induced apoptosis. As these plots also indicated that the

percentage of cells stuck in G_1 arrest actually fell, in could be concluded that the growth-arrest induced by AA in the [^{3}H]thymidine studies was actually due to apoptotic cells. The question therefore remains, if CD40-treatment is capable of reversing AA-induced apoptosis, why was there no rescue in [^{3}H]thymidine samples co-treated with anti-CD40. It was not due to cells rescued from apoptosis entering a state of G_1 arrest, as G_1 arrest levels were less in samples treated with AA and anti-CD40, which also enhances mitogenic ERK-MAPKinase activity, than AA alone. Nevertheless, the ability of CD40 ligation to rescue from AA-induced apoptosis suggests that CD40 ligation activates signalling cascades to block downstream effectors of AA signalling. Indeed, recent studies within our lab (Katz et.al., Journal of Immunology, in press) have confirmed the ability of anti-CD40 to rescue from AA-induced apoptosis by reversing AA-mediated decreases in the mitochondrial membrane potential. The ability of AA to drive cells through the cell-cycle, in addition to apoptosis is confirmed by time course studies investigating ERK-MAPKinase activation. Unlike BCR-ligation, AA treatment was incapable of completely abolishing basal ERK-MAPKinase signals which have previously been shown to be essential for WEHI-231 cell proliferation. AA treatment, therefore, may lead to the selective activation of apoptotic signals, although not all cells undergo this process, and unlike anti-Ig stimulated cells continue to proliferate, explaining the inability of AA stimulation to downregulate basal ERK-MAPKinase activity.

AA-induced apoptosis was also shown to possibly involve ERK-MAPKinase activation as the ERK-MAPKinase inhibitor U0126 was effective in reducing AA-induced apoptosis, albeit marginally. Previous data suggested that AA treatment was ineffective in inducing the activation of an early ERK-MAPKinase signal previously shown to be essential in BCR-induced apoptosis. However, AA stimulation did appear to enhance ERK-MAPKinase activation at a later time point (approximately 2 hours post stimulation) In addition, studies within our group have shown that AA treatment, possibly via a positive feedback mechanism, leads to an increase in the generation of intracellular AA. Therefore, AA, as part of a positive feedback loop, may induce the activation of ERK-MAPKinase/cPLA$_2$ at time periods out with the 60 minute window investigated in figure 48B. This would account for the ability of U0126 to weakly inhibit AA-induced apoptosis in WEHI-231 cells.

One puzzling event uncovered when examining the effect of AA treatment on WEHI-231 cells was its apparent ability to induce both growth and apoptosis. As AA is the main substrate for COX activity leading to the generation of intracellular PGs and PGs have been shown to have mitogenic properties in certain cells types it is tempting to speculate that some of the exogenously added AA is broken down by basal COX activity into PGs. We therefore decided to examine the what role was played by PGs in WEHI-231 cell fate. Initial studies treating cells with exogenous PGE_2 suggested a

negative role for PGE_2 in both immature and mature B lymphocyte proliferation. However, these results may not truly represent the role of PGE_2 produced intracellularly via receptor-mediated activation of COX. Accordingly, we measured the levels of intracellular PGE_2 produced under both normal (untreated), death (anti-Ig), and rescue (anti-CD40) conditions. These results showed that PGE_2 production was suppressed by BCR-ligation, either by inhibition of COX activity or perhaps activation of the lipoxygenase pathway, converting AA into anti-apoptotic second messengers. However, co-treatment with anti-CD40 could help restore PGE_2 production to near basal levels. As CD40 activation has previously been shown to upregulate both COX-2 protein expression and activation (125), it seems feasible to predict that BCR-ligation leads to the suppression of COX activity preventing breakdown of newly generated AA into PGE_2. However, co-treatment with CD40 could feasibly re-establish COX activity allowing for the breakdown of BCR-mediated AA generation into PGE_2.

In order to confirm a role for COX in WEHI-231 cell fate the non-selective COX inhibitor indomethacin was used to dissect the involvement of COX under both positive and negative conditions. These results suggested that indomethacin treatment was marginally effective in reducing basal proliferation in addition to receptor-mediated proliferation via CD40 or IL-4 treatment. However, no effect on CD40-mediated rescue from BCR-induced growth arrest was observed. Although these results suggest that COXs may play a role in normal WEHI-231 cell proliferation but no role in CD40-mediated rescue, the involvement of COX in the latter response cannot be directly ruled out. The ability of indomethacin to prevent all COX activity was not established in these studies. Therefore, even in the presence of indomethacin, a residual COX activity may still be present. This may account for the inability of indomethacin to block CD40-mediated rescue from BCR-induced growth-arrest. Indomethacin was capable of marginally reducing [^{3}H]thymidine incorporation of basal WEHI-231 cells, however, cells treated under such conditions will have little or no intracellular AA. However, cells treated with anti-Ig will have elevated AA levels, therefore, any residual COX activity will presumably convert this into PGE_2. The ability of indomethacin to induce growth-arrest under some stimuli, but not others, may be due to the availability of AA as a substrate for residual COX activity.

Taken together, these results suggest that AA plays a pivotal role in determining WEHI-231 cell fate (**figures 49 and 50**). It also highlights the ability of CD40-ligation to utilise lipid metabolites produced under negative, BCR-mediated responses, suggesting that the balance of positive and negative lipid metabolities plays a vital role in both the death and survival of the cell.

Figure 41. Primary prostanoid production. Role of COX in the conversation of AA to PGE_2. Cyclooxygenase (COX-1 (constitutive) and COX-2 (inducible)) catalyses the first two steps of prostaglandin (PG) synthesis from the substrate, arachidonic acid (AA). The first step involves the oxidation of AA to the hydroperoxy endoperoxide PGG_2 and then its subsequent reduction to the hydroxy endoperoxide, PGH_2. PGH2 is then transformed by a range of enzymatic and nonenzymatic mechanisms into the primary prostanoids, PGE_2. PGD_2, PGF_2, PGI_2 and TXA_2.

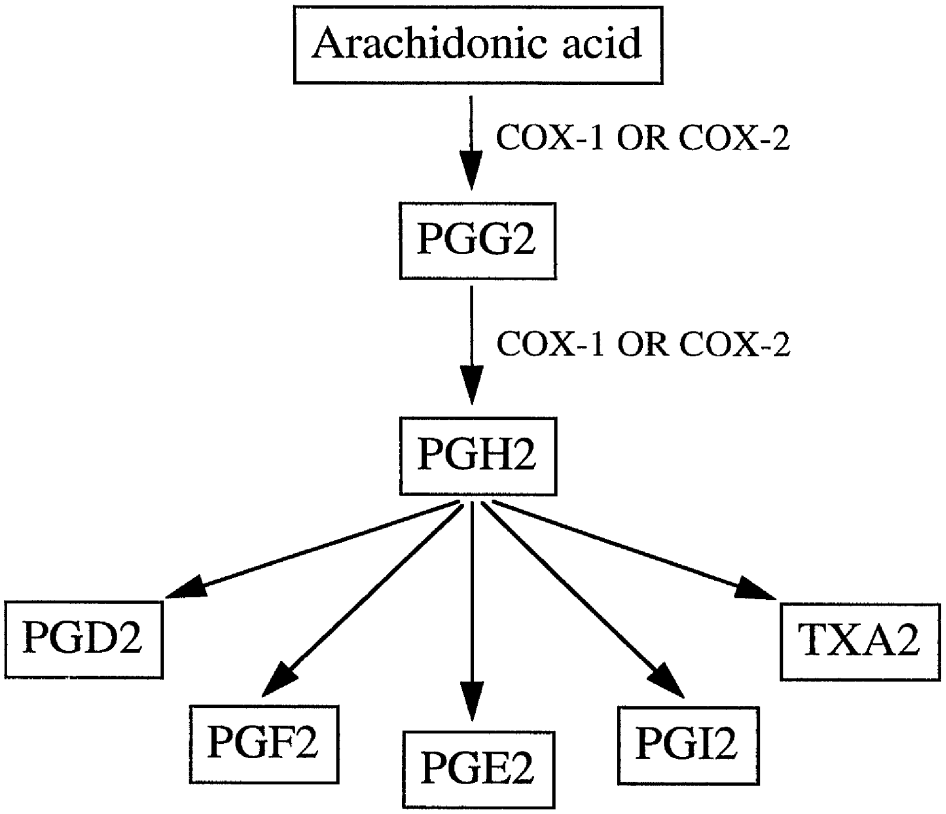

Arachidonic acid
COX-1 OR COX-2
PGG2
COX-1 OR COX-2
PGH2
PGD2
PGF2
PGE2
PGI2
TXA2

Figure 41

Figure 42. AA induces moderate growth-arrest and down-regulation of ERK MAPKinase in WEHI-231 cells. **A.** WEHI-231 cells (10^4/well) were left untreated, or treated with anti-Ig (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml). In addition, cells were also treated with AA (25 μM) or AA plus anti-CD40 (10 μg/ml). Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells. **B.** WEHI-231 cells (5 x 10^5/ml) were stimulated as follows: **lane 1**, cells left untreated for 0 hours, **lane 2**, cells left untreated for 1 hour, **lane 3**, cells left untreated for 2 hours, **lane 4**, cells left untreated for 4 hours, **lane 5**, cells left untreated for 8 hours, **lane 6**, cells left untreated for 24 hours, **lane 7**, cells left untreated for 48 hours, Levels of pERK/wERK expression were determined by western blotting (30 μg/lane). **C.** WEHI-231 cells (5 x 10^5/ml) were stimulated as follows: **lane 1**, cells left untreated for 0 hours, **lane 2**, cells treated with AA (25 μM) for 1 hour, **lane 3**, cells treated with AA (25 μM) for 2 hours, **lane 4**, cells treated with AA (25 μM) for 4 hours, **lane 5**, cells treated with AA (25 μM) for 8 hours, **lane 6**, cells treated with AA (25 μM) for 24 hours, **lane 7**, cells treated with AA (25 μM) for 48 hours, **lane 8**, cells treated with AA (25 μM) plus anti-CD40 (10 μg/ml) for 1 hour, **lane 9**, cells treated with AA (25 μM) plus anti-CD40 (10 μg/ml) for 2 hours, **lane 10**, cells treated with AA (25 μM) plus anti-CD40 (10 μg/ml) for 4 hours, **lane 11**, cells treated with AA (25 μM) plus anti-CD40 (10 μg/ml) for 8 hours, **lane 12**, cells treated with AA (25 μM) plus anti-CD40 (10 μg/ml) for 24 hours, **lane 13**, cells treated with AA (25 μM) plus anti-CD40 (10 μg/ml) for 48 hours, **lane 14**, cells left untreated for 48 hours. Levels of pERK/wERK expression were determined by Western blotting (30 μg/lane).

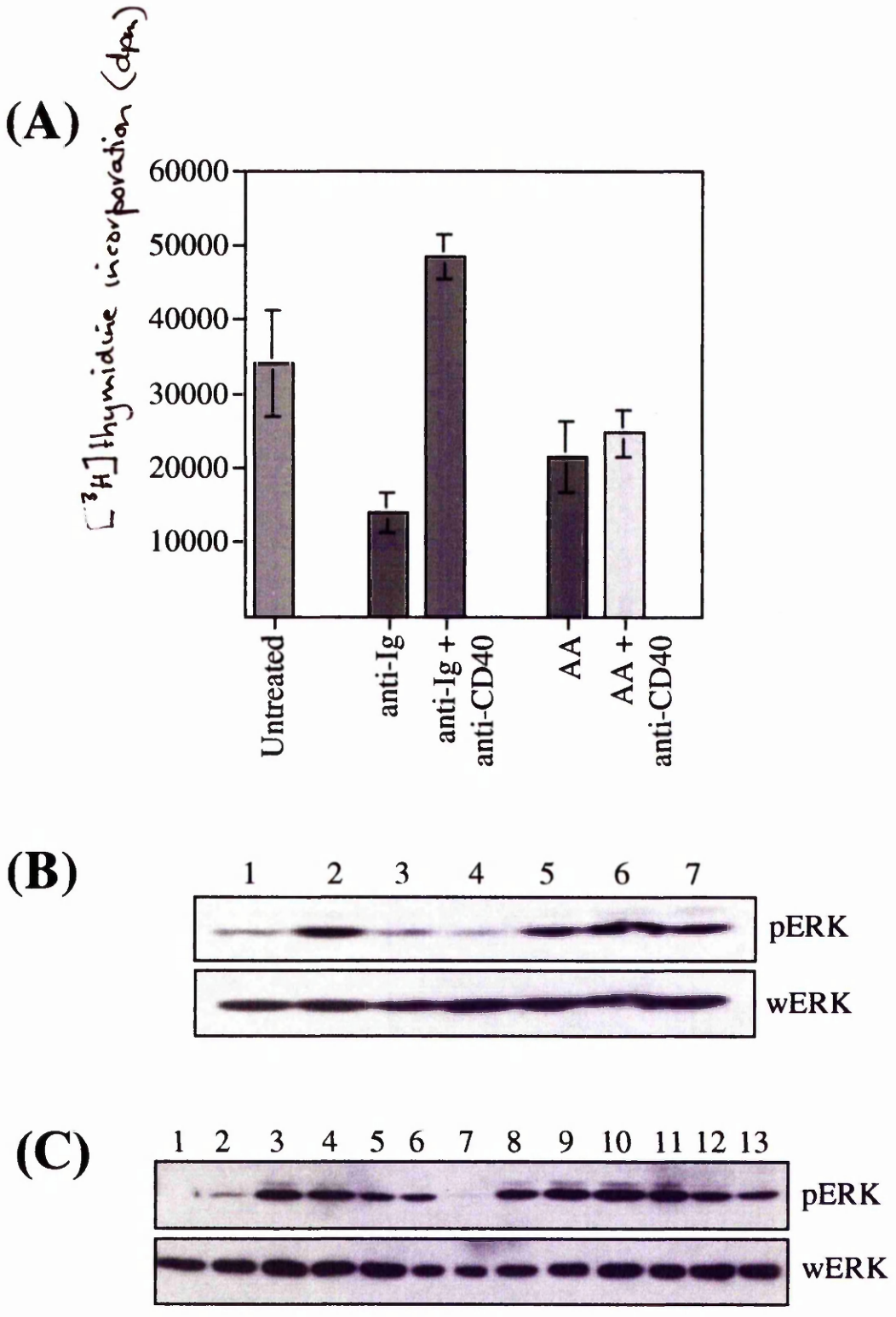
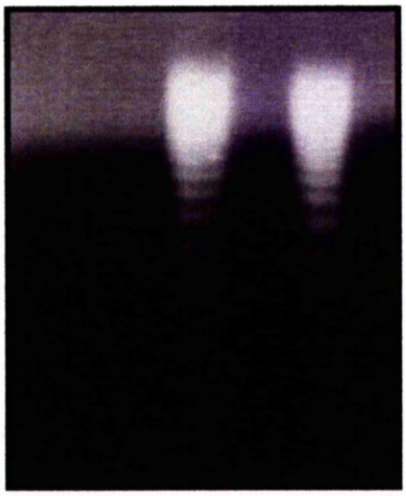

(A)
[3H] thymidine incorporation (dpm)
60000
50000
40000
30000
20000
10000
Untreated
anti-Ig
anti-Ig + anti-CD40
AA
AA + anti-CD40
(B)
1 2 3 4 5 6 7
pERK
wERK
(C)
1 2 3 4 5 6 7 8 9 10 11 12 13
pERK
wERK

Figure 42

Figure 43. AA treatment induces apoptosis, but not activation of the three members of the MAPkinase family in WEHI-231 cells. A. Cells were left untreated or treated with anti-Ig (10 μg/ml) or AA (25 μM). Levels of apoptosis were measured by DNA laddering after 48 hours cell stimulation. **B.** WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1,** cells left untreated for 0 minutes, **lane 2,** cells treated with AA (25 μM) for 1 minute, **lane 3**, cells treated with AA (25 μM) for 5 minutes, **lane 4**, cells treated with AA (25 μM) for 10 minutes, **lane 5**, cells treated with AA (25 μM) for 30 minutes, **lane 6**, cells treated with AA (25 μM) for 60 minutes, **lane 7**, cells left untreated for 60 minutes. Levels of pERK/wERK; pp38/wp38; pJNK/wJNK expression were determined by Western blotting (15 μg/lane).

(A)



None Anti-Ig AA

(B)

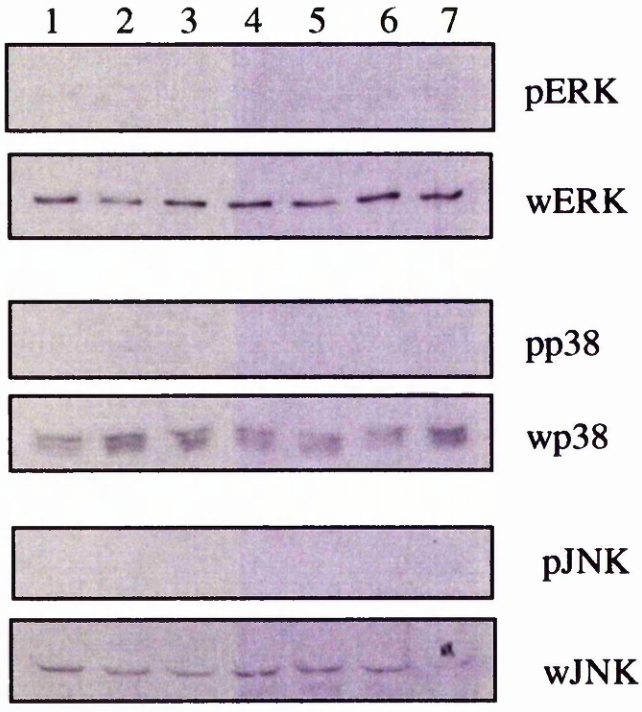


Figure 43

Figure 44. Exogenous AA stimulation promotes further generation of intracellular AA.

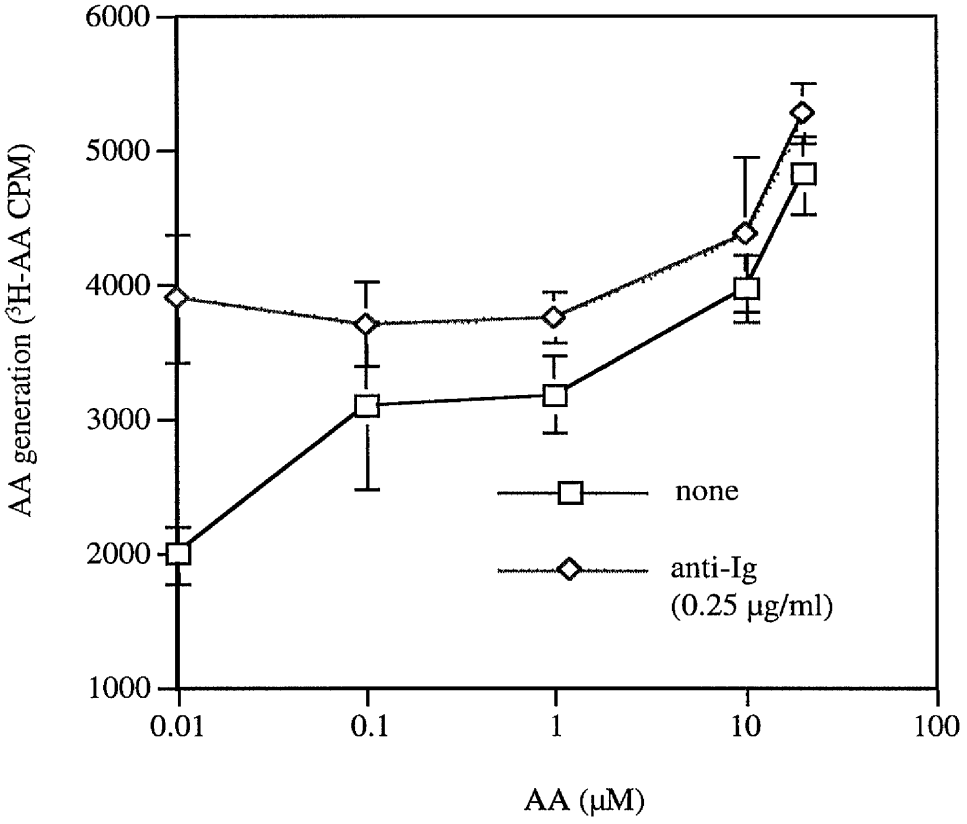

6000
5000
4000
3000
2000
1000
AA generation (^{3}H-AA CPM)
0.01
0.1
1
10
100
AA (μM)
none
anti-Ig
(0.25 μg/ml)

figure 44

Figure 45. AA treatment induces apoptosis in WEHI-231 cells that can be reversed by co-treatment with anti-CD40. WEHI-231 cells (5 x 10^5/ml) were treated with the indicated stimuli (anti-Ig and anti-CD40 both 10 μg/ml). Levels of apoptosis were determined by PI staining and FACS analysis after 48 hours as described in Materials and Methods.

Figure 45

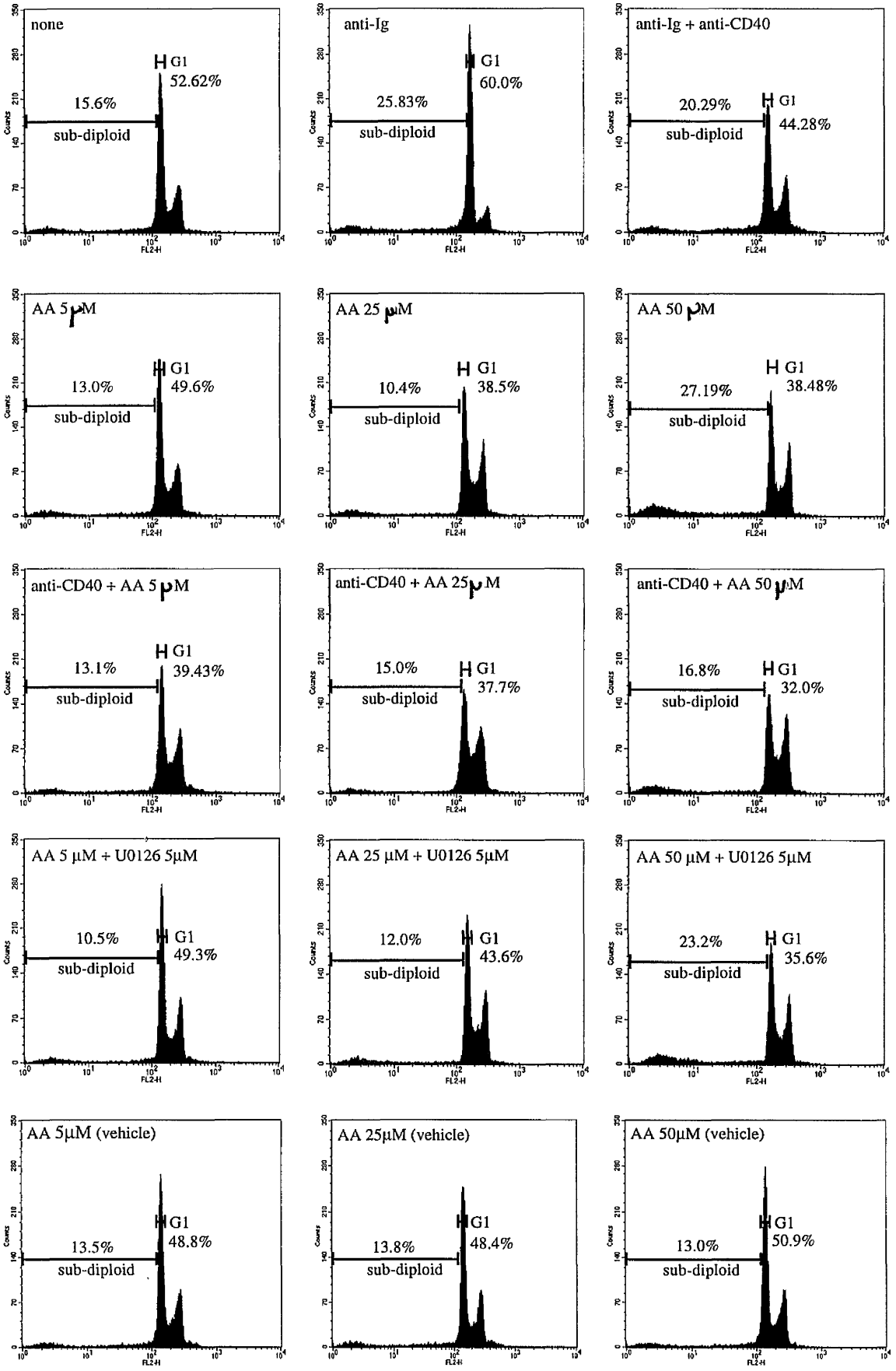
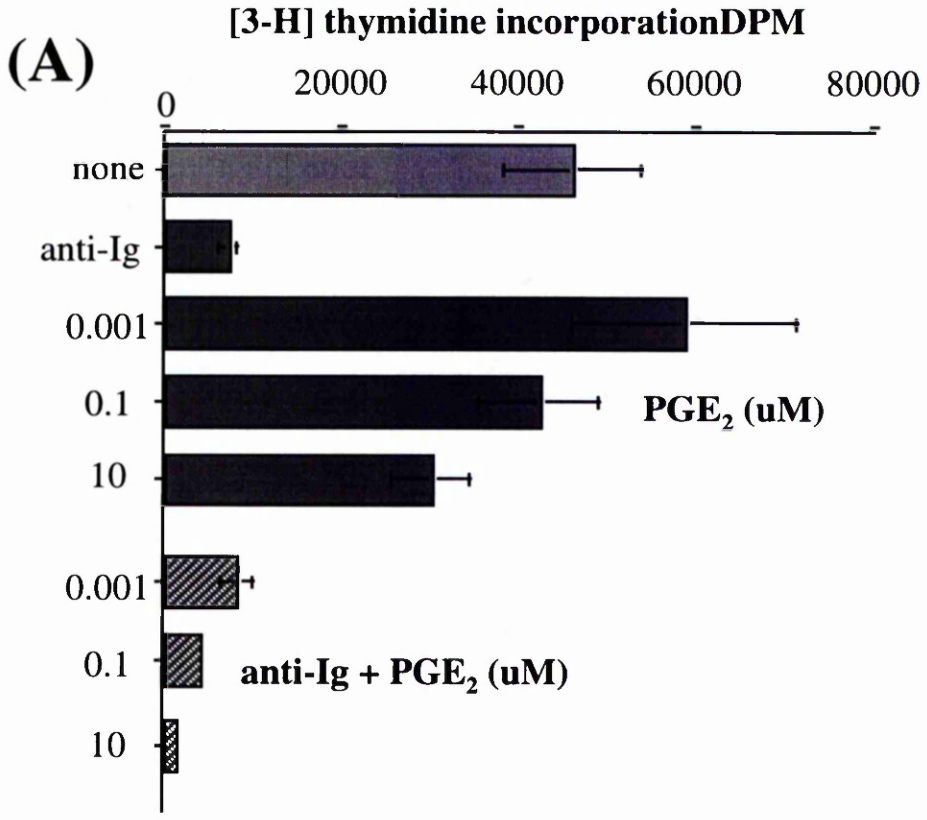

none
G1
52.62%
15.6%
sub-diploid
anti-Ig
G1
60.0%
25.83%
sub-diploid
anti-Ig + anti-CD40
20.29%
G1
44.28%
sub-diploid
AA 5 μM
13.0%
G1
49.6%
sub-diploid
AA 25 μM
10.4%
G1
38.5%
sub-diploid
AA 50 μM
27.19%
G1
38.48%
sub-diploid
anti-CD40 + AA 5 μM
13.1%
G1
39.43%
sub-diploid
anti-CD40 + AA 25 μM
15.0%
G1
37.7%
sub-diploid
anti-CD40 + AA 50 μM
16.8%
G1
32.0%
sub-diploid
AA 5 μM + U0126 5μM
10.5%
G1
49.3%
sub-diploid
AA 25 μM + U0126 5μM
12.0%
G1
43.6%
sub-diploid
AA 50 μM + U0126 5μM
23.2%
G1
35.6%
sub-diploid
AA 5μM (vehicle)
13.5%
G1
48.8%
sub-diploid
AA 25μM (vehicle)
13.8%
G1
48.4%
sub-diploid
AA 50μM (vehicle)
13.0%
G1
50.9%
sub-diploid

Table 2. AA treatment induces apoptosis in WEHI-231 cells. WEHI-231 cells (5 x 10^5/ml) were treated as figure 45. Cell cycle progression was determined by PI staining and FACS analysis after 48 hours as described in Materials and Methods.

Sample	Sub-diploid (%)	G1/G0 (%)	S (%)	G2/M (%)
none	15.2	51.1	18.1	14.9
anti-Ig	25.8	58.3	10	6.2
anti-Ig + anti-CD40	20.7	41.8	20.4	14.3
AA (5 μM)	12.6	49.3	19.9	16.5
AA (25 μM)	10.6	40.7	24.3	22.6
AA (50 μM)	24.9	34.6	23.8	14.6
AA (5 μM) + anti-CD40	12.8	39.8	24.5	17.2
AA (25 μM) + anti-CD40	10.7	37.7	31.6	19.7
AA (50 μM) + anti-CD40	17	31.8	30.2	20.6
AA (5 μM) vehicle	13.7	49.5	19.2	16
AA (25 μM) vehicle	14.2	49.5	17	16.4
AA (50 μM) vehicle	13.5	51.6	18.4	15.4

Table 2

Figure 46. Exogenous PGE_2 has negative effects on both immature and mature B lymphocyte proliferation. A. WEHI-231 cells (10^4/well) were left untreated, or treated with anti-Ig (10 μg/ml) with or without PGE_2, or treated with PGE_2 alone. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells. **B.** Mature splenic B lymphocytes ($5x10^5$/well) were left untreated, or treated with anti-Ig (50 μg/ml) with or without PGE_2, or treated with PGE_2 alone.


(A)
[3-H] thymidine incorporationDPM
0
20000
40000
60000
80000
none
anti-Ig
0.001
0.1
10
PGE2 (uM)
0.001
0.1
10
anti-Ig + PGE2 (uM)

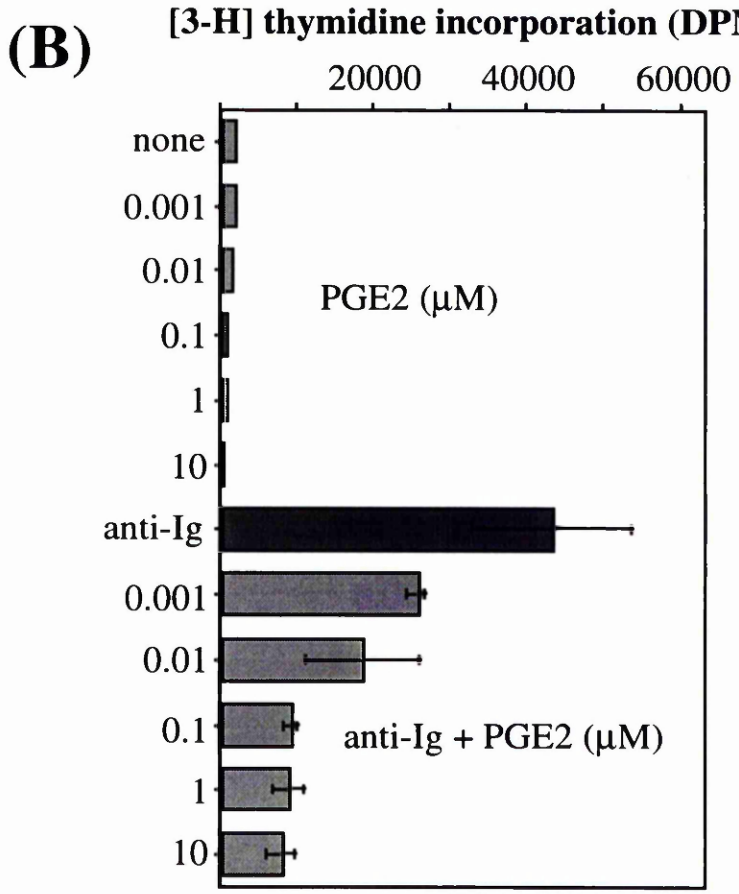

(B)
[3-H] thymidine incorporation (DPM)
20000
40000
60000
none
0.001
0.01
0.1
1
10
PGE2 (μM)
anti-Ig
0.001
0.01
0.1
1
10
anti-Ig + PGE2 (μM)

Figure 46

Figure 47. Intracellular production of PGE_2 in WEHI-231 under apoptotic or rescue conditions. WEHI-231 cells ($5x10^5$) were left untreated, or treated with anti-Ig (1 μg/ml) or anti-Ig and anti-CD40 (10 μg/ml) for the times indicated. Intra- and extracellular PGE_2 levels were measured by immuno-assay (R&D Systems) according to the manufacturers protocol (see Material and Methods).

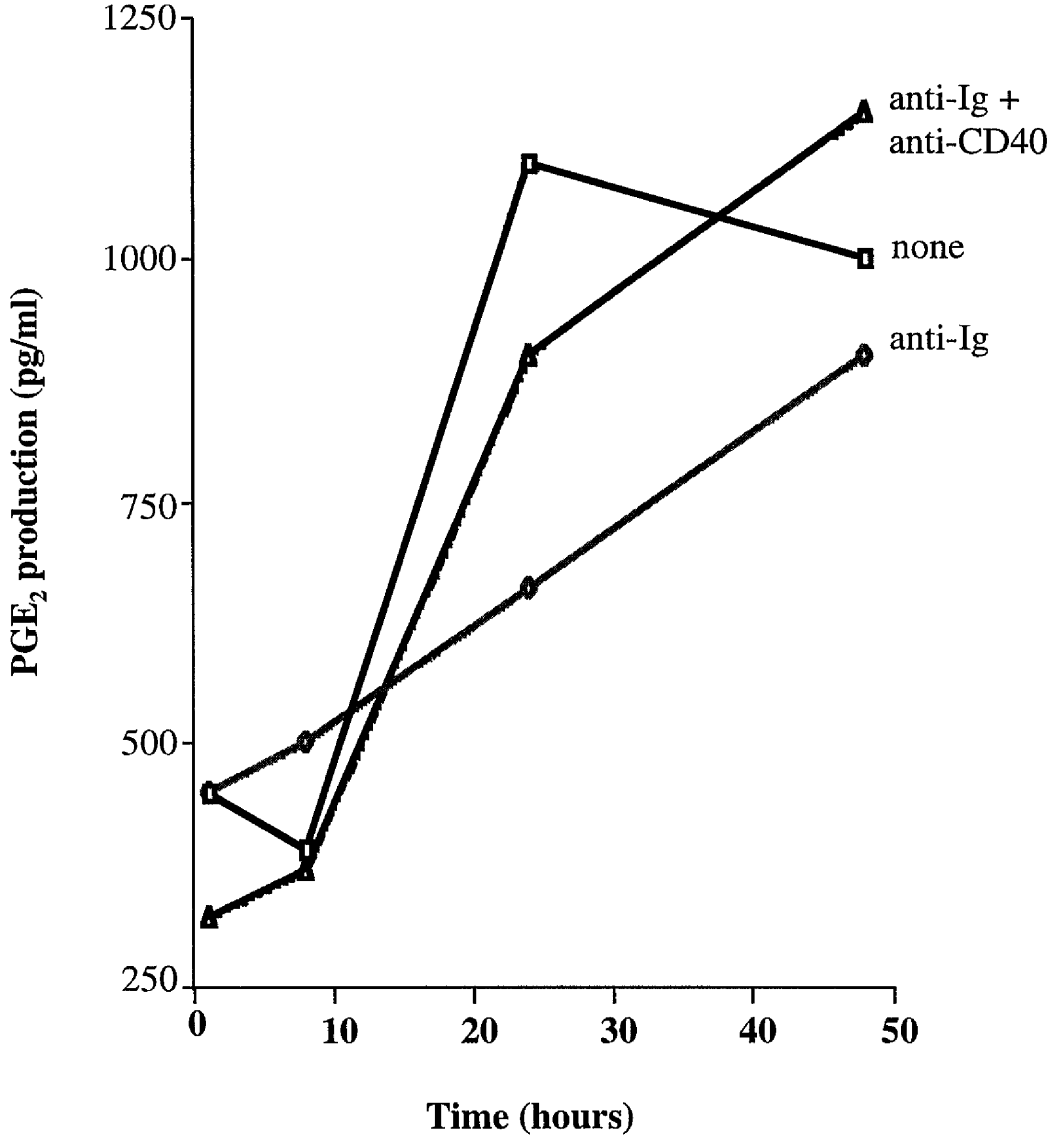

1250
1000
750
500
250
PGE2 production (pg/ml)
0
10
20
30
40
50
Time (hours)
anti-Ig +
anti-CD40
none
anti-Ig

Figure 47

Figure 48. Indomethacin inhibits basal and CD40-stimulated proliferation, but does not affect CD40-mediated rescue from BCR-induced growth-arrest. WEHI-231 cells (10^4/well) were left untreated, or treated with anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml), IL-4 (1 μg/ml) or anti-Ig plus anti-CD40 with or without pre-treatment with indomethacin (15 μM). Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells.

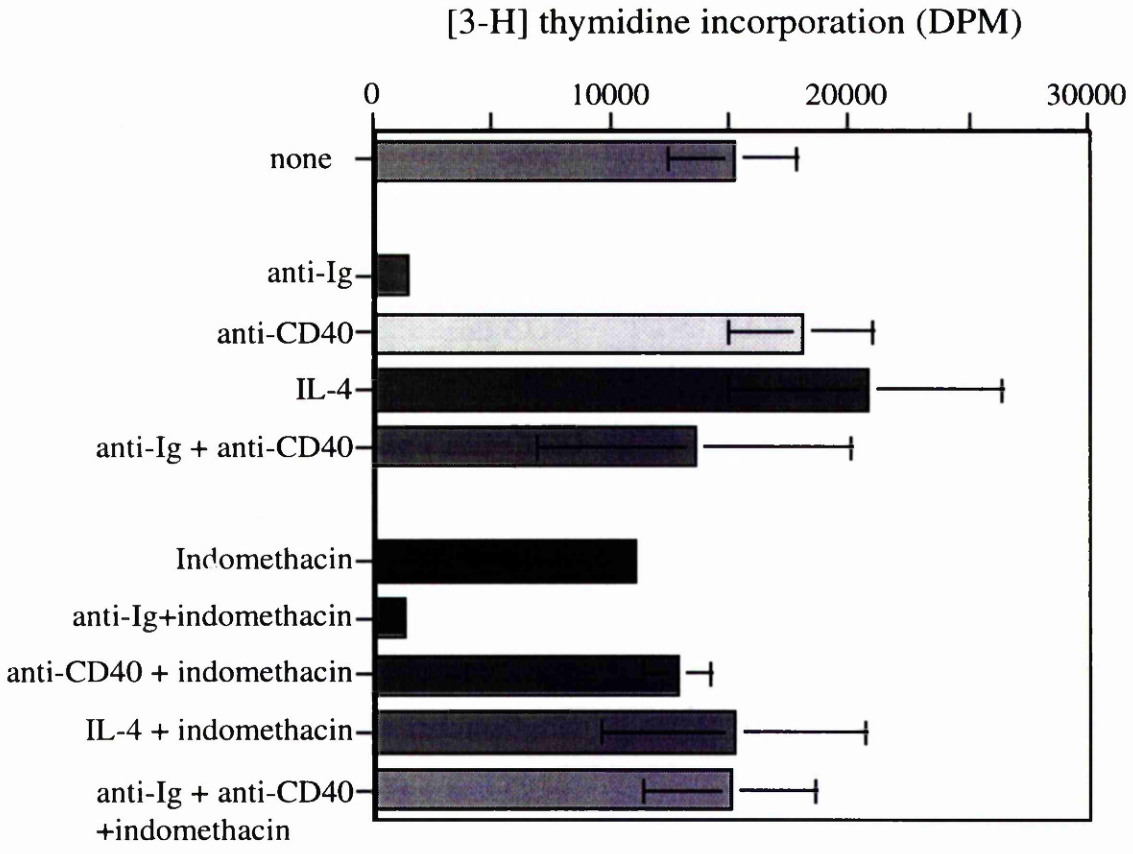

[3-H] thymidine incorporation (DPM)
0
10000
20000
30000
none
anti-Ig
anti-CD40
IL-4
anti-Ig + anti-CD40
Indomethacin
anti-Ig+indomethacin
anti-CD40 + indomethacin
IL-4 + indomethacin
anti-Ig + anti-CD40
+indomethacin

Figure 48

Figure 49. Model for the role of intracellular AA generation in WEHI-231 cell apoptosis. BCR-ligation induces $cPLA_2$ activation, AA generation and the induction of apoptosis. BCR ligation also downregulates PGE_2 production, presumably through inhibition of COX activity. CD40 mediated rescue from apoptosis reduces AA generation and re-establishes COX activity.

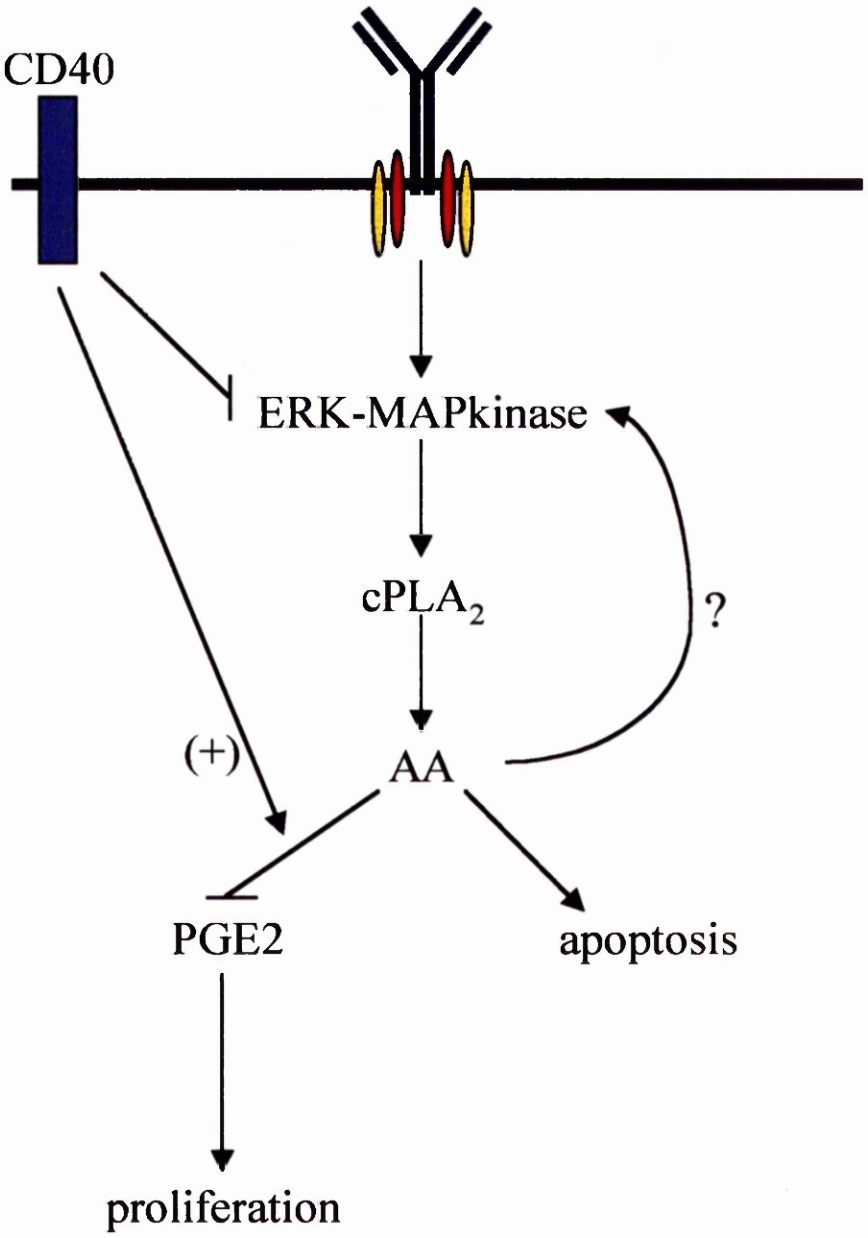


figure 49

Figure 50. Exogenous AA stimulation induces both positive and negative responses in WEHI-231 cells. AA stimulation leads to the induction of both apoptotic and proliferative responses. AA-mediated apoptosis has been shown to result from the disruption of the mitochondrial membrane potential and subsequent cytochrome c release. However, AA stimulation also induces cell cycle progression, possibly via the conversion of AA into PGE_2 by basal COX activity.

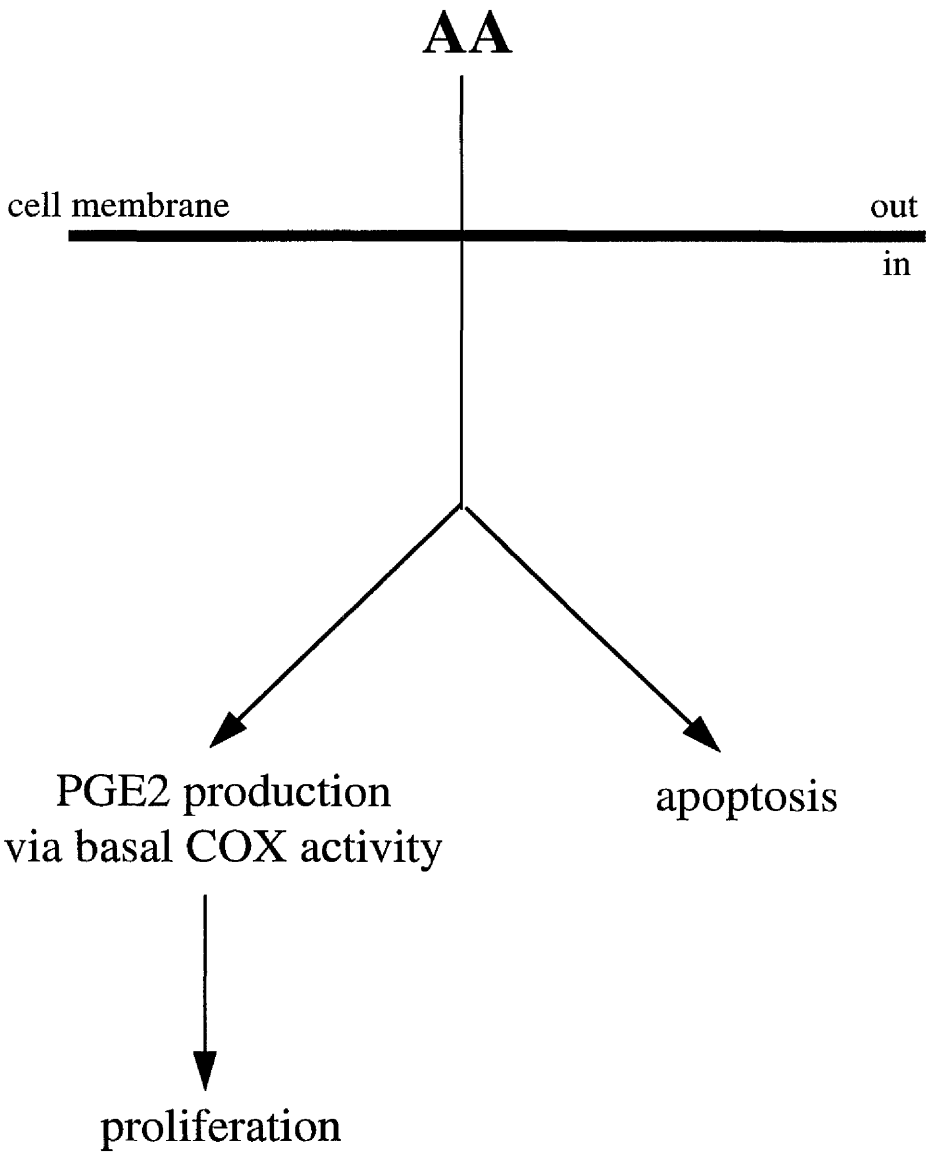

AA
cell membrane
out
in
PGE2 production
via basal COX activity
apoptosis
proliferation

figure 50

CHAPTER 6 - Regulators of MAPKinase activity in WEHI-231 cells.

6.0 Introduction.

The MAPKinases play crucial roles in determining cellular responses activated by a wide range of receptor types. Previous studies in this project have shown both proliferative and apoptotic responses to be modulated by ERK-MAPKinase in WEHI-231 cells. This chapter aims to address the mechanisms utilised by WEHI-231 cells to regulate ERK-MAPKinase activity, both positively and negatively, to help dissect the signalling mechanisms behind these differential biological responses.

As many signalling elements are known to regulate ERK-MAPKinase activity, this chapter shall be split into two sections. Section 6.1 will discuss the classical regulators of BCR mediated ERK-MAPKinase patterns, whilst section 6.2 investigates regulation of ERK-MAPKinase activity by cross-talk with other signalling pathways, including the roles played by lipid kinases.

6.1 Classical regulators of ERK-MAPKinase activity.

Negative feedback mechanisms leading to the inhibition of ERK-MAPKinase activities have been widely reported and encompass molecules that compete as upstream substrates of the Ras-Raf signalling cassette. These molecules include the recently described Raf-kinase inhibitor protein or RKIP, which prevents MEK activation by Raf-1 (128), Ras-GTPases such as p120GAP or Rap1 which inactivates Ras. In addition, a number of protein tyrosine phosphatases such as PAC-1, MKP1, MKP-2 and PP2A have been shown to directly downregulate ERK-MAPKinase activity. These molecules may therefore play key roles in the mechanisms of BCR-mediated down-regulation of ERK-MAPKinase in WEHI-231 cells in addition to modulating other BCR-mediated responses.

6.1.1 Phosphatases.

Protein phosphorylation is central to almost all cellular events. However, in order to prevent aberrant signalling events this mechanism is tightly regulated. Abnormalities in these check-points can lead to severe physiological disorders, such as autoimmune diseases, immunodeficiency and neoplasia (129). Originally, protein tyrosine kinases were thought to be the major regulators of tyrosine phosphorylation. However, the discovery of a large number of protein tyrosine phosphatases (PTPs) has shown protein tyrosine de-phosphorylation to play a key role in determining protein phosphorylation levels within the cell.

Presently, three families of PTPs have been classified:

1. Classical PTPs.
2. Dual specific PTPs.
3. Atypical PTPs.

Although all three families have similar tertiary structures and similar catalytic mechanisms, they differ greatly in their relative sequence homologies (129).

6.1.1.1 CD45

Classical PTPs can exist as transmembrane (receptor) and non-transmembrane forms, however, perhaps the most widely studied PTP is the transmembrane molecule CD45 which is expressed on all leukocytes. Early views suggested a positive role for CD45 in B and T lymphocyte signalling (reviewed in (130)). These studies suggested that C-terminal Src kinase (Csk) phosphorylated the C-terminal negative regulatory tyrosine residue of the src-family PTKs. But, upon antigen receptor ligation, CD45 could dephosphorylate this negative regulatory site allowing the Src-PTKs to become activated. A number of studies have since challenged this view, including work using CD45-deficient WEHI-231 cells. These studies investigated the basal phosphorylation state of the Src family PTK Lyn, showing Lyn to be both constitutively active, and hyper-phosphorylated at regulatory tyrosine residues (130) suggesting that Src family PTKs can be activated even with phosphorylated C-terminal tyrosine residues. Other studies have addressed the importance of CD45 in B lymphocyte development/function (reviewed in (129)). CD45-/- mice have been shown to possess a mature B cell population, but normal B cell development is disturbed. This may reflect the potential role for CD45 in setting the threshold for BCR responsiveness. Indeed, recent studies have shown CD45-/- B lymphocytes have raised BCR thresholds for activation (131). Further evidence to support this comes from studies in which mice expressing transgenic, HEL-specific BCRs, were crossed onto a CD45-/- background. Subsequently, upon antigen (HEL) exposure, a marked decrease in HEL-induced deletion was observed, again suggesting a positive role for CD45 in setting BCR threshold levels for the deletion of auto-reactive B cells.

6.1.1.2 SH2 domain containing tyrosine PTP (SHP-1)

In addition to the transmembrane phosphatase CD45, other non-transmembrane PTPs are known to play key roles in B lymphocyte development and function. These include the two SH2 domain containing tyrosine PTPs (SHP-1 and SHP-2). The role of SHP-1 in B lymphocyte development/function is now more clearly understood due to studies on motheaten (*me*) and motheaten viable (me^v) mice. These are natural

mouse mutants which are SHP-1 null or express catalytically inactive SHP-1 respectively (129). These mice have defects in nearly all hematopoietic cell lineages and physiologically, have high serum immunoglobulin and autoantibody levels suggesting defects in B lymphocyte development/function. An important role for SHP-1 in regulating BCR-mediated responses was shown in studies using purified splenic B lymphocytes from *me* and *me^v* mice. Stimulation of these cells with LPS induced normal proliferative responses, whereas anti-IgM stimulation induced proliferative responses comparable to wild-type cells at one-tenth the normal dose of anti-IgM (132). In addition, developing B cells from *me^v* mice crossed onto a HEL-transgenic background were shown to be hyperresponsive to anti-HEL mediated deletion, with normally sub-lethal doses of anti-HEL sufficient to induce cell death. Taken together, these results suggest SHP-1 plays an important role in regulating both BCR-mediated proliferative and negative selection processes. Studies have addressed the signalling pathways targeted by SHP-1 in B lymphocytes under such conditions. Recent work by Dustin et. al., 1999 (133) showed that expression of catalytically inactive SHP-1 increased Ca^{2+} mobilisation, extracellular signal-regulated kinase activation, and homotypic adhesion after BCR engagement suggesting that SHP-1 regulates both early and late events in B lymphocyte activation.

How BCR-mediated signalling events are regulated by SHP-1 is still relatively unclear. However, SHP-1 has been shown to be recruited to the B lymphocyte-restricted surface molecule CD22 upon BCR-ligation by binding to an ITIM motif in its cytoplasmic tail (17, 60). CD22 has been shown to negatively regulate BCR-mediated responses, with B cells from CD22 -/- displaying enhanced intracellular Ca^{2+} responses and characteristics of chronic BCR stimulation (134). Further evidence confirming a negative role for CD22 has emerged from studies suggesting that CD22 may remove SHP-1 from the BCR complex following BCR ligation (129). Further evidence to suggest such a theory comes from studies by Fujimoto *et. al.* (134) who have shown that CD19, which is required for optimal CD22 phosphorylation after BCR-ligation, regulates SHP-1 binding to CD22. CD22 has also been shown to be a key regulator of BCR-mediated MAPKinase activation. Studies (reviewed in (17)) have shown that crosslinking of CD22 with the BCR reduce BCR-mediated activation of both ERK-MAPKinase and JNK, but not p38 MAPKinase. These studies used biotinylated Fab fragments of anti-Ig and CD22 antibodies and crosslinking was induced by the addition of avidin. In contrast to these experiments, studies have shown that pre-treating cells with anti-CD22 antibodies prior to BCR ligation actually enhanced ERK-MAPKinase and JNK activation. This contradictory report has been attributed to the ability of the anti-CD22 antibody to sequester CD22 away from the BCR complex so removing any

negative regulatory effect it might yield, in turn decreasing the threshold for BCR signalling.

SHP-1 has been shown to be constitutively associated with the BCR in resting B cells, but rapidly dissociates after BCR stimulation (132). However, additional roles for SHP-1 in modulating BCR-mediated responses have arisen from studies showing the inducible association of SHP-1 with other transmembrane molecules. The first of these processes involves the Fc receptor, FcγRIIB. Crosslinking of this inhibitory receptor with the BCR by immune complexes is known to abrogate BCR-mediated proliferative responses in mature splenic B lymphocytes. Early experiments showed the recruitment of SHP-1 (and SHP-2) to the cytoplasmic tail of phosphorylated (ITIM) FcγRIIB suggesting a role for SHP-1 in FcγRIIB-mediated inhibition of BCR stimulation (17). However, the absolute involvement of SHP-1 in FcγRIIB-mediated inhibitory responses is in doubt. Firstly, FcγRIIB-mediated suppression of BCR-induced calcium influxes remained even in SHP-1-/- DT40 cells. Secondly, under these circumstances the lipid phosphatase, SHIP, is still recruited by FcγRIIB. Only in studies where SHIP was also deficient, did FcγRIIB-mediated inhibition of BCR-induced calcium influxes become reversed (17). However, FcγRIIB-mediated inhibition of BCR-induced proliferation in mature B lymphocytes has been shown to be impaired in SHP-1 deficient motheaten mice (60) suggesting that SHP-1 is not redundant in all FcγRIIB mediated signalling events.

6.1.1.3 Specific MAPKinase phosphatases

In the last decade a number of MAPKinase specific, nuclear phosphatases have also been isolated, cloned and functional studies carried out. These include the MAPKinase phosphatases, MKP-1 and MKP2, and also PAC-1. All three phosphatases are dual-specific, that is, they all dephosphorylate ERK-MAPKinase on tyrosine and threonine residues. MKP-1 was the first MAPKinase phosphatase to be discovered, and is encoded by the human CL100 and mouse 3CH134 immediate early genes and was shown to be activated following oxidative stress and mitogenic stimulation (135). PAC-1, a 32kDa nuclear PTPase, was originally discovered as a nuclear product of an early-response gene induced by T-cell activation, and has been subsequently shown to be predominantly expressed in hematopoietic tissue (136). Recombinant PAC-1 possesses strong substrate specificity for MAP kinase and has been shown to inhibit MAPKinase activity stimulated by epidermal growth factor, phorbol myristyl acetate, or T-cell receptor crosslinking (137). Recent studies have also indicated a role for PAC-1 in B lymphocyte biology. A paper by Grumont *et. al.* (138) has shown that PAC-1 mRNA expression is suppressed in resting mature B

lymphocytes, however, mitogenic concentrations of anti-Ig, LPS and anti-CD40 were all shown to upregulate PAC-1 mRNA expression. These results suggest that PAC-1 may be involved in the regulation of proliferative B lymphocyte responses.

The *in vivo* substrates of MKP-1, MKP-2 and PAC-1 are also distinct, with MKP-1 dephosphorylating ERK-MAPKinase and p38, MKP-2 dephosphorylating ERK-MAPKinase and JNK, whilst PAC-1 dephosphorylates ERK-MAPKinase, JNK and p38 MAPKinase respectively (139). This therefore allows for greater regulation of MAPKinase activation and cross-talk following cell stimulation.

6.1.2 Ras-like GTPases and GAPs.

The Ras-like GTPases belong to a family of proteins which contain a characteristic effector domain. This region undergoes a substantial conformational change during the conversion of a GDP-bound form into the active GTP-bound form which has been likened to a molecular switch. The Ras GTPase protein is the most widely studied member of this family as it frequently occurs in a mutated, constituitively active, form in human tumours (140). Ras activation has been shown to play key roles in antigen receptor signalling events in both B and T lymphocytes. Regulation of Ras activation occurs via two opposing pathways: activation by upstream Ras guanine nucleotide exchange factors (GEFs) and inhibition by GTPase-activating proteins (GAPs) (**figure 51**). A number of GAPs are known to be responsible for the downregulation of Ras, including p120GAP and neurofibromin (140). p120GAP is known to associate with the RasGAP-binding protein $p62^{dok}$ and this molecule has recently been shown to be a key mediator of inhibitory FcγRIIB signals in B lymphocytes (68). This study also highlighted the ability of SHIP to act as an adaptor molecule. Briefly, the study by Tamir *et. al.* (68) suggested BCR- FcγRIIB co-aggregation led to phosphorylation of FcγRIIB on its ITIM motif which in turn led to the recruitment of SHIP. This in turn creates a binding site for $p62^{dok}$ and Shc whilst also bringing them in close proximity to the BCR. $p62^{dok}$ could then be phosphorylated and recruit RasGAP which in turn converts BCR-stimulated Ras into its GTP-bound inactive form (**figure 52**).

In addition to RasGAPs, the Ras-like GTPase, Rap1, has been shown to be responsible for negatively regulating Ras-MAPKinase activation. Rap1 consists as two isoforms, Rap1a and Rap1b, differing in only a few amino-acids at the C-terminus (140). Rap1 has been shown to have an intrinsic GTPase activity approximately 10-fold lower that Ras, and unlike Ras, does not transform cells (140). Rap1 activity has not been extensively studied in B lymphocytes, however, its role as a negative regulator of T lymphocytes responses has been studied (96). The only known GEF for Rap1 is

C3G. A model of Rap1 activation by the TCR is shown in **figure 53**. Rap1 activity has been shown to negatively regulate the ERK-MAPKinase signalling cascade in lymphocytes due to the ability of Rap1 to compete with Ras for Raf-1 binding. However, these studies have utilised Rap1 mutants where Rap1 is transiently overexpressed and it is not known whether normal cellular levels of Rap1 are sufficient to induce such a response (96). It has been suggested however, that Rap1 expression is upregulated during the induction of anergy in T lymphocytes (96) again confirming a role for this GTPase in negative T lymphocyte signalling events. In addition to the antagonistic effects of Rap1, it should also be noted that recent studies have indicated a positive role for Rap1 in the activation of sustained ERK-MAPKinase signals. These studies highlighted the role of Rap1 in the differentiation of PC12 cells with nerve growth factor (NGF) which is associated with sustained ERK-MAPKinase activation. Subsequent investigation revealed that Rap1 formed a stable complex with B-Raf leading to ERK-MAPKinase activation (42, 141, 142). Therefore, it appears Rap1 can mediate both positive and negative signals by activating cAMP-dependent B-Raf, but inhibiting Raf-1 activity.

6.1.3 RKIP

Recent studies by Yeung *et. al.*, 1999 (128), have highlighted the role of a new protein-kinase-inhibitor protein to suppress the activity of the Raf/MEK/ERK signalling cascade. The technique of yeast two-hybrid screening, using the structural Raf-1 kinase domain (BXB) as bait, was used to isolate scaffolding and regulatory proteins involved in regulating the Raf/MEK/ERK signalling cascade. One recovered clone was shown to bind to both kinase-active and kinase-negative BXB and was subsequently termed RKIP. RKIP, now known to be widely expressed in a range of cell types, was shown to suppress ERK-MAPKinase activity by disrupting the ERK-MAPKinase pathway primarily at the Raf/MEK interface both *in vitro* and *in vivo* (128) by directly disrupting the interaction between these two kinases.

6.1.4 Aim of studies.

The aim of this study is to investigate BCR-induced growth-arrest, and in particular, the mechanisms underlying the long-term down-regulation of ERK-MAPKinase.

In particular, we will focus on whether:

1. BCR-ligation uncouples elements of the Ras/Raf/MAPKinase pathway by recruitment of RasGAPs or other negative regulatory elements.

2. known ERK-MAPKinase phosphatases, especially PAC-1 are regulated during BCR-mediated signalling cascades.

6.1.5 Results.

6.1.5.1 BCR ligation increases the association of ERK-MAPKinase with the nuclear phosphatase PAC-1.

In order to determine a possible mechanism by which BCR ligation down-regulates ERK-MAPKinase activation, we decided to investigate whether PAC-1 played any role in this process. PAC-1 is an inducible nuclear phosphatase which has been shown to possess strong substrate specificity towards ERK-MAPKinase. To investigate a possible association between ERK-MAPKinase and PAC-1, whole cell lysates were immuno-precipitated with an antibody recognising ERK-MAPKinase. Samples were then subjected to SDS-PAGE and probed with an antibody recognising PAC-1. Western-blot analysis revealed an association with ERK-MAPKinase and PAC-1 which increased with time following BCR ligation (**figure 54A**). This suggests that BCR ligation leads to an increase in the association of these two enzymes which may result in the dephosphorylation of ERK-MAPKinase. Interestingly, co-treatment with CD40 prevents this increase in association, suggesting that CD40 treatment reverses BCR-mediated growth arrest by re-establishing a proliferative ERK-MAPKinase by suppressing the association of PAC-1 with ERK-MAPKinase. In order to rule out the possibility of BCR-mediated increases in PAC-1 protein expression, whole cell lysates were probed with anti-PAC-1 antibody and compared to wERK expression used as a loading control for whole protein levels (**figure 54B**). Interestingly western blot analysis showed that PAC-1 protein levels varied with time (0-48 hours) by both anti-Ig and anti-Ig plus anti-CD40 treatment. However, the most pronounced effect on PAC-1 protein expression resulted from co-stimulation with anti-Ig and anti-CD40. This observation is in agreement with other studies which observed increases in PAC-1 mRNA levels following CD40 stimulation in mature B lymphocytes (138). However, together, these observations suggest that although PAC-1 protein expression is upregulated by anti-CD40 stimulation, the association with ERK-MAPKinase is decreased.

Additional studies have examined whether ERK-MAPKinase associates with the phosphatase PP2A. Initial experiments suggest that ERK-MAPKinase and PP2A are constituitively associated with each other, and this association remains unchanged regardless of cell stimulation. Whether PP2A activity is modulated by BCR-ligation has not yet been addressed in immature B cells and warrants further study.

6.1.5.2 Role of GAPs in WEHI-231 cell signalling.

Having shown that early, BCR-mediated ERK-MAPKinase signals correlated with Ras activation in WEHI-231 cells (**figure 25C**), similar studies were carried out to examine whether long-term ERK-MAPKinase signals (0-48 hours) were also activated in a Ras-dependent manner (**figure 55A**). WEHI-231 cells left untreated were shown to express a basal Ras activity. Ras activity, unlike ERK-MAPKinase (**figure 55B**), was also observed over the whole 48 hour time course in anti-Ig treated cells suggesting that BCR-induced suppression of ERK-MAPKinase occurs downstream of Ras. Cells treated with anti-Ig and anti-CD40 were shown to have substantial elevations in Ras activity, suggesting that CD40 mediated rescue from BCR-induced growth arrest involves the enhanced activation of Ras.

In addition, as Rap1 has previously been shown to uncouple TCR signalling through the Ras/Raf-1/MAPKinase, the activity of Rap1 was measured following BCR ligation. Due to the specific nature of Raf-RBD (Ras) and Ral-RBD (Rap1) substrates, activated Ras and Rap1 could be immunoprecipitated from the same source, allowing for the direct comparison of their relative activities. As previously shown, untreated WEHI-231 cells express a basal level of Ras activity which appears to be cyclic in nature and is probably linked to basal proliferation of this cell line (**figure 55A and 56A**). Interestingly, untreated cells also expressed a high amount of activated Rap1 protein (**figure 56A**). This may reflect a negative feedback mechanism utilised by WEHI-231 to prevent excessive ERK-MAPKinase activation during normal, unstimulated, proliferation. However, basal Rap1 activity could also represent an additional model of basal Raf/ERK-MAPKinase activation which results in the activation of B-Raf by Rap1 (42, 141, 142).

Previous studies had demonstrated that long-term BCR-ligation led to the down-regulation of a basal, proliferative, ERK-MAPKinase signal which is presumably central to BCR-mediated growth-arrest in WEHI-231 cells. However, long-term BCR-ligation was ineffective in down-regulating Ras activity in these cells (**figures 55A and 56A**). Therefore, some other mechanism must be activated upon BCR-ligation to uncouple Ras-activity from ERK-MAPKinase activation. We therefore examined whether Rap1 activity was induced by BCR ligation and hence down-regulate ERK-MAPKinase activity. Initial studies (**figure 56A**) indicated that BCR ligation did not induce Rap1 activation suggesting that this pathway is not utilised in the down-regulation of ERK-MAPKinase activity following BCR ligation.

Earlier findings had determined a role for CD40 in both re-establishing and strengthening ERK-MAPKinase activity in cells co-stimulated with anti-Ig to ligate the BCR. This was also seen to induce a late, but large increase in Ras activity compared

to untreated cells (**figures 55A and 56A**). Studies also indicated that Rap1 activity was not upregulated in these samples (**figure 56A**).

These results suggest that BCR ligation does not utilise Rap1 activity in the down-regulation of ERK-MAPKinase activity in WEHI-231 cells. However, these results do suggest that Rap1 activity may be important for the basal proliferation of these cells. In addition, the results indicate that co-stimulation with CD40, whilst substantially enhancing Ras activity compared to untreated cells, down-regulates the basal Rap1 activity present in untreated cells.

6.1.5.3 BCR-ligation uncouples ERK-MAPKinase from its upstream mediators.

In order to address the possibility that BCR ligation physically uncouples Ras from the Raf/ERK-MAPKinase signalling cassette, Raf-RBD pull down samples from **figure 56A** were stripped and re-probed with an antibody recognising whole ERK-MAPKinase (**figure 56B**). Cells left untreated, or co-treated with both anti-Ig plus anti-CD40, both proliferative signals, were shown to express wERK over the whole time range which followed the cyclic nature of Ras activation. However, cells stimulated with anti-Ig alone, although Ras activation could be detected (**figure 56A**), ERK-MAPKinase protein expression was markedly decreased indicating that BCR-ligation, in addition to regulating ERK-MAPKinase activity, promotes the dissociation of the Ras/Raf/MAPKinase signalling cascade. These results are therefore suggestive of a possible role for RKIP in the uncoupling of the Ras/ERK-MAPKinase signalling cascade.

6.1.5.4 RKIP-mediated suppression of ERK-MAPKinase by BCR-ligation is yet undetermined in WEHI-231 cells.

In order to determine whether RKIP could be responsible for BCR-mediated suppression of ERK-MAPKinase in WEHI-231 cells we examined whether RKIP/Raf-1 association was modulated by BCR, or indeed CD40 ligation. Whole cell lysates of WEHI-231 cells left untreated or treated with anti-Ig or anti-Ig and anti-CD40 over a 48 hour period were immuno-precipitated with Raf-1 and subjected to SDS-PAGE. Membranes were then probed with RKIP (kindly provided by W. Kolch, Beatson Institute for Cancer Research, Glasgow). Unfortunately, the results indicated that the antisera to RKIP recognised multiple, non-specific, proteins and no accurate analysis of RKIP/Raf-1 association could be determined. However, these results do not rule out a role for RKIP in modulating ERK-MAPKinase activities in B lymphocytes.

6.1.6 Discussion.

The results in this section aimed to address the role of a number of classical regulators of ERK-MAPKinase activation in order to dissect the signalling mechanisms involved in the downregulation of ERK-MAPKinase activities following BCR-ligation. This phenomenon is likely to result from the activation of a wide range of signalling molecules following BCR-ligation and not solely one particular signalling element. This is likely because ERK-MAPKinase is known to act as both a cytoplasmic protein kinase, but also translocates to the nucleus upon activation where it acts as a nuclear protein kinase activating transcription factors. Therefore termination of this signal probably involves the activation of both nuclear and cytoplasmic regulators of ERK-MAPKinase.

Initial studies examined the role of the nuclear phosphatase PAC-1 in the regulation of ERK-MAPKinase activity following BCR ligation. These results suggest that BCR ligation promotes the association of PAC-1 with ERK-MAPKinase suggesting that PAC-1 may be involved in the dephosphorylation, and hence inactivation, of this kinase. Interestingly, the co-association between PAC-1 and ERK-MAPKinase appeared to be enhanced approximately 4-8 hours post anti-Ig treatment, however, it remains to be determined whether these interactions are localised to the nucleus. This fits the kinetics of previous experiments which showed the down-regulation of basal, proliferative ERK-MAPKinase signals at approximately the same time. Interestingly, this co-association was reversed by co-treatment with anti-CD40 indicating that CD40 ligation leads to the regulation of nuclear- aswell as cytoplasmic-signalling events. Additional experiments have suggested that co-stimulation of anti-Ig treated cells with anti-CD40 results in upregulated PAC-1 protein expression. This is in agreement with other studies (138) which described increased expression of PAC-1 mRNA in resting splenic B lymphocyte following mitogenic stimulation, including anti-CD40. However, why PAC-1 expression should increase during the rescue of WEHI-231 cells from BCR-induced growth-arrest is unclear, but suggests that PAC-1 may regulate ERK-MAPKinase responses during cell proliferation.

In addition to studying the role of PAC-1 in WEHI-231 cell signalling we examined the ability of BCR ligation to regulate Ras activity and hence down-regulate ERK-MAPKinase activity. Earlier studies in this section had described the inability of BCR ligation to down-regulate Ras activation. We therefore decided to examine Rap1 GTPase activity as the effector binding domain of this can compete with Ras-GTP for downstream effectors such as Ras. A previous study by McLeod et. al., 1998 (44) had confirmed the ability of BCR-ligation to activate Rap1 in WEHI-231 cells. However, these studies used extremely high levels of anti-Ig over relatively a short time course.

We therefore wanted to examine whether BCR-ligation activated Rap1 at lower anti-Ig concentrations and whether its activity was also modulated by CD40-ligation.

Our results indicated that BCR-ligation does not induce Rap1 activation under conditions favourable to the induction of growth arrest and apoptosis in WEHI-231 cells. In comparison, and initially rather surprising, untreated cells were shown to express high levels of active Rap1 in a cyclic pattern. However, recent studies have suggested a role for Rap1 in the sustained activation of ERK-MAPKinase by B-Raf. This suggests that basal ERK-MAPKinase activity, which is vital for cell cycle progression, may be activated via a Rap1/B-Raf dependent mechanism (**figure 57**), however, basal Ras activity suggests that it is not the sole pathway leading to ERK-MAPKinase activation. The loss of Rap1 activity in anti-Ig stimulated cells may therefore be due to the uncoupling of this mechanism, resulting in the loss of basal ERK-MAPKinase activity and hence growth arrest, all of which we have observed following BCR-ligation. Interestingly, co-stimulation of anti-Ig treated cells with anti-CD40 did not restore basal Rap1 activity, but has previously been shown to restore ERK-MAPKinase activity, in addition to enhancing Ras activation.

In order to investigate whether BCR-ligation lead to the dissociation of the Ras/Raf/ERK signalling cascade, Ras assay blots (**figure 56A**) were stripped and re-probed with an antibody to ERK-MAPKinase. Surprisingly, these results indicated that that GTP-bound Ras was complexed to ERK-MAPKinase in cells left untreated, or stimulated with anti-Ig plus anti-CD40 (both proliferative in nature). In contrast to this, ERK-MAPKinase was not complexed with GTP-bound Ras in cells treated with anti-Ig. These results indicate that the Ras/Raf/MEK/ERK signalling cascade may exist as a pre-formed complex in WEHI-231 cells and that BCR ligation promotes the dissociation of this complex and inhibition of ERK-MAPKinase signalling. The recently described Raf binding protein, RKIP (128), has been shown to suppress ERK-MAPKinase activity by competitively disrupting Raf/MEK interactions. We therefore examined whether this molecule was involved in the disruption of the Ras/Raf/ERK cascade following BCR ligation. Unfortunately, initial studies have been unable to address this hypothesis.

6.2 Cross-talk regulation of ERK-MAPKinase activity by BCR-activated signalling pathways.

In addition to the mechanisms discussed in section 5.1, other signalling pathways are known to directly regulate ERK-MAPKinase activity. Unlike the regulatory processes described above, many of these pathways are initiated upon receptor ligation and regulate ERK-MAPKinase activity through the activation of cross-talk mechanisms. This section will concentrate on three known signalling elements which are known to regulate ERK-MAPKinase activity. These include cAMP and the lipid kinases, PI(3)K and sphingosine kinase.

6.2.1 cAMP and the phosphodiesterases.

Adenosine-3', 5'-cyclic monophosphate (cAMP) is known to a play central role in many biological responses. However, its role as a negative regulator of cell growth is perhaps the most widely documented of these functions. Elevation of intracellular cAMP levels has been shown to promote growth arrest in growth-factor stimulated fibroblasts (including rat-1 and NIH 3T3 cells), smooth muscle cells and adipocytes (142). In addition, cAMP has also been shown to play a key role as a negative regulator of proliferation in lymphocytes (143). Indeed, early studies indicated that cAMP generating agents such as cholera toxin, forskolin and prostaglandin E_2, were able to inhibit BCR-mediated proliferation of both human and murine lymphocytes (144).

cAMP is generated by the membrane bound adenylyl cyclases which catalyse the generation of cAMP from ATP. Presently, there are more than eight isoforms of adenylyl cyclase, each genetically unique, being encoded from separate genes (143). A wealth of literature has arisen examining the downstream effectors of cAMP generation, where subsequently, the cAMP-dependent protein kinase, PKA, has been shown to play a key role. PKA is a holoenzyme consisting of two regulatory and two catalytic sub-units. cAMP binding to the regulatory subunits causes the release and subsequent activation of the two catalytic subunits. The catalytic subunits act as serine/threonine kinases with both cytoplasmic and nuclear substrates (143, 145). The nuclear targets of PKA are transcription factors, consisting of members of the cAMP responsive element-binding factor (CREB) family, whilst cytoplasmic targets include PKC activation and the inhibition of PIP_2 breakdown by PLCγ. In addition, the role of cAMP/PKA as a negative regulator of ERK-MAPKinase activity has been reviewed in T lymphocytes (143) where PKA activity has been shown to suppress Raf-1 activity stimulated through the TCR/CD3 receptor complex.

Intracellular cAMP levels are tightly regulated to help maintain the normal homeostasis of the cell. This process is achieved by both the regulation of cAMP generation, but also by cAMP degradation by the cyclic nucleotide phosphodiesterases (PDEs). The PDEs form part of a multigene family and have been shown to have roles in vascular relaxation, cardiac muscle contraction and inflammation (143). They can be separated into at least seven functional classes, depending on their substrate specificity, inhibitor sensitivity and molecular sequence (146). Catalytically, the PDEs promote the hydrolysis of cAMP and cGMP to their respective 5' nucleoside monophosphates. In recent years, the mechanisms underlying PDE activity and expression have been studied extensively. Reports suggest that anti-CD3 or TCR-mAbs induce a slow, but sustained activation of PDE4. In addition, this was shown to be PKC-dependent as selective inhibitors of PKC could block this effect, but could be mimicked by phorbol ester treatment (143). More recent reports have suggested roles for both ERK-MAPKinase and PKA itself in regulating PDE activity and expression. Studies by Lui and Maurice,1999 (146), provided evidence suggesting that PKA caused the phosphorylation and activation of the PDE4D3 and PDE4D5 isoforms in vascular smooth muscle. PDE4D3 was also activated by PKC-mediated ERK-MAPKinase activity in the same cells, whilst, co-activation of both PKA and ERK-MAPKinase allowed for both the activation, but also translocation of PDE4D3 from the particulate to the cytosolic fraction of the same cells.

The role played by cAMP/PKA in lymphocyte signalling remains controversial. However, a number of lines of evidence exist to suggest that cAMP my play role in lymphocyte responses. A range of stimuli regulating B lymphocyte function including anti-Ig, IL-4 and IL-10, which induce DNA synthesis and differentiation, have been shown to be tightly influenced by cAMP (144). An emerging area of cAMP/PKA signalling in B lymphocytes is via CD40 ligation, although evidence for this is remains controversial. A number of studies have observed CD40-mediated increases in cAMP levels in both human tonsillar B cells activated with anti-CD40 antibodies and also murine splenic B cells treated with activated T cell clones or membranes (145). However, reports also suggest that cAMP/PKA signalling events regulate, but are not activated by, CD40-ligation. For example, studies by Goldstein *et. al.* (145) provided evidence for the role of cAMP/PKA in CD40 signalling by using a tetracycline-repressible dominant-negative form of PKA in the B lymphoma line M12. Expression of a dominant-negative form of PKA inhibited cAMP mediated growth arrest which was reversed upon tetracycline treatment. However, expression of the dominant-negative PKA had no effect on CD40-mediated growth arrest suggesting that CD40-mediated growth arrest in M12 cells is cAMP/PKA independent. Other studies examining a role for PKA in CD40 mediated signalling events have shown that unlike BCR-ligation, CD40-mediated activation of ERK-MAPKinase uses an unidentified

PKA-insensitive MEKK (ie. other than Raf-1) to regulate ERK-MAPKinase activation (97).

6.2.2 Lipid kinases

Important roles for sphingophospholipid- and glycerophospholipid-derived second messengers in determining cell fate have been shown in a wide range of studies over many years. Perhaps the most intensely studied of all lipid second messengers are ceramide and 1,2-diacylglycerol (DAG). Not only do these second messengers act as signalling intermediates, but that also have key structural roles as they form the backbone of all phospholipids, existing as bound or free forms making up 1-2% of total phospholipid levels in mammalian cells (147).

The structural roles of the glycerophospholipid-derived second messenger DAG are well established. However, its generation and ability to activate protein kinase C (PKC) remain less well understood. DAG is produced from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by the activation of phospholipase C (PLC) (148). Currently, three PLC isoforms have been discovered and include PLCβ, PLCγ and PLCδ. All three PLC isoforms share similarities in their structure. Subsequently, each isoform is known to contain two regions of high homology, preceded by a lipid-binding pleckstrin homology (PH) domain. To-date a number of mechanisms exist explaining the activation of PLC. These include the activation of PLC-β by hetertrimeric G-proteins, whilst PLC-γ is recruited to the cell membrane by binding to receptor tyrosine kinases via its SH2 domains and is activated by subsequent phosphorylation (147).

In addition to the above mentioned sphingophospholipid and glycero-phospholipid lipid pathways, other phospholipid generating pathways have been shown to play keys roles in a wide range of cellular responses. Perhaps the most important of these was the discovery of phosphoinositide-3-OH kinase (PI(3)K) over a decade ago.

6.2.2.1 Structure, function of PI(3)K.

PI(3)K was originally discovered due to its association with the polyoma middle T antigen, $pp60^{v\text{-}src}$ and $pp68^{v\text{-}ros}$, and with anti-phosphotyrosine immunoprecipitates from PDGF-stimulated fibroblasts (149). These studies also suggested a potential role for PI(3)K in mitogenic responses. The kinase activity of PI(3)K is known to phosphorylate the D3-hydroxy-group of the inositol headgroup of phosphoinositides (**figure 58A**). Subsequently, PI(3)K has been shown to phosphorylate PtdIns(4)P and PtdIns(4,5)P_2 to produce PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively (150).

Use of modern molecular biology techniques has identified PI(3)K to consist of a number of family members. Subsequently, three sub-families of PI(3)K have been described, each based on its specific *in vitro* substrate specificity (149). These sub-families can be summarised as follows:

- **Class I PI(3)Ks** - heterodimers of approximately 200kDa (110-120kDa catalytic subunit and a 50-100kDa adaptor subunit) that phosphorylate PtdIns and PtdIns(4,5)P_2 (preferred substrate).
- **Class II PI(3)Ks** - 170-210kDa proteins with no identified adaptor molecules that phosphorylate PtdIns and PtdIns 4-P.
- **Class III PI(3)Ks** - exclusively phosphorylate PtdIns.

Class I PI(3)Ks can be divided further into class IA and class IB (151). Class IA PI(3)Ks (consisting of p110α, p110β or p110γ catalytic subunits) associate with a p85 adaptor protein essential for interaction with receptor tyrosine kinases. Class IB PI(3)K is activated by heterotrimeric G-protein sub-units and associates via its N-terminus with a p101 adaptor subunit which is believed to confer Gβγ sensitivity to PI(3)Kγ (149).

The structure of class IA PI(3)Ks has been extensively studied by many groups and has produced many insights into its activation process. The p85 prototype adaptor (or regulatory) sub-unit associated with the class IA PI(3)Ks is a multi-domain molecule consisting of N-terminal SH3 domains, a BH domain flanked by two proline rich regions and two C-terminal SH2 domains and also a inter-SH2 domain that mediates the association of p85 with the catalytic sub-unit (149) (**figure 58B**).

This structural information has helped in the understanding of the mechanisms underlying class IA PI(3)K activation. In quiescent cells, class I PI(3)K is inactive, but in the presence of extracellular stimuli, is rapidly activated. Although the precise mechanisms for both maintaining PI(3)K in an inactive state and subsequent activation are still under much investigation some theories have come to light (reviewed in Wymann et. al., 1998 (149)). It has been suggested that class I PI(3)K is kept inactive due to the intrinsic protein serine kinase activity of its associated catalytic subunit. This allows regulatory subunits to become phosphorylated on Ser residues (Ser^{608} of p85α) which in turn reduces lipid kinase activity.

Mechanisms of class I PI(3)K activation have been suggested that involve binding of different molecules to the different domains in the structure of PI(3)K. One such model has suggested that class IA PI(3)Ks can be activated by four co-operating processes:

1. PI(3)K translocation to the plasma membrane by targeting the SH2 domains of p85 subunits to pYXXM motives of receptor protein tyrosine kinases or their substrates.
2. Binding of GTP-loaded Ras to a Ras-binding domain (RBD) in the catalytic subunit.
3. Binding of proline-rich regions of Shc, Cbl and dynamin to p85 SH3 domains.
4. Binding of Lyn, Fyn, Grb2, v-Src, Abl and Lck SH3 domains to p85 proline rich regions.

Downstream targets of PI(3)K include a wide range of protein kinases leading to physiological responses such as actin rearrangement, vesicular trafficking, cell adhesion, growth or survival (**figure 59**). The protein Ser/Thr kinase Akt/PKB has received much attention as a major downstream substrate of PI(3)K activity. Much of this attention is attributable to the survival signal provided by Akt/PKB in many cell systems. Akt/PKB was first discovered in the early 1990s as a retroviral oncogene which was amplified in many human tumours (152). Further analysis of the cellular function of Akt has revealed that it acts as a strong mediator of growth-factor-induced survival and has been shown to suppress apoptosis induced by a wide range of stimuli (32). Akt is activated both upon membrane localisation and phosphorylation of Ser^{473} and Thr^{308} residues. PI(3)K is known to be crucial for these events because membrane localisation of Akt is dependent upon binding to PtdIns(3,4)P_2/ PtdIns(3,4,5)P_3 and phosphorylation on Thr^{308} and Ser^{473} requires the activity of PtdIns(3,4,5)P_3-dependent protein kinases (PDKs). PDK-1, known to be directly activated by PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (150) phosphorylates Akt on Thr^{308} only. Therefore, additional protein kinases are required for full activation of Akt, and these may also be under the control of PI(3)K lipid products. PI(3)K activation, and in particular PIP_3 generation, has also been shown to be important for the activation of both PLCγ and Btk in lymphocytes. Under these circumstances PIP_3 accumulation at the plasma membrane acts as a specific membrane binding site for the PH domains of both PLCγ and Btk. This allows these enzymes to then come into contact with membrane associated tyrosine kinases that are required for their full activation (36). In addition, PI(3)K has been shown to exert its anti-apoptotic response by suppressing ceramide generation in TNF-α treated MCF-7 cells suggesting cross-talk between PI(3)K and SMase pathways (153).

6.2.2.2 A dual role for PI(3)K as both a lipid and a protein kinase.

An emerging area of PI(3)K interest lies in its role as both a lipid and a protein kinase. Mutations to the catalytic core of PI(3)Kα and PI(3)Kγ corresponding to a region of the activation loop in protein kinases have allowed the generation of protein kinase-active, but lipid kinase-inactive mutant enzymes (149). Studies by Lopez-Ilasaca *et. al.* (154) have shown that lipid-kinase inactive PI(3)Kγ mutants are still able to activate ERK-MAPKinase in a G-protein dependent manner. In addition, studies by Bondeva *et. al.* (155) have shown that PI(3)Kγ lipid kinase-inactive mutants are as effective as wild-type PI(3)Kγ in LPA-mediated ERK-MAPKinase activation. These studies highlight a new area for PI(3)K signalling.

6.2.2.3 Role of PI(3)K in B lymphocyte signalling.

PI(3)K activation is also known to play key roles in BCR mediated signalling pathways leading to B lymphocyte differentiation (156) and survival (36). Indeed, ablation of PI(3)K signalling in B lymphocytes by targeted disruption of the p85α regulatory subunit has been shown to lead to impaired B cell development at the pro-B cell stage and reduced numbers of mature B cells with decreased proliferative responses to BCR-ligation, LPS and anti-CD40 stimulation, a phenotype similar to that of xid (Btk)-deficient mice (157). This similarity in phenotypes between p85α -/- and xid mice is due to the involvement of PI(3)K activity in Btk activation. Studies have shown that the PH domain of Btk binds to PtdIns(3,4,5)P_3 (generated by PI(3)K) in the plasma membrane, and that this membrane recruitment is required for the enzymatic activation of Btk. Therefore in the case of p85α -/- mice, the reduced generation of PtdIns(3,4,5)P_3 results in decreased Btk activity. In addition, the mutation to Btk in xid mice is known to be located in the PH domain, which also results in decreased Btk activation, but this time due to the defective binding of Btk to membrane PtdIns(3,4,5)P_3.

BCR-mediated activation of PI(3)K has been shown to occur via Ras-dependent mechanisms, where active GTP-bound Ras binds the catalytic sub-unit of PI(3)K. In addition, the activation of PI(3)K has been shown to result from the phosphorylation of CD19 following BCR ligation. This mediates the association and activation of PI(3)K due to SH2 domains in its p85 regulatory sub-unit which bind pTyr residues on the cytoplasmic tail of CD19 (7, 38, 156). In addition, the binding of PI(3)K to CD19 following BCR-ligation also serves to localise PI(3)K to the plasma membrane where its phosphoinositide substrates are located.

Although PI(3)K activity and PIP_3 generation are crucial for normal B lymphocyte development, PIP_3 levels generated through BCR ligation are attenuated through coaggregation of the BCR with the low affinity Fc receptor, FcγRIIB. This inhibition is due to the recruitment of the Src homology 2 domain-containing 5' inositol phosphatase (SHIP) which hydrolyses the 5' phosphate from PtdIns(3,4,5)P_3 to produce PtdIns(3,4)P_2. This in turn leads to the disruption of BCR-mediated PLCγ and Btk signalling as both these molecules preferentially bind PtdIns(3,4,5)P_3 (67). Physiologically, this leads to decreases in BCR-mediated proliferative signalling in mature B lymphocytes due to decreased calcium influx resulting from impaired PLCγ signalling (69) and impaired B lymphocyte development (16).

6.2.2.4 Sphingolipid signalling pathways.

Although sphingolipid-derived second messengers have been shown to play key roles in mediating both proliferative and apoptotic responses they still remain relatively unstudied. Perhaps the best known of the sphingolipid-derived second messengers is ceramide, which is generated through the sphingomyelin pathway and has been shown to have key roles in the induction of apoptosis by agents such as TNF-α or FasL (158). Sphingomyelin is preferentially located on the outer leaflet of the plasma membrane and is hydrolysed by the activation of a phospholipase C (PLC)-like enzyme, sphingomyelinase (SMase). Three classes of SMase are currently known, classified according to their pH optima and Mg^{2+} -dependence. As well as the production of ceramide, the immediate product of SMase activity, other metabolities of sphingomyelin are known to function as second messengers. These include sphingosine (formed by the ceramidase-catalysed deacylation of ceramide), and sphingosine-1-phosphate (SPP) (formed by the sphingosine kinase-catalysed phosphorylation of sphingosine) (159). Interestingly, although ceramide is believed to be a negative regulator of cell fate by inducing apoptosis in a wide range of cell types, other sphingolipid-derived second messengers such as SPP have been shown to be mitogenic (147).

6.2.2.5 Structure and function of sphingosine kinase and its metabolite sphingosine-1-phosphate.

Sphingosine-1-phosphate (SPP) is a highly polar lipid second messenger metabolised from sphingosine by the activation of sphingosine kinase (**figure 60**) and like many lipid messengers, has been shown to possess both intra- and extra-cellular actions (160). Evidence as an intracellular messenger is wide-spread with suggested roles in a wide range of cell types, from T lymphocytes to monocytes (159). SPP is

believed to play a key role in many mitogenic responses including those activated via platelet-derived growth factor or fetal calf serum (161). It has also been shown to play a key role in anti-apoptotic responses by rescuing HL60 promyelocytic cells, U937 monoblastic leukaemia cells and Fas-expressing Jurkat T lymphocytes from apoptosis induced by dimethylsphingosine, TNF-α, ceramide, sphingomyelinase, PKC inhibitors and Fas (159). The mechanisms behind these anti-apoptotic mechanisms are still poorly understood. Suggestions have been put forward suggesting that the balance of sphingosine to SPP may play key roles in determining cell fate. In addition, PKC may play a role in the suppression of ceramide-mediated apoptosis by the activation of sphingosine kinase. Studies by Cuviller *et. al.* (162) provided evidence suggesting that not only could activation of PKC reverse ceramide induced apoptosis in HL-60 cells, but PKC mediated rescue was prevented using the competitive inhibitor of sphingosine kinase, N,N-dimethylsphingosine.

SPP, secreted by activated platelets (163), is also known to signal extracellularly through a group of orphan G_i-protein coupled receptors called Edg-1, Edg-3 and H218 (SPPR1, SPPR3 and SPPR2 respectively) (164).

G-protein coupled receptors comprise of seven membrane spanning domains, of which one interacts with the G-protein. G-proteins exist as trimers, consisting of αβ and γ subunits, although it is the α subunit which possess GTPase activity. In the resting state, GDP is associated with the α subunit, however, when the receptor becomes occupied by an agonist molecule a number of conformational changes occur resulting in the activation of target proteins (**figure 61**). Firstly, upon agonist binding, the αβγ trimer associates with the receptor which causes the GDP bound to the α subunit to dissociate and become replaced with GTP which in turn causes the dissociation of α-GTP from the βγ subunits. α-GTP, now in its active form now acts as an effector activating downstream signalling elements. However, it should be noted that βγ subunits have also been implicated in the activation of downstream effectors. G-proteins can be divided into 4 families; G_q, G_{12}, G_s and G_i. The G-proteins G_s and G_i have been shown to stimulate or inhibit adenylyl cyclase activity respectively and are perhaps the most widely studied of all G-protein families. This is perhaps due to the ability of cholera toxin and pertussis toxin to inhibit signalling through G_s and G_i coupled receptors respectively, enabling the investigation of the G-proteins involved in a number of biological systems to be explored.

The signals activated by SPP following the ligation of G_i-coupled Edg-receptors have been shown to include the activation of PLD, leading to Ca^{2+} mobilisation (163), the activation of ERK-MAPKinase via a pertussis toxin-sensitive pathway (164) and also the inhibition of forskolin induced cAMP generation (165). Studies have also suggested that SPP-mediated protection of T lymphocytes from apoptosis may involve

signalling through distinct Edg receptors leading to the suppression of the pro-apoptotic molecule, Bax (166).

The cloning and characterisation of the first mammalian sphingosine kinase was carried out by the work of Kohama et. al. (160). In this study they reported on the structure of the murine sphingosine kinase. Their studies indicated that murine sphingosine kinase consisted of two isoforms with a reported molecular mass of only 49 kDa. The authors also discussed that sequence analysis of these enzymes suggested that murine sphingosine kinases (SPHK1 and SPHK2) belonged to a novel family of kinases, but did show evolutionary conserved regions common to other kinases.

6.2.3 Aims of study.

The aims of this investigation are:

1. To examine the role of cAMP in immature B lymphocyte responses to BCR and CD40 ligation.
2. To investigate the coupling of the BCR to PI(3)K activation under conditions of apoptosis and to examine the effect of co-stimulation with anti-CD40 on PI(3)K responses.
3. To investigate the coupling of the BCR to SPP production under apoptotic and proliferative conditions.

6.2.4 Results.

Previous reports have suggested a negative role for cAMP in lymphocyte signalling. In addition, cAMP has been shown to negatively regulate key enzymes involved in signalling cascades, including ERK-MAPKinase by suppressing Raf-1 activity (143). Due to these results, and additional studies suggesting that cAMP levels could modulate BCR signals (144) we hypothesised that cAMP may play a role in BCR-mediated growth-arrest/apoptosis in WEHI-231 cells and may indeed mediate these signals by regulating ERK-MAPKinase activity.

6.2.4.1 Pharmacological increases in cAMP levels correlate with the induction of growth-arrest in WEHI-231 cells.

Initial studies were carried out to examine the role of increasing cAMP levels on basal WEHI-231 cell proliferation (**figure 62**). Cells treated with forskolin, a direct activator of adenyl cyclase, showed a dose-dependent increase in growth arrest indicating a potential negative role for cAMP in WEHI-231 cell growth. Additional studies indicated that increased cAMP levels could act in synergy with anti-Ig to increase growth-arrest , whilst anti-CD40 or anti-Ig plus anti-CD40 treated cells also witnessed a dose-dependent increase in growth-arrest with forskolin treatment (**figure 62**).

6.2.4.2 Inhibition of PDE activity promotes growth-arrest in WEHI-231 cells.

In order to investigate a role for cAMP in the regulation anti-Ig mediated WEHI-231 cell growth arrest, cells were treated with a range of PDE inhibitors which act intracellularly to prevent cAMP breakdown and therefore enhance intracellular cAMP levels. Previous studies from other groups have suggested that specific PDE isoforms are differentially activated under different conditions. For example, PDE4 has been shown to play an important role in the regulation of cAMP degradation in $CD4^+CD8^+$ thymocytes, whereas the role of PDE3 was redundant (143). In this study we therefore utilised the specific PDE inhibitors Rolipram (PDE4) and Cilostamide (PDE3) and the non-specific PDE inhibitor IBMX to study the role of the PDEs in WEHI-231 cell growth. Rolipram treatment (**figure 63A**) appeared to induce a concentration-dependent increase in growth-arrest. However, at low concentrations, thymidine uptake appeared to be enhanced, compared to basal, untreated cells. Thymidine incorporation levels from vehicle studies however, suggested that these increases may be due to cell stimulation from the vehicle used (DMSO). Treatment of WEHI-231 cells

with the PDE3 specific inhibitor cilostamide (**figure 63B**) also induced growth-arrest in a concentration-dependent manner. Additional studies using the non-specific PDE inhibitor IBMX (**figure 63C**), also caused a concentration dependent increase in growth arrest. Once again, increased thymidine incorporation shown at lower IBMX concentrations may be attributable to the apparent stimulation of cell proliferation shown in vehicle controls. Taken together, these results suggest, but do not definitively confirm, that cAMP can negatively regulate basal proliferation of WEHI-231 cells.

6.2.4.3 BCR ligation suppresses PDE4 activity in WEHI-231 cells.

As previous studies (**figures 62 and 63**) addressed the role of cAMP in WEHI-231 cells by pharmacological enhancement of cAMP levels, additional investigations were carried out to address whether the BCR was coupled to pathways regulating cAMP levels/PDE activity under conditions of growth-arrest/apoptosis. Subsequent studies (**figure 64; unpublished data by Sandra Seatter**) compared the effect of BCR ligation or LPS treatment on PDE4 activity with time. PDE4 activity was maintained at a basal level in untreated (control) cells over a 24 hour period. However, BCR ligation was shown to cause an early, sustained decrease in PDE4 activity, which recovered back to basal levels after approximately 7 hours. In contrast, LPS treatment, a mitogenic signal for WEHI-231 cells, enhanced PDE4 activity over the same time scale in which it was suppressed by BCR-ligation. These results confirm that the BCR is coupled to a pathway capable of suppressing PDE activity. In addition, the ability of LPS to enhance PDE4 activity suggests that other, proliferative pathways such as CD40, may be positively coupled to PDE activity.

6.2.4.4 Anti-CD40 co-stimulation can rescue from IBMX-induced growth arrest.

Previous studies in this thesis had postulated that BCR-mediated suppression of growth was due to the down-regulation of a vital ERK-MAPKinase signal. Anti-CD40 treatment is known not only to restore growth, but also restore ERK-MAPKinase activity. Since cAMP is known to downregulate ERK-MAPKinase activity by suppression of Raf-1 activity in other systems, and the BCR is known to down-regulate PDE activity in WEHI-231 cells, does CD40-mediate some of its rescue signals by reversing cAMP-mediated effects? In order to investigate this hypothesis, the ability of the non-selective PDE inhibitor, IBMX, to block CD40-mediated effects was examined.

Treatment of WEHI-231 cells with IBMX induced growth-arrest, and also low levels of apoptosis (**figures 65A and 65B**). These correlate with previous findings suggesting a role for cAMP as a negative regulator of WEHI-231 cell growth. In

addition, the effect of IBMX pre-treatment on BCR-mediated Ras and ERK-MAPKinase activation was also examined (**figure 66A and 66B**). These results indicated, that although IBMX at 10 μM caused a small decrease in Ras activation it was relatively ineffective in modulating BCR-mediated ERK-MAPKinase activation. In contrast, treatment of cells with 20 μM IBMX, previously shown to induce apoptosis, enhanced BCR-mediated ERK-MAPKinase activation.

Interestingly, co-treatment of cells with anti-CD40 antibodies could reverse IBMX-induced growth arrest (**figure 65A**). In addition, anti-CD40-mediated rescue of BCR-induced growth-arrest was unaffected by co-treatment with IBMX. This latter point would be consistent with ideas put forward by Purkerson et. al., 1998 (97) who, as previously mentioned, suggested that CD40-mediated ERK-MAPKinase activation was cAMP/PKA-independent/insensitive. It would therefore be interesting to examine whether cAMP generation downregulated basal ERK-MAPKinase activity in long-term (0-48 hour) samples during the induction of growth arrest and whether CD40 costimulation could override this signal and re-establish ERK-MAPKinase activity.

6.2.4.5 Pharmacological inhibition of PKA does not reverse BCR-mediated growth arrest.

In order to examine the role of cAMP/PKA in WEHI-231 signalling, we investigated the effect of PKA inhibition in WEHI-231 cells treated under both negative and positive conditions. **Figure 67** shows the effect of PKA inhibition using the pharmacological inhibitor H-89. Perhaps as expected, PKA inhibition did not inhibit the basal proliferation of WEHI-231 cells. Additionally, PKA inhibition did not reverse BCR-mediated growth arrest, suggesting that PKA is not involved in BCR-induced growth arrest. In addition, CD40-mediated rescue from BCR induced growth-arrest was unaffected by PKA inhibition.

These results would appear to suggest that BCR-induced growth arrest and the suppression of basal ERK-MAPKinase activities does not involve PKA activity.

6.2.4.6 BCR ligation leads to PtdIns(3,4,5)P_3 generation in WEHI-231 cells.

In order to determine if the BCR is coupled to PI(3)K activation, levels of PtdIns(3,4,5)P_3 generation were measured following anti-Ig treatment over a time period of 60 minutes (**figure 68A**). The results show that anti-Ig stimulation induces a rapid increase in PtdIns(3,4,5)P_3 production, peaking at 60 minutes. Interestingly, co-stimulation with anti-CD40 antibodies was shown to alter the kinetics of anti-Ig-

induced PtdIns(3,4,5)P_3 production, inducing an early enhanced, yet transient rise in PtdIns(3,4,5)P_3 production, peaking at 5 minutes, which was downregulated with time there after. In addition, cells treated with only anti-CD40 produced PtdIns(3,4,5)P_3 generation profiles similar to those treated with anti-Ig plus anti-CD40. These results suggest that the BCR is coupled to PI(3)K activation and that late (30-60 minute) increases in PtdIns(3,4,5)P_3 generation may play a role in BCR-mediated growth-arrest and apoptosis. Indeed, the kinetics of PtdIns(3,4,5)P_3 production mirror that of BCR-induced ERK-MAPKinase activation, and its subsequent down-regulation by co-stimulation with anti-CD40. **Figure 68B** shows the percentage of PtdIns(3,4,5)P_3 generation following anti-Ig and anti-CD40 co-stimulation relative to the levels obtained by anti-Ig treatment alone in a repeat experiment confirming that BCR-ligation induces a late (60 minute) PtdIns(3,4,5)P_3 signal which is suppressed following co-treatment with anti-CD40.

6.2.4.7 PI(3)K inhibition prevents BCR-mediated activation of ERK-MAPKinase.

Although Ras is known to lie upstream of PI(3)K in a wide range of signalling cascades, evidence is also emerging for a role of PI(3)K in the activation of ERK-MAPKinase. Due to the similarity in terms of kinetics of ERK-MAPKinase activation and PtdIns(3,4,5)P_3 generation following anti-Ig and/or anti-CD40 treatment the ability of specific PI(3)K inhibitors to modulate BCR-mediated ERK-MAPKinase activation was examined (**figure 69**). Cells were pre-treated with either wortmannin (Wm) or Ly294002 (Ly) for different time periods and at different concentrations prior to anti-Ig stimulation for 30 minutes, which has previously been shown to induce a strong ERK-MAPKinase signal. Interestingly, both the Wm and Ly compounds were partially effective in reducing BCR-mediated ERK-MAPKinase activation. This suggests that PI(3)K activity lies upstream of ERK-MAPKinase activation following BCR ligation and that PI(3)K may play some role in BCR-mediated responses such as apoptosis. These results are consistent with other findings in which wortmannin blocked acute PDGF-dependent activation of Raf in CHO cells and also the inhibition of Raf kinase activation by nerve growth factor (167). However, wortmannin-mediated inhibition of MAPKinase is not universal and appears to be both cell- and stimuli-specific.

6.2.4.8 PI(3)K plays a non-essential role in BCR mediated apoptosis.

In order to address the hypothesis that PI(3)K may play a role in transducing negative BCR-mediated signalling events the ability of the PI(3)K inhibitors wortmannin (Wm) and Ly294002 (Ly) to prevent BCR-mediated apoptosis was

examined. Therefore, following pre-incubation with either Wm or Ly, cells were treated with anti-Ig and the level of apoptosis measured by PI staining and measurement of DNA content. Anti-Ig treatment was shown to increase basal apoptosis levels from 25.7% to 47.3% and this was partially reversed with co-treatment with anti-CD40 antibodies, reducing apoptosis levels to 36.3% (**figure 70**). However, pre-incubation with either Wm or Ly, previously shown to partially block BCR-mediated ERK-MAPKinase activation, was ineffective in blocking BCR-induced apoptosis. Although this would appear to suggest that PI(3)K plays no role in the induction of apoptosis following BCR ligation in WEHI-231 cells, this may be explained due to the inability of PI(3)K inhibition to completely block BCR-induced ERK-MAPKinase activation. It could also suggest that other apoptotic mechanisms are activated following BCR-ligation and that PI(3)K mediated ERK-MAPKinase activation is not essential for the induction of $cPLA_2$ activity or indeed other apoptotic signals. It would therefore be of interest to examine whether Wm or Ly treatment modulates BCR-mediated cPLA2 activity.

6.2.4.9 Long-term BCR-ligation down-regulates p85α-subunit protein expression.

Having ruled out an essential role for PI(3)K in early, BCR-mediated apoptotic responses, it still remained to be determined whether PI(3)K played a role in the long-term rescue of WEHI-231 cells from growth-arrest by anti-CD40 stimulation. Western blot analysis of whole cell lysates suggested that protein levels of the p85α-regulatory subunit of PI(3)K were affected by BCR ligation (**figure 71**). These results suggested that long-term BCR ligation down-regulates p85 α protein expression, which would in turn reduce PI(3)K activation due to disruption of the adaptor (regulatory) and catalytic sub-unit association required for PI(3)K activation (personal communication, Lewis Cantley). p85 α protein levels were restored in samples co-stimulated with anti-CD40 antibodies indicating a role for CD40 in regulating BCR-mediated changes in PI(3)K signalling. These studies examined protein expression, it would also be interesting to examine whether p85α mRNA expression is modulated under similar conditions. What mechanisms the BCR activates in order to down-regulate p85 α protein expression are unclear, indeed, no reports exist suggesting the regulation of PI(3)K activity by modulating p85 α protein levels.

6.2.4.10 PI(3)K plays no apparent role in CD40-mediated rescue from BCR-mediated growth-arrest.

As the above study suggests a positive, possibly proliferative role for PI(3)K in WEHI-231 cells, we wanted to determine whether PI(3)K played a role in the regulation of basal ERK-MAPKinase activity and in CD40-mediated rescue of WEHI-231 cells. Cells were therefore pre-treated with either Wm or Ly and levels of proliferation/growth-arrest measured by thymidine uptake and subsequent DNA synthesis. Pre-incubation with either inhibitor (**figure 72A and 72B**) had no effect on either anti-CD40 treated cells or cells treated with anti-Ig plus anti-CD40 suggesting that PI(3)K does not play an essential role in CD40 mediated rescue from anti-Ig-induced growth-arrest. Due to previous observations (**results not shown**) suggesting that the ERK-MAPKinase inhibitors PD98059 and U0126 had relatively short half-lives in WEHI-231 cells, additional studies were carried out in which both Wm and Ly were added at 1 hour pre-stimuli and then at the following time intervals; 1, 2, 4, 8, 12, 16, 20, 24 and 28 hours post-stimuli to maintain PI(3)K inhibition over a long time period. Prolonged additions of both Wm and Ly were effective in inducing substantial growth arrest of unstimulated cells indicating that PI(3)K activity may be important for the basal proliferation of WEHI-231 cells (**figure 73**). Prolonged additions also reversed CD40-mediated rescue, although this may be purely be due to the ability of Wm and Ly to inhibit basal proliferation in WEHI-231 cells.

6.2.4.11 Anti-CD40 mediated rescue from growth-arrest does not involve long-term PtdIns(3,4,5)P_3 production.

The above results in this section have suggested roles for PI(3)K activity in the basal proliferation of WEHI-231 cells and possibly in CD40-mediated rescue from growth arrest. Therefore, long-term studies were carried out to examine the profiles of the PtdIns(3,4,5)P_3 generation in WEHI-231 cells during these responses and also during BCR-stimulation. Unlike ERK-MAPKinase, which has previously been shown to possess a cyclic pattern of activation in untreated (basal) cells, PtdIns(3,4,5)P_3 levels were maintained at a steady-state with very little fluctuation over time (**figure 74**) suggesting tight regulation of PtdIns(3,4,5)P_3 generation. In contrast, anti-Ig stimulated cells appeared to increase PtdIns(3,4,5)P_3 levels with time, whilst cells treated with anti-Ig and anti-CD40 suppressed PtdIns(3,4,5)P_3 profiles similar to untreated cells. The finding suggesting that long-term BCR ligation, but not other stimuli, increases PtdIns(3,4,5)P_3 levels appears contradictory to earlier studies as similar treatment has also been shown to cause substantial down-regulation of p85α protein expression. However, reports (personal communication, Lewis Cantley)

suggest that other regulatory sub-units such as p85β or p55γ may be recruited under such circumstances restoring PI(3)K activity. These studies also indicate that BCR-stimulated PtdIns(3,4,5)P_3 generation may be involved in the promotion of growth arrest in WEHI-231 cells.

Although these results suggest that BCR-induced PtdIns(3,4,5)P_3 generation may play a role in the induction of growth arrest, results similar to those described in the human RL B cell line (17), they do not rule out positive roles for PI(3)K in both the basal proliferation, as indicated in previous studies, and in CD40-mediated rescue. It would be interesting to examine the generation or suppression of other PI(3)K generated phosphoinositides such as PtdIns(3,4)P_2, but also the role of other PI(3)K isoforms such as the G-protein sensitive p110γ PI(3)K in the basal proliferation of WEHI-231 cells.

6.2.4.12 Akt is weakly activated by apoptotic and proliferative signals in WEHI-231 cells.

One major downstream effector of PI(3)K activity is the anti-apoptotic protein kinase PKB/Akt which has been shown to suppress apoptosis induced by growth-factor withdrawal, cell-cycle discordance, loss of cell adhesion and DNA damage (32).

As Akt requires dual phosphorylation on both Thr^{318} and Ser^{473} residues for full activation (168) we utilised antibodies recognising p-Thr or p-Ser residues to establish the relative activation state of Akt under both apoptotic and rescue conditions. **Figure 75A** presents the data obtained from these studies. In addition to pThr and pSer antibodies, the membrane was also probed with $pThr^{308}$-Akt and a antibody recognising the whole form of Akt to confirm the identity of observed bands as Akt. The phospho-specific (Thr^{308}) Akt antibody indicated that Akt was weakly phosphorylated in untreated cells. Cells treated with anti-Ig plus anti-CD40 also expressed weak Thr phosphorylation which decreased with time. In contrast, anti-Ig treatment enhanced Thr phosphorylation of Akt becoming maximal after 30 minutes. Interestingly, an antibody specific to p-Thr residues produced a different pattern of pThr-Akt expression than the $pThr^{308}$ specific Akt antibody. Under these conditions, untreated cells expressed low, but detectable levels of Thr-phosphorylation. Treatment with anti-Ig, enhanced Thr-phosphorylation relative to untreated (basal) cells after 30 minutes, but was suppressed to below basal after 60 minutes. Perhaps most striking was anti-Ig plus anti-CD40 mediated increases in Thr phosphorylation which were previously undetected by the $pThr^{308}$ specific antibody suggesting that anti-Ig plus anti-CD40 treatment phosphorylates a Thr residue other than 308 on Akt. Levels of Ser-phosphorylation were also examined and indicated that both anti-Ig and anti-Ig plus

anti-CD40 treatments lead to similar levels of Ser-phosphorylation with similar kinetics. Taken together, these results suggest that anti-Ig treatment appears to be the only stimuli leading to dual Thr/Ser phosphorylation in WEHI-231 cells, occurring approximately 30 minutes post-BCR ligation.

Further studies to investigate the long-term kinetics of Akt activation were also examined. Results shown in **figure 75B** suggested that long-term (1-48 hours) anti-Ig, anti-CD40 and most interestingly anti-Ig plus anti-CD40 treatment were all in-effective in promoting Akt Thr-phosphorylation suggesting that Akt is not involved in CD40-mediated rescue from BCR-mediated growth arrest and apoptosis. These results are compatible with previous findings which suggested anti-Ig plus anti-CD40 co-stimulation were ineffective in induced PtdIns(3,4,5)P_3 production. However, they also suggest that the late generation of PtdIns(3,4,5)P_3 following BCR-ligation is unlikely to be involved in the regulation of Akt activity.

Taken together, these results provide weak evidence to suggest that Akt may play a role in modulating early, BCR-mediated signalling events, but does not play a role in late survival signals induced by CD40 treatment. BCR-induced apoptosis may therefore result, at least in part, from the inability of BCR-ligation to sustain Akt activity, therefore preventing cell survival. Further studies examining Akt enzyme activity using the specific Akt substrate, crosstide, would more accurately determine the activation state of Akt following cell stimulation and would help confirm whether or not Akt is involved in CD40-mediated rescue of WEHI-231 cells.

6.2.4.13 BCR-ligation is coupled to sphingosine-kinase in WEHI-231 B cells.

Sphingosine kinase activity has been shown to play a positive, mitogenic role in a number of cell types. In addition, a wide range of cell surface receptors have been shown to be coupled to sphingosine-kinase including FcγRI (169). Currently, no evidence is available suggesting a role for SPP as a lipid second messenger in B lymphocytes . In this study, we aimed to identify whether signalling events activated through BCR-ligation in WEHI-231 cells were coupled to sphingosine kinase activation and whether SPP could signal via extracellular mechanisms.

In order to determine if there was differential receptor coupling of sphingosine kinase under apoptotic or proliferative signals, WEHI-231 cells were treated with either anti-Ig or both anti-Ig and anti-CD40 (**figure 76**). Anti-Ig treatment caused a rapid rise in SPP levels, however, by 10 minutes this was suppressed to below basal levels. In contrast, co-treatment of anti-Ig treated cells with anti-CD40 antibodies maintained SPP levels above basal over the 10 minute time course. These results suggest that BCR

ligation uncouples activation of a sphingosine kinase pathway and that CD40 mediated rescue from growth-arrest/apoptosis may involve the maintenance of this signal.

In order to determine whether BCR-mediated suppression of SPP levels after 10 minutes of stimulation was maintained over longer time periods further time course studies were carried out (**figure 77A**). Anti-Ig stimulation over a 60 minute time period induced a rapid increase in SPP levels, maximal at 15 minutes, which then rapidly dropped back to basal levels where it remained after 30 minutes. Although these results do not show suppression of SPP levels to below basal as seen in **figure 76**, they confirm that BCR ligation induces an early, transient, activation of sphingosine kinase.

Previous studies in this investigation have shown the ability of anti-CD40 co-stimulation to uncouple the BCR from the induction of apoptotic signals via ERK-MAPKinase and $cPLA_2$. We therefore investigated the effect of anti-CD40 co-stimulation on BCR-mediated SPP generation. Our results indicated that CD40 co-stimulation lead to an elevated and sustained generation of SPP (**figures 76 and 77B**) suggesting that CD40 mediated rescue was positively coupled to sustained SPP generation.

6.2.4.14 The BCR of mature splenic B lymphocytes is also coupled to sphingosine kinase activation.

In order to determine if the coupling of the BCR to sphingosine kinase activation was a maturation state-dependent phenomenon, mature splenic B lymphocytes were stimulated with anti-Ig and SPP levels determined. Unlike WEHI-231 cells, mature splenic B lymphocytes undergo an activation process following BCR ligation, with concurrent increases in cell proliferation measured by tritiated thymidine uptake. As for WEHI-231 cells, splenic B lymphocytes were incubated with [^{3}H]serine to label phospholipid pools. Cells were then stimulated with a mitogenic dose (50 μg/ml) of anti-Ig and a time course study examined changes in SPP generation (**figure 78**). These results indicated that BCR-ligation induces an early rise in sphingosine kinase activity after 30 minutes, which then drops back to basal. Results recorded from 1 hour post anti-Ig treatment suggested that untreated splenic B lymphocytes have the ability to regulate SPP levels, although this is as yet unconfirmed. Sphingosine kinase activation therefore also appears to be coupled to BCR-ligation in mature B lymphocytes. Our results also indicated that the BCR of WEHI-231 cells was also coupled to sphingosine kinase activation. However, the kinetics of SPP generation between these two developmental stages of B lymphocytes were shown to differ, yet were still transient in nature.

BCR-induced SPP generation may therefore represent a primary signal in both immature and mature B lymphocytes which requires costimulation for sustained generation. These costimulatory signals may include CD40 stimulation or additional activation signals generated in mature B lymphocytes following BCR-ligation. This may explain the rapid suppression of SPP generation, following its initial activation, in immature B lymphocytes following BCR-ligation.

6.2.4.15 Exogenous Sphingosine-1-phosphate (SPP) has no extracellular role in determining WEHI-231 cell fate.

Having determined that both BCR- and CD40-ligation can regulate intracellular SPP levels in WEHI-231 cells, its role as an extracellular lipid second messenger was examined. SPP is known to signal through Edg-receptor family members with important roles in the activation of multiple signalling cascades such as the ERK-MAPKinase family. In order to examine an extracellular role two separate approaches were made. Firstly, supernatant from stimulated cells was examined for the presence of SPP and secondly, exogenous SPP was added to cells in culture and subsequent affects on growth-arrest/proliferation were examined. Studies revealed (**figure 79A**) that no SPP could be detected in the supernatant under any of the experimental conditions over a 60 minute time range, indicating that SPP was not secreted by WEHI-231 cells following stimulation. In addition, exogenously added SPP caused little change in DNA synthesis of otherwise untreated cells, and in particular, was ineffective in rescuing WEHI-231 cells from anti-Ig mediated growth arrest (**figure 79B**). Taken together, these results suggest that SPP is acting purely as a intracellular second messenger and appears to play no extracellular role in regulating WEHI-231 cell fate.

6.2.4.16 CD40 treatment restores long-term suppression of SPP levels following BCR-ligation.

Previous studies in this investigation had examined the regulation of SPP levels over a one hour period. As previous studies have shown SPP to be a downstream mediator of proliferative responses by fetal calf serum, studies were extended to further examine any correlation between growth arrest/proliferation and SPP levels in WEHI-231 cells (**figure 80**). Untreated cells, over a 48 hour time course, produced peak SPP levels between 8-24 hours, suggesting a potential role for SPP in the basal proliferation of WEHI-231 cells. Interestingly, long-term anti-Ig treatment of WEHI-231 cells, suppressed SPP levels well below that of untreated cells although SPP levels still peaked at 8-24 hours. BCR ligation may therefore promote growth arrest by uncoupling of a sphingosine kinase dependent mitogenic signal or regulate SPP levels

directly via lipid phosphate phosphatase activity (163). In addition, co-treatment with anti-CD40 induced a slight elevation of SPP levels relative to anti-Ig alone, especially at later time points (24-48 hours) where anti-Ig mediated suppression of SPP levels was at its greatest. These results indicate that SPP levels are tightly controlled by cell stimuli and removal of such signals may have a negative effect on WEHI-231 cell growth. Indeed, it has been suggested the balance between SPP and ceramide may determine the physiological fate of a cell (165). Therefore removal of SPP may tip the balance in the favour of ceramide and lead to the induction of apoptosis. It would therefore be interesting to examine whether WEHI-231 cell death results in the accumulation of intracellular ceramide that mirrors decreases in SPP levels.

6.2.4.17 Sphingosine kinase inhibition promotes growth-arrest and apoptosis.

In order to substantiate the hypothesis that SPP is an essential proliferative signal for WEHI-231 cells, we utilised the potent sphingosine kinase inhibitor *N,N*-dimethylsphingosine (DMS) which has been shown to inhibit sphingosine kinase with few non-specific toxic effects (170). Thymidine incorporation studies confirmed that blockage of SPP production induced complete growth-arrest in WEHI-231 cells (**figure 81**). In addition, DMS was shown to act in synergy with anti-Ig to promote increased growth-arrest and apoptosis over cells treated with anti-Ig alone.

In order to determine whether the growth-arrest induced by sphingosine kinase inhibition was due to the induction of apoptosis, additional studies were carried out to measure DNA content (**figure 82**). Cells treated with DMS were shown to be strongly driven into apoptosis. In addition, DMS appeared to act in synergy to enhance BCR-induced apoptosis. DMS treatment also prevented CD40-mediated rescue from BCR-induced apoptosis, although the addition of anti-CD40 to cells treated with DMS and anti-Ig did reduce the number of apoptotic cells compared to those treated with DMS and anti-Ig only. This suggests that CD40 ligation may be able to restore some sphingosine kinase activity abolished by DMS treatment, data consistent with earlier studies examining SPP production.

6.2.5 Discussion.

A wide range of signalling cascades are activated upon receptor ligation, which through cross-talk mechanisms, regulate the activity of other signalling elements such as ERK-MAPKinase. This section aimed to address the roles of three signalling pathways all of which are known to regulate ERK-MAPKinase activities.

Firstly, we examined the role of cAMP which has been shown to suppress Raf-1 activity and hence ERK-MAPKinase activity. In order to determine whether modulation of intracellular cAMP could affect WEHI-231 cell growth a number of pharmacological agents were used to artificially enhance cAMP levels. Both forskolin, a potent activator of adenylyl cyclase, and a number of PDE inhibitors were effective in promoting WEHI-231 cell growth-arrest. Interestingly , BCR-ligation was also shown to be capable of suppressing PDE activity over a 1-7 hour period of time. These results suggested that cAMP elevation could play important roles BCR-mediated growth-arrest, possibly due to PKA-mediated suppression of Raf-1.

Therefore, in addition to examining the role of cAMP in WEHI-231 cell signalling, the cAMP-dependent enzyme, PK-A, was also examined. Inhibition of PK-A with H-89 was ineffective in inhibiting basal proliferation or inducing apoptosis in WEHI-231 cells. These results confirmed, as expected, that PK-A plays no role in basal WEHI-231 proliferation, or indeed, is not activated during basal proliferation possibly due to low cAMP levels. However, PK-A inhibition had no effect on BCR-mediated growth-arrest, apparently ruling out a role for PK-A mediated suppression of Raf-1 during BCR-induced growth arrest.

As LPS treatment, a proliferative signal in WEHI-231 cells, was shown to enhance PDE activity, we hypothesised that CD40-mediated rescue of WEHI-231 cells may involve the recovery of PDE activity. Consequently, we examined the ability of CD40 treatment to rescue IBMX-induced growth-arrest. CD40 treatment was effective in reversing IBMX-induced growth-arrest suggesting that CD40 acts independently, or downstream, of PDE activity to restore cell proliferation. IBMX treatment was also shown to be ineffective in preventing CD40-mediated rescue of BCR-induced growth-arrest again suggesting that CD40 acts independently of, or acts below, PDE activity. As previously mentioned this data is consistent with recent data by Purkerson *et. al.* (97) suggesting that CD40 activated ERK-MAPKinase in a PKA-insensitive manner at the level of MEK. Although previous studies in this report suggest that both BCR- and CD40- mediated ERK-MAPKinase activation are Ras-dependent, Ras is known to activate MEK kinases other than Raf-1 (97). Our results are therefore not inconsistent with the model proposed by Purkerson *et. al.* in which they suggest CD40 may be coupled to a Ras-dependent, PKA insensitive MEKK other than Raf-1 (**figure 83**). It

would therefore be of interest to examine the kinase activity of MEK and Raf-1 in WEHI-231 cells under conditions of growth-arrest/rescue to examine possible differences in their activation.

In addition to cAMP pathways which have been shown to down-regulate ERK-MAPKinase activity, a number of pathways have been implicated in positively regulating ERK-MAPKinase, including PI(3)K which is a known intermediate in the signal transduction of growth factor signals in a wide range of cell types. In addition, a number of newly emerging lipid second messengers including SPP are emerging as key regulators of cell fate and regulators of ERK-MAPKinase activity.

PI(3)K activity has been reported to be activated in both immature and mature B lymphocytes, with important roles in determining the rate of positive selection of immature B lymphocytes (16) as SHIP-/- B lymphocyte were shown to develop in an accelerated manner. However, its role in the transduction, or prevention, of apoptotic signalling pathways is unclear. In addition, although the coupling of numerous growth factor receptors to sphingosine kinase is well documented, the coupling of the BCR to sphingosine kinase has never been shown. The results in this study therefore indicate a novel signalling mechanism activated by BCR ligation on both immature, and mature B lymphocytes.

Initial data (**figure 68**) suggested that PI(3)K may play an role in mediating BCR-induced apoptosis by coupling proximal-BCR signalling events to the activation of an early, ERK-MAPKinase signal in WEHI-231 cells (**figure 84**). Evidence for this was obtained from studies investigating the generation of PtdIns $(3,4,5)P_3$ following BCR-ligation which appeared to closely mirror the known kinetics of BCR-induced ERK-MAPKinase activation (**figure 25A**). In addition, studies have shown that the PI(3)K inhibitors Wm and Ly can block BCR-mediated ERK-MAPKinase activation (**figure 69**). However, these inhibitors were ineffective in preventing BCR-mediated apoptosis (**figure 70**). These results suggest that early PI(3)K signals generated through BCR-ligation are not essential for the induction of apoptosis in WEHI-231 cells suggesting that other apoptotic pathways are activated by BCR-ligation which may or may not be ERK-MAPKinase dependent. However, the generation of PtdIns $(3,4,5)P_3$ following BCR-ligation may play additional roles in WEHI-231 signalling events by recruiting signalling elements containing lipid binding PH domains such as Btk or PLCγ to the cell membrane. Indeed PI(3)K mediated activation of PLCγ may play a role in the generation of Ca^{2+} influx required for $cPLA_2$ activation following BCR-ligation (**figure 84**). Additional studies aimed to address the effect of long-term BCR-ligation and CD40-mediated rescue on PI(3)K activity. These studies indicated that PI(3)K inhibition could block basal proliferation of WEHI-231 cells. However, additional studies have revealed that PtdIns $(3,4,5)P_3$ levels remain constant

during basal proliferation of WEHI-231 cells. The ability of prolonged PI(3)K inhibition to block basal proliferation may therefore result from the decrease in generation of other lipid species regulated by PI(3)K activity such as PtdIns(3,4)P_2. Interestingly, PtdIns(3,4)P_2 is known to activate ERK-MAPKinase via a PKC-sensitive pathway (150), suggesting this may play a role in the basal proliferation of WEHI-231 cells.

The ability of BCR-ligation to induce PtdIns(3,4,5)P3 generation suggests that PI(3)K activity may be involved in the induction of growth arrest. Indeed, it has been suggested that PI(3)K mediates JNK activation through a Rac/PAK/MEKK/MKK4/JNK pathway (149). Previous data has suggested the possible induction of JNK activity 1-2 hours after BCR-ligation in WEHI-231 cells. We were, however, unable to measure any Rac activity in BCR-stimulated cells. Interestingly, a recent review article (171) has described the ability of sphingo- and phospho-lipid species to activate PAK, and hence JNK, in a Rac-independent manner suggesting that BCR-stimulated PtdIns(3,4,5)P_3 generation may mediate the induction of growth arrest through the activation of JNK.

In summary, it would appear that basal PI(3)K activity, possibly through the generation of lipid species other that PtdIns(3,4,5)P_3 may play positive roles in regulating WEHI-231 cell proliferation. However, our results also indicate that BCR-stimulated PtdIns(3,4,5)P_3 generation has an opposite effect and may promote growth arrest. These differential effects may result from the activation of different downstream signalling elements from PI(3)K but may also result from the activation of different PI(3)K pools.

Interestingly, PtdIns (3,4,5)P_3 generation was only observed in anti-Ig treated samples. These results confirm that PtdIns(3,4,5)P_3 plays no, or a minor, role in the rescue of WEHI-231 cells from BCR-induced apoptosis by anti-CD40 co-stimulation. This may therefore be linked to the inability to observe any Akt signals under such rescue conditions. Ironically, studies in this chapter and other published results (Su et al 1999 ; Gold et al , 1999) have suggested that BCR-ligation induces Akt activation. Although this would seem immediately contradictory, the induction of apoptotic signals is undoubtedly a balance between both positive and negative signals. The activation of Akt following BCR-ligation in immature B lymphocyte may therefore form part of the proposed "rescue window" (50) which allows Ag-stimulated cells to undergo RAG gene reactivation and receptor editing. However, what is clear from our studies in this section, is that Akt appears to play a minor role, if any, in CD40-mediated rescue of WEHI-231 cells from BCR-induced apoptosis.

However, our results do not rule out a role for PI(3)K in the survival of WEHI-231 cells. As previously discussed, PI(3)K has been shown to exert both lipid and protein kinase functions. PI(3)K may therefore play a role in the long-term re-

establishment of ERK-MAPKinase activity by CD40 ligation. Mutant studies utilising lipid- or protein kinase inactive PI(3)K would help address this hypothesis.

PI(3)K and Akt have been shown to induce key survival signals in a number of cells types. However, data in this study suggests that its role, if any, in CD40-mediated survival signals is limited. We therefore examined whether other known survival signals were activated in WEHI-231 cells by CD40 ligation. One emerging lipid metabolite with roles in cell survival is SPP. However, there are no reports as to whether SPP generation is coupled to BCR, or CD40 ligation. Previous studies within our group and others (54) have highlighted the ability of BCR-ligation to induce increases in intracellular ceramide levels under apoptotic conditions. Interestingly, the balance between cellular concentrations of ceramide and SPP have been proposed to determine cell fate in what has been termed the ceramide/SPP rheostat (165). Our results indicate that not only is the BCR directly linked to the suppression of SPP generation, but co-treatment with anti-CD40 antibodies may reverse this. This result indicates that SPP levels may play a key role in determining cell fate in WEHI-231 cells with a shift in the balance towards ceramide levels promoting the activation of apoptotic mechanisms within the cell. In contrast, elevation of SPP levels by CD40 treatment may prevent this shift, keeping the balance of ceramide:SPP levels more equal, or even reducing ceramide levels. Longer term studies examining the effect of both BCR-ligation and CD40-mediated rescue indicated that SPP may also play an important role in determining growth-arrest levels. Cells left untreated over a 48 hour period did not maintain SPP levels at a constant level, instead, SPP levels were shown to peak after 8 hours, falling back to lower levels after 24 hours. As SPP has been linked to the intra-cellular activation of ERK-MAPKinase, sphingosine kinase may represent a route for the basal ERK-MAPKinase signal expressed in WEHI-231 cells. In contrast, BCR-ligation partially reduced levels of SPP over the 4-48 hour period. Co-treatment with anti-CD40 however, appeared to reverse some of the BCR-mediated suppression of SPP generation, albeit at later time points (24-48 hours). These results suggest that long-term BCR-ligation acts to suppress sphingosine kinase activity. As SPP has been proposed regulate the ERK-MAPKinase pathway, suppression of this pathway may account for the ability of BCR-ligation to suppress basal ERK-MAPKinase signals. Indeed, inhibition of sphingosine kinase activity with DMS was shown to promote both growth-arrest and apoptosis, confirming its role as a mitogenic signal in WEHI-231 cells. In addition SPP has been shown to negatively regulate both JNK and caspase activities and cAMP generation (165). Early (0-60 minute) increases in SPP levels induced by CD40 ligation may therefore play a role in the suppression of these key apoptotic signals although our data currently suggests that JNK activity is not activated under apoptotic conditions, but does not rule out a role for SPP in downregulating BCR-mediated increases in cAMP.

In order to determine if the coupling of the BCR to sphingosine kinase activity was a maturation-state dependent phenomenon, similar studies were carried out on mature splenic B lymphocytes. These results indicated that sphingosine kinase activity, as observed in WEHI-231 cells, was also coupled to the BCR in mature B lymphocytes. Why sphingosine kinase activation is coupled to BCR-ligation in both WEHI-231 cells and mature B cells at first seems strange. BCR-ligation initiates negative signalling in WEHI-231-231 cells, yet activation in mature B cells. However, although the BCR was coupled to transient sphingosine kinase activity in both these cells, the kinetics of SPP generation differed. BCR-ligation induced an early, yet short-lived activation of sphingosine kinase which was suppressed by about 10-15 minutes. In contrast, BCR-ligation of mature B lymphocytes activated a later sphingosine kinase signal, peaking at 30 minutes. BCR-induced sphingosine kinase activation may therefore represent a primary signal activated at both immature and mature developmental stages. The later, and slightly more sustained nature of sphingosine kinase activation in mature B cells may result from additional costimulatory signals activated during BCR-ligation. The failure to activate these additional costimulatory signals following BCR-ligation in WEHI-231 cells may account for the short, transient activation of sphingosine kinase in these cells. However, co-stimulation of WEHI-231 cells with anti-CD40 was shown to elevate and maintain sphingosine kinase activity, again suggesting that costimulation may be required for sustained activation of this enzyme. Why the BCR is coupled to a short, transient increase in sphingosine kinase activity in WEHI-231 cells is still unclear. However, this may relate to the ability of SPP to induce Ca^{2+} influxes which may be required for $cPLA_2$ activity which is known to occur following BCR-ligation. Another interesting factor observed in these studies was the evidence suggesting that SPP acted intracellularly and not extracellularly as observed in some systems.

These results suggest that lipid metabolities play important roles in the regulation of WEHI-231 cell fate. They also highlight the emergence of a new, but potentially important , signalling pathway coupled to BCR-ligation which may have key roles in B lymphocyte maturation.

Figure 51. Regulation of Ras activation by upstream Ras guanine nucleotide exchange factors (GEFs) and inhibition by GTPase-activating proteins (GAPs).

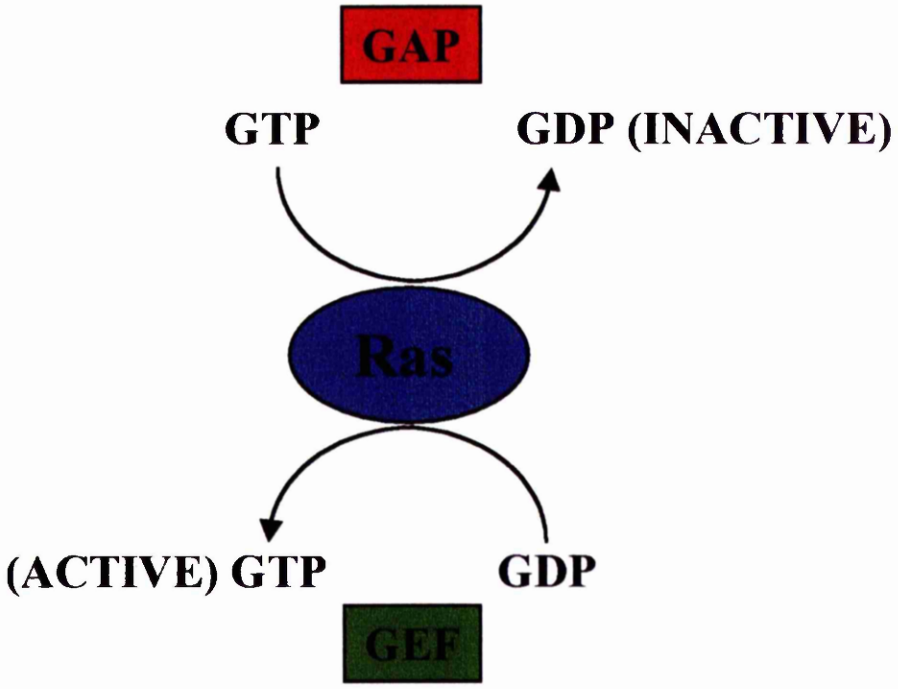


figure 51

Figure 52. Role of SHIP as an adaptor molecule. BCR- FcγRIIB co-aggregation leads to phosphorylation of FcγRIIB on its ITIM motif which in turn leads to the recruitment of SHIP. This in turn creates a binding site for $p62^{dok}$ and Shc whilst also bringing them in close proximity to the BCR. $p62^{dok}$ can then be phosphorylated and recruit RasGAP which in turn converts BCR-stimulated Ras into its GDP-bound inactive form.

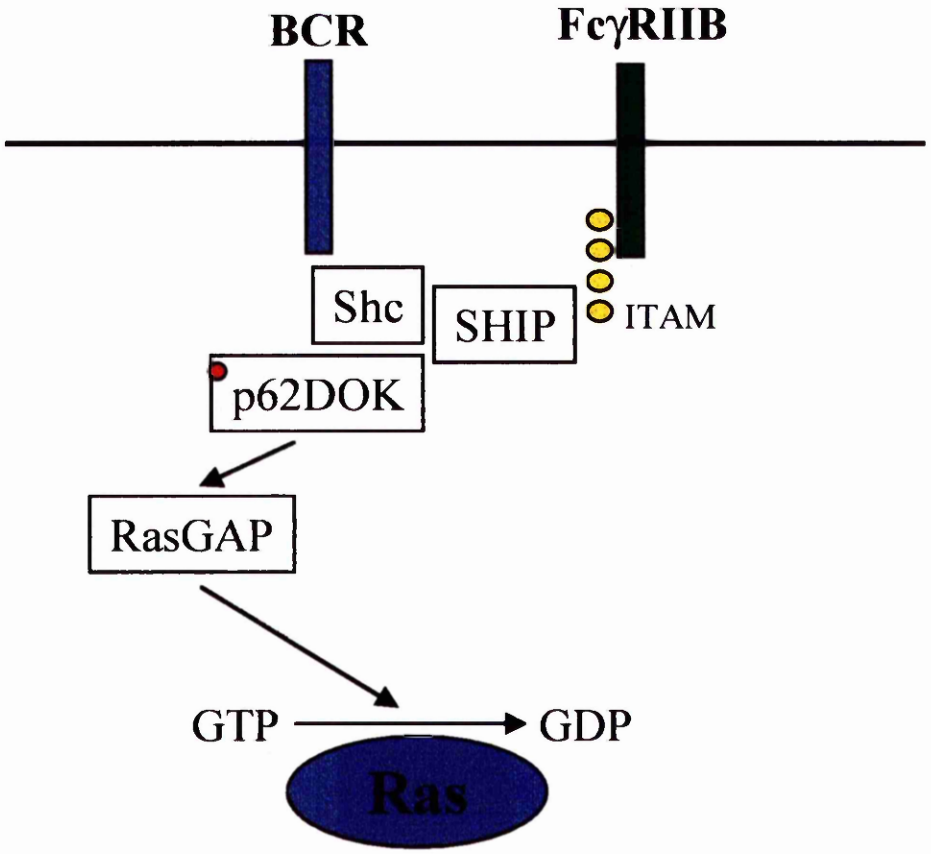


figure 52

Figure 53. Mechanisms involved in Rap1 activation in T lymphocytes. TCR-induced Lyn activation promotes formation of a Cbl-CrkL-C3G complex. This leads to increased interactions between the GEF C3G and its downstream target Rap1 leading to GTPase activity and hence Rap1 activation. Active Rap1 then competes with Ras for Raf-1 downregulating the activity of the Ras/Raf/MEK/ERK-MAPKinase pathway.

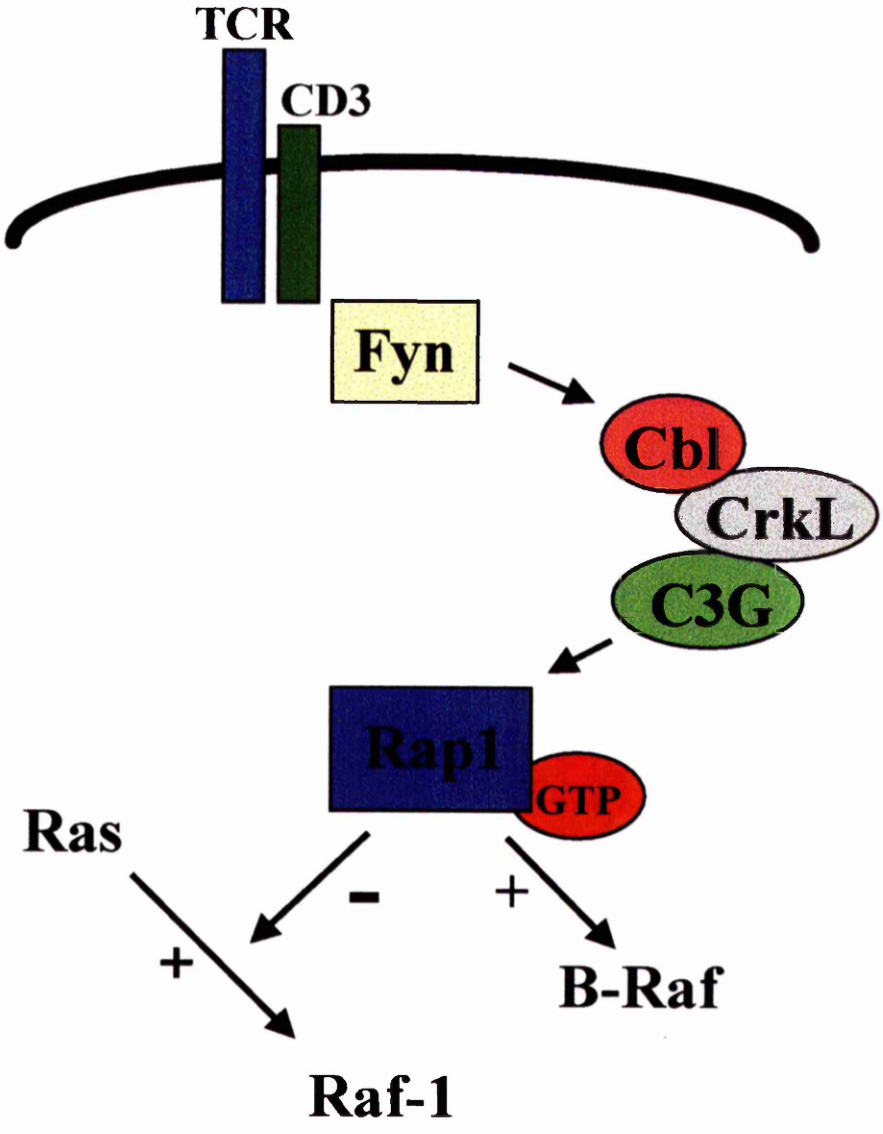
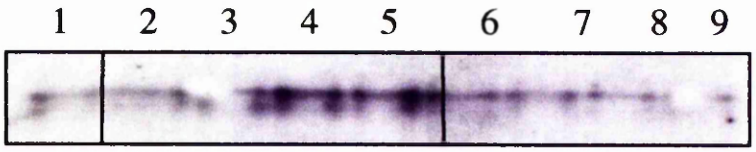


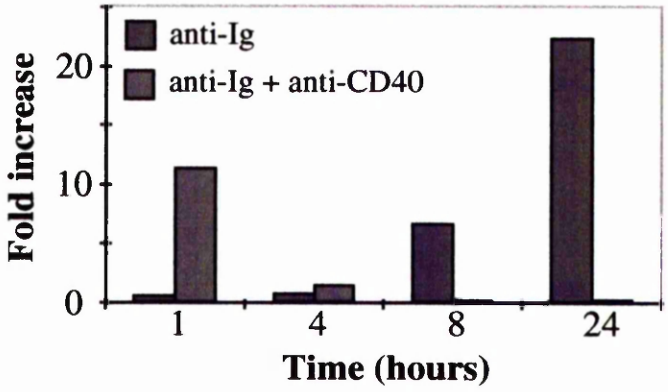
figure 53

Figure 54. BCR-ligation promotes PAC-1 association with ERK-MAPKinase but does not induce new PAC-1 protein synthesis. A. WEHI-231 whole cell lysates were immunoprecipitated with wERK antibody as described in Materials and Methods. Immunoprecipitates were subjected to SDS-PAGE and immuno-blotted with an anti-Pac1 antibody. Densitometry shows relative band density analysed on Gel-Pro software. Lanes were loaded as follows: : **lane 1**, cells left untreated for 0 hours, **lane 2**, cells treated with anti-Ig (1 μg/ml) for 1 hour, **lane 3**, cells treated with anti-Ig (1 μg/ml) for 4 hours, **lane 4**, cells treated with anti-Ig (1 μg/ml) for 8 hours, **lane 5**, cells treated with anti-Ig (1 μg/ml) for 24 hours, **lane 6**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 1 hour, **lane 7**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 4 hours, **lane 8**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 8 hours, **lane 9**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 24 hours, Levels of PAC-1 expression were determined by western blotting. **B.** WEHI-231 cells (5 x 10^5/ml) were stimulated as follows: **lane 1**, cells left untreated for 0 hours, **lane 2**, cells treated with anti-Ig (1 μg/ml) for 1 hour, **lane 3**, cells treated with anti-Ig (1 μg/ml) for 2 hours, **lane 4**, cells treated with anti-Ig (1 μg/ml) for 4 hours, **lane 5**, cells treated with anti-Ig (1 μg/ml) for 8 hours, **lane 6**, cells treated with anti-Ig (1 μg/ml) for 24 hours, **lane 7**, cells treated with anti-Ig (1 μg/ml) for 48 hours, **lane 8**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 1 hour, **lane 9**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 2 hours, **lane 10**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 4 hours, **lane 11**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 8 hours, **lane 12**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 24 hours, **lane 13**, cells treated with anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 48 hours, **lane 14**, cells left untreated for 48 hours. Levels of PAC-1 and wERK expression were determined by Western blotting (30 μg/lane).

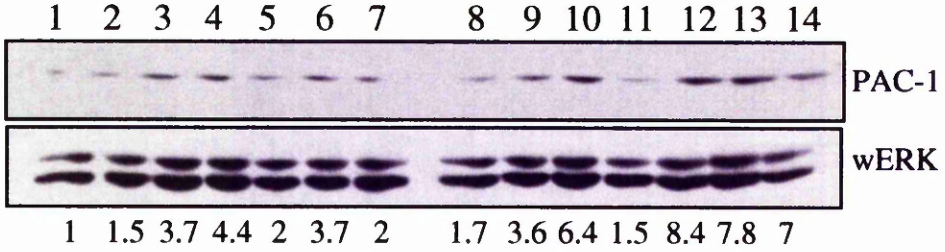
(A)



ERK ip : Probed anti-PAC-1



(B)



1 1.5 3.7 4.4 2 3.7 2 1.7 3.6 6.4 1.5 8.4 7.8 7

Fold incease
(normalised to wERK)

figure 54

Figure 55. Ras activity is present in both resting and apoptotic conditions, but is enhanced in the rescue of WEHI-231 cells by anti-CD40. **A.** WEHI-231 cells (5 x 10^5/ml) were stimulated as follows, **lane 1**, cells stimulated as indicated for 1 hour, **lane 2**, cells stimulated as indicated for 2 hours, **lane 3**, cells stimulated as indicated for 4 hours, **lane 4**, cells stimulated as indicated for 8 hours, **lane 5**, cells stimulated as indicated for 24 hours, **lane 6**, cells stimulated as indicated for 48 hours. GTP-bound Ras was then immunoprecipitated using Raf-1-RBD as bait. Ras activation was then determined by Western blotting using a anti-Ras antibody to equal levels of cell sample. Densitometry shows relative band density measured on Gel-Pro Analysis software. **B.** WEHI-231 cells (5 x 10^5/ml) were stimulated as follows, **lane** 1, cells left untreated for 0 hours, **lane 2**, cells stimulated as indicated for 1 hour, **lane 3**, cells left untreated for 2 hours, **lane 4**, cells stimulated as indicated for 4 hours, **lane 5**, cells stimulated as indicated for 8 hours, **lane 6**, cells stimulated as indicated for 24 hours, **lane 7**, cells stimulated as indicated for 48 hours. Levels of pERK/wERK expression were determined by western blotting (30 μg/lane).

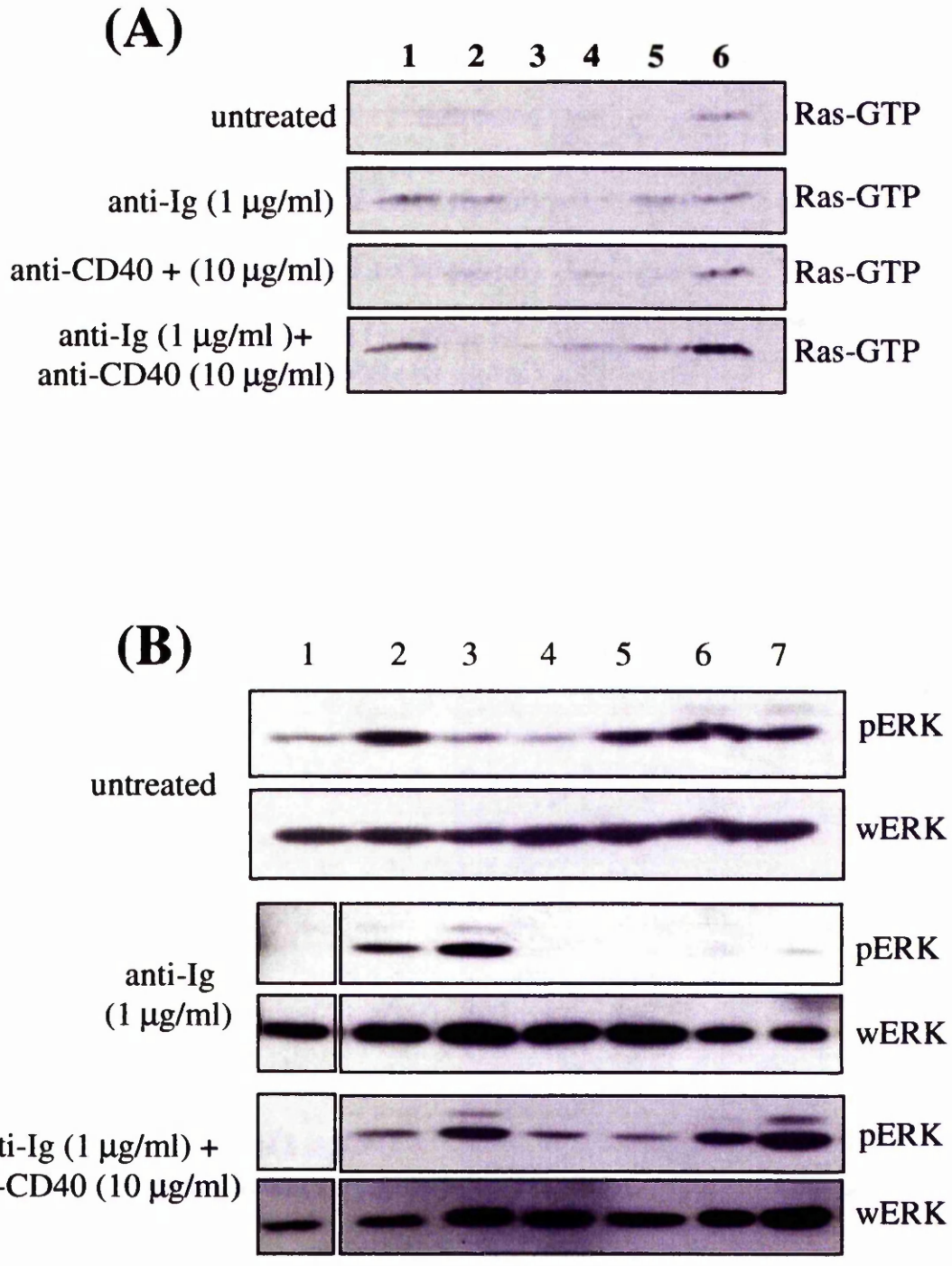

(A)
1 2 3 4 5 6
untreated
Ras-GTP
anti-Ig (1 μg/ml)
Ras-GTP
anti-CD40 + (10 μg/ml)
Ras-GTP
anti-Ig (1 μg/ml)+
anti-CD40 (10 μg/ml)
Ras-GTP
(B)
1 2 3 4 5 6 7
untreated
pERK
wERK
anti-Ig
(1 μg/ml)
pERK
wERK
anti-Ig (1 μg/ml) +
anti-CD40 (10 μg/ml)
pERK
wERK

figure 55

Figure 56. Basal Rap1 activity is suppressed by anti-Ig and anti-CD40 treatment. **A.** WEHI-231 cells (5 x 10^5/ml) were stimulated as follows, **lane 1**, cells stimulated as indicated for 5 minutes, **lane 2**, cells stimulated as indicated for 30 minutes, **lane 3**, cells stimulated as indicated for 1 hour, **lane 4**, cells stimulated as indicated for 2 hours, **lane 5**, cells stimulated as indicated for 4 hours, **lane 6**, cells stimulated as indicated for 8 hours, **lane 7**, cells stimulated as indicated for 18 hours, **lane 8**, cells stimulated as indicated for 24 hours, **lane 9**, cells stimulated as indicated for 32 hours, **lane 9**, cells stimulated as indicated for 48 hours. GTP-bound Ras and GTP-bound Rap1 were then immunoprecipitated using Raf-1-RBD or Ral-RBD, respectively, as bait. Ras or Rap1 activity was then determined by Western blotting using a anti-Ras or anti-Rap1 antibody to equal volumes of cell sample. **B.** Ras activity membranes from figure 56A were stripped of bound antibody and re-probed with an antibody recognising wERK.

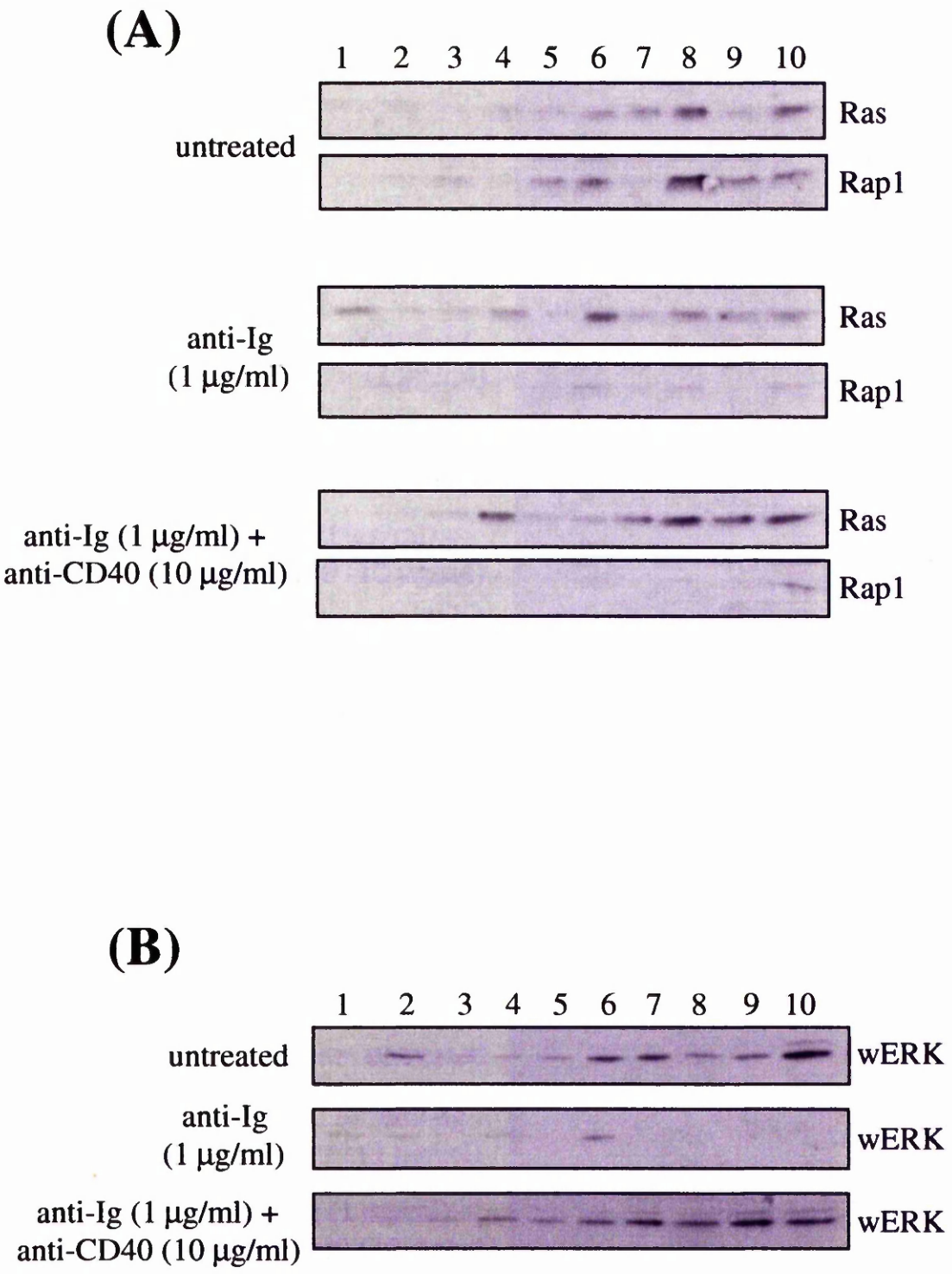

(A)
1 2 3 4 5 6 7 8 9 10
untreated
Ras
Rap1
anti-Ig
(1 μg/ml)
Ras
Rap1
anti-Ig (1 μg/ml) +
anti-CD40 (10 μg/ml)
Ras
Rap1
(B)
1 2 3 4 5 6 7 8 9 10
untreated
wERK
anti-Ig
(1 μg/ml)
wERK
anti-Ig (1 μg/ml) +
anti-CD40 (10 μg/ml)
wERK

figure 56

Figure 57. Model for basal ERK-MAPKinase activity in WEHI-231 cells mediated by Rap1 activity. GTP-bound Rap1 is known to induce the long-term activation of ERK-MAPKinase through stimulation of B-Raf. BCR-ligation in WEHI-231 cells downregulates basal Rap1 activity suggesting Rap1 may be involved in the maintenance of basal ERK-MAPKinase activity in proliferating WEHI-231 cells.

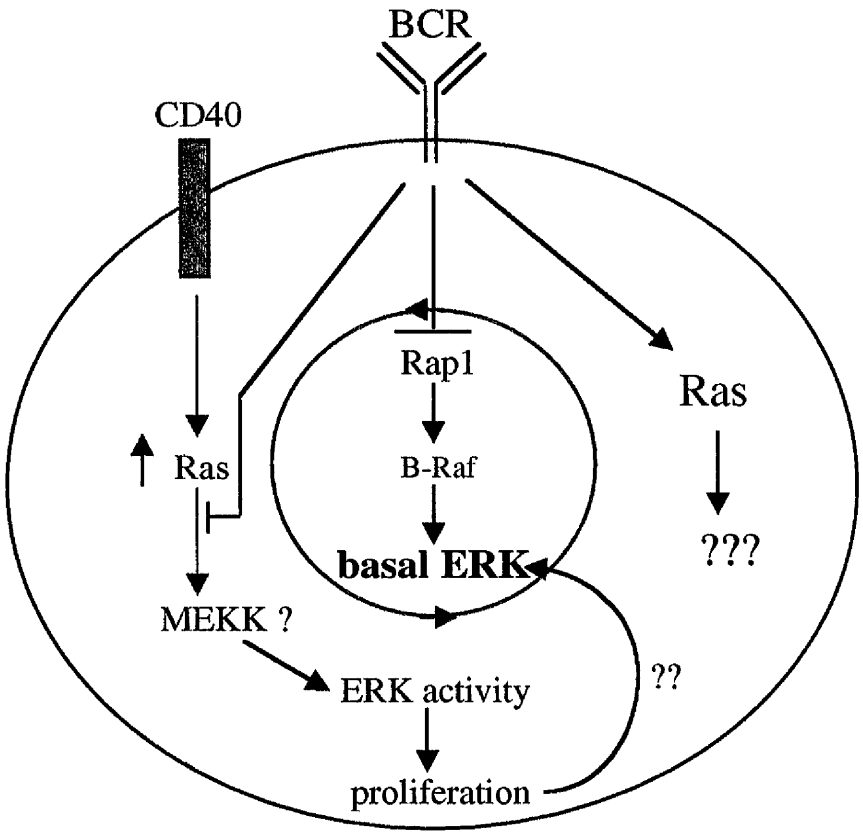
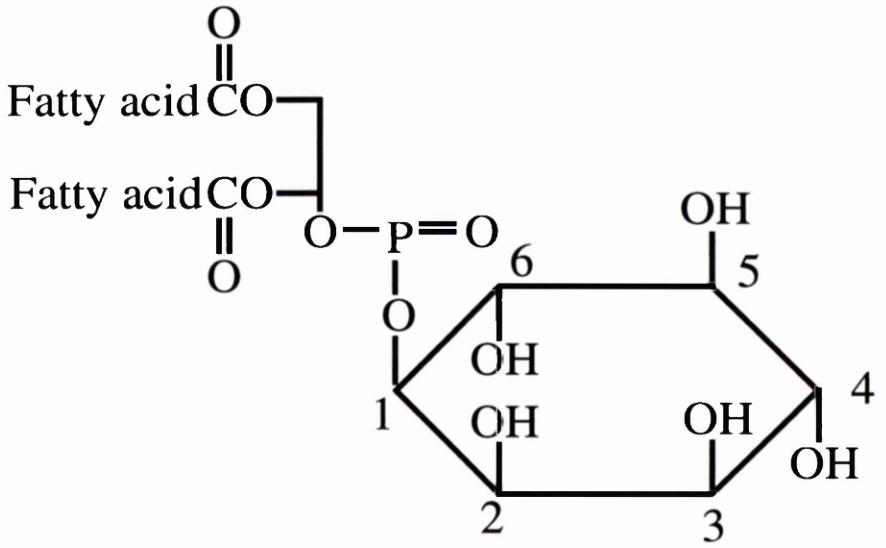

BCR
CD40
Rap1
Ras
Ras
B-Raf
???
basal ERK
MEKK ?
??
ERK activity
proliferation

figure 57

Figure 58. A. Structure of Phosphatidylinositol. **B.** Structure of p85α regulatory sub-unit of PI(3)K.

(A)



Phosphatidylinositol (PtdIns)

(B)

figure 58

Figure 59. Downstream targets of PI(3)K activity. Figure taken from (150).

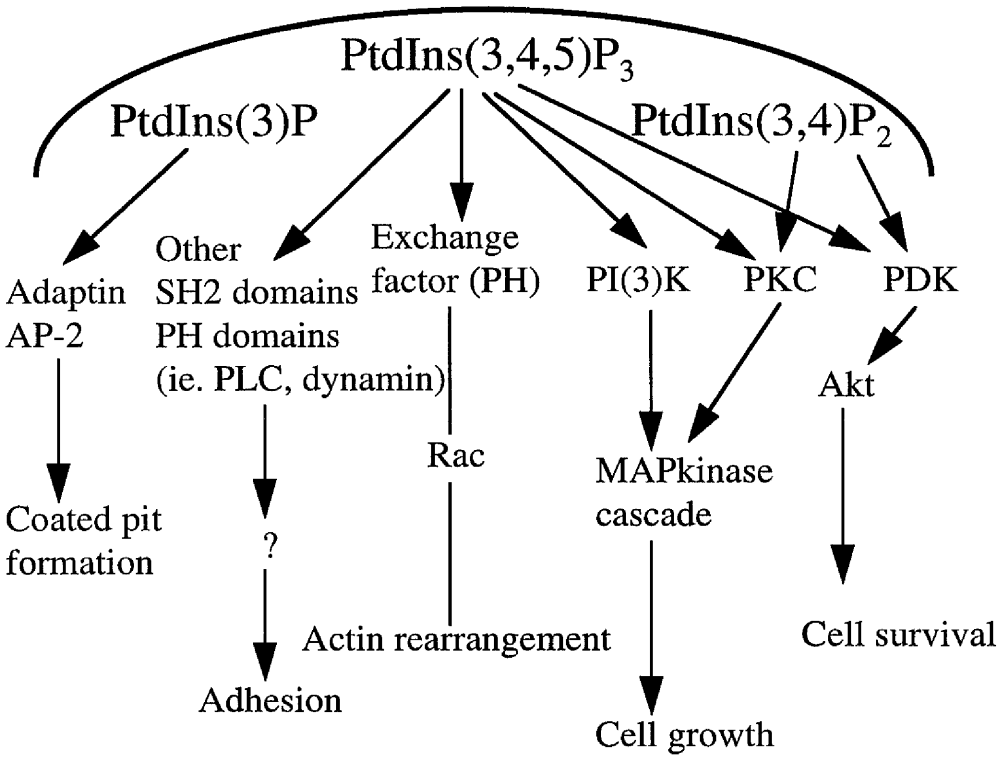

PtdIns(3,4,5)P3
PtdIns(3)P
PtdIns(3,4)P2
Adaptin
AP-2
Other
SH2 domains
PH domains
(ie. PLC, dynamin)
Exchange
factor (PH)
PI(3)K
PKC
PDK
Akt
Coated pit
formation
?
Rac
MAPkinase
cascade
Actin rearrangement
Cell survival
Adhesion
Cell growth

figure 59

Figure 60. Ceramide/SPP rheostat. Pathways showing the generation of ceramide and SPP.

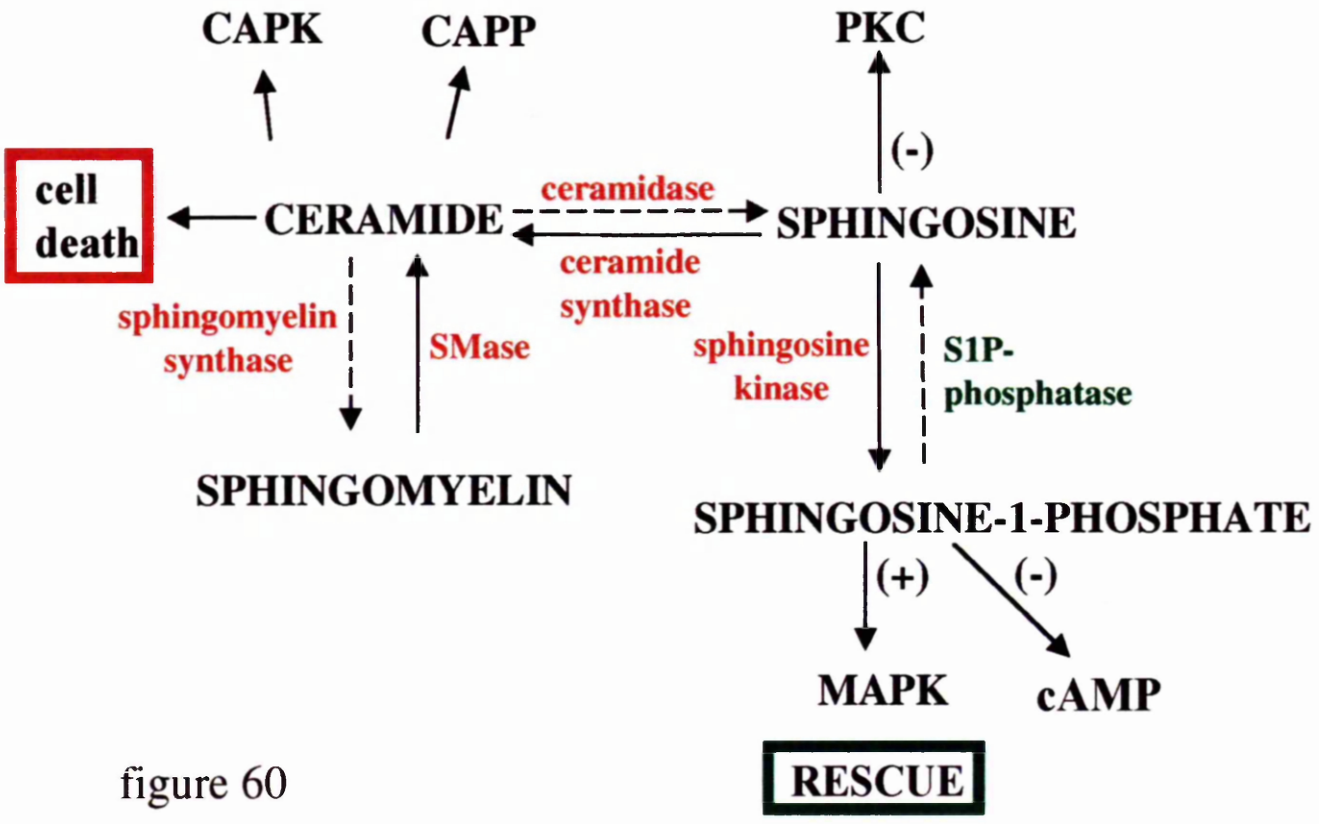

CAPK
CAPP
PKC
cell death
CERAMIDE
ceramidase
SPHINGOSINE
(-)
ceramide synthase
sphingomyelin synthase
SMase
sphingosine kinase
S1P-phosphatase
SPHINGOMYELIN
SPHINGOSINE-1-PHOSPHATE
(+)
(-)
MAPK
cAMP
RESCUE

figure 60

Figure 61. Model of G-protein activation following receptor-agonist binding. Receptor agonist binding leads to the association of the αβγ trimer with the receptor which causes the GDP bound to the α subunit to dissociate and become replaced with GTP. This in turn, causes the dissociation of α-GTP from the βγ subunits. α-GTP, now in its active form now acts as an effector activating downstream signalling elements. However, it should be noted that the dissociated βγ subunits are also known to activate downstream signalling elements.

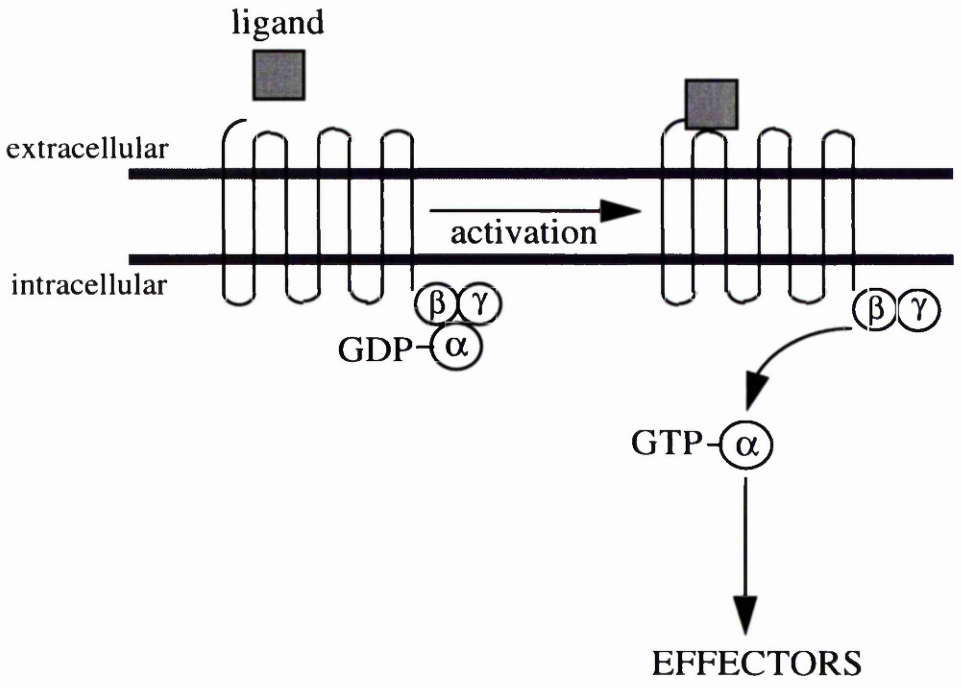

ligand
extracellular
intracellular
activation
β γ
GDP-α
β γ
GTP-α
EFFECTORS

figure 61

Figure 62. Pharmacological increases in cAMP levels by forskolin correlate with induction of growth-arrest in WEHI-231 cells. WEHI-231 cells (10^4/well) were left untreated or treated with anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml). In addition, cells were treated with increasing concentrations of forskolin (as indicated) either alone, or in the presence of anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml). Proliferation was assessed by measuring cell measuring DNA synthesis as indicated by [^{3}H]thymidine uptake at 48 hours. Values shown are means ± SD of quadruplicate wells.

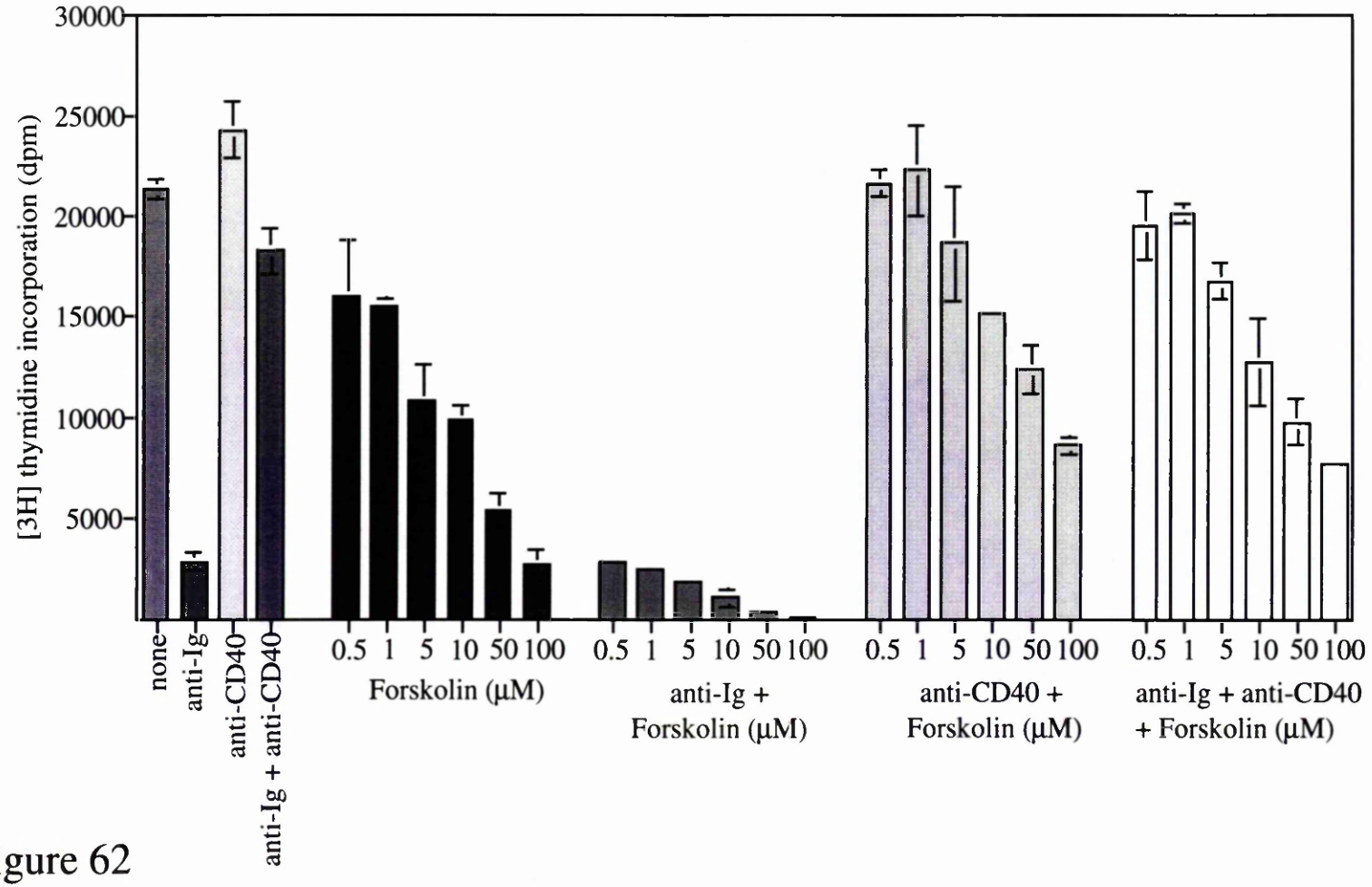

30000
25000
20000
15000
10000
5000
[3H] thymidine incorporation (dpm)
none
anti-Ig
anti-CD40
anti-Ig + anti-CD40
0.5 1 5 10 50 100
Forskolin (μM)
0.5 1 5 10 50 100
anti-Ig +
Forskolin (μM)
0.5 1 5 10 50 100
anti-CD40 +
Forskolin (μM)
0.5 1 5 10 50 100
anti-Ig + anti-CD40
+ Forskolin (μM)

figure 62

Figure 63. Inhibition of PDE activity promotes growth-arrest in WEHI-231 cells. A. WEHI-231 cells (10^4/well) were left untreated, treated with anti-Ig (10 μg/ml) or with increasing concentrations with rolipram (0.01 μM-100 μM) or vehicle controls. Proliferation was assessed by measuring DNA synthesis as indicated by [^{3}H]thymidine uptake at 48 hours. Values shown are means ± SD of quadruplicate wells. **B.** WEHI-231 cells (10^4/well) were left untreated, treated with anti-Ig (10 μg/ml) or with increasing concentrations with cilostamide (0.001 μM-10 μM) or vehicle controls. Proliferation was assessed by measuring DNA synthesis as indicated by [^{3}H]thymidine uptake at 48 hours. Values shown are means ± SD of quadruplicate wells. **C.** WEHI-231 cells (10^4/well) were left untreated, treated with anti-Ig (10 μg/ml) or with increasing concentrations with IBMX (0.2 μM-100 μM) or vehicle controls. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are means ± SD of quadruplicate wells.

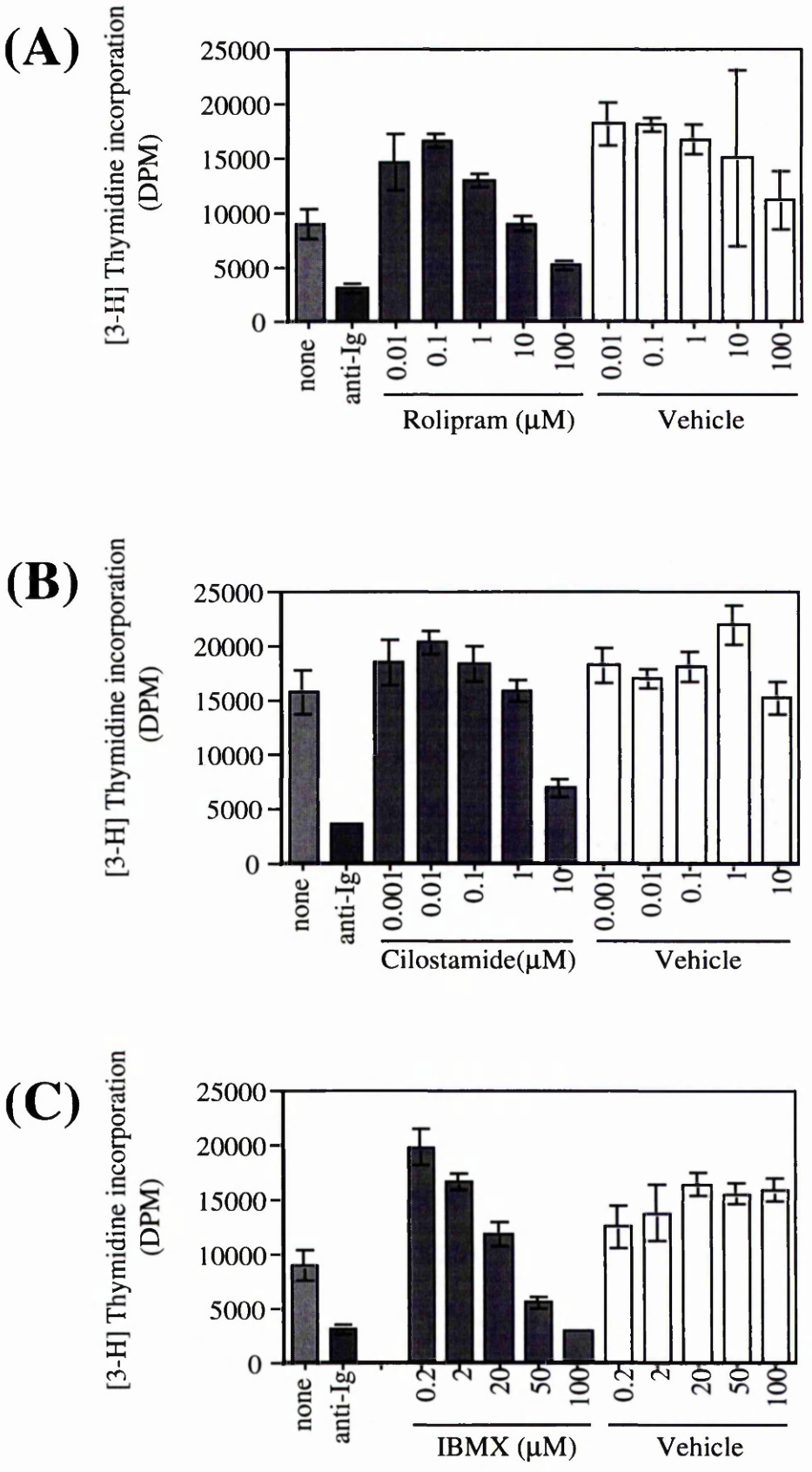

(A)
[3-H] Thymidine incorporation (DPM)
25000
20000
15000
10000
5000
0
none
anti-Ig
0.01
0.1
1
10
100
0.01
0.1
1
10
100
Rolipram (μM)
Vehicle
(B)
[3-H] Thymidine incorporation (DPM)
25000
20000
15000
10000
5000
0
none
anti-Ig
0.001
0.01
0.1
1
10
0.001
0.01
0.1
1
10
Cilostamide(μM)
Vehicle
(C)
[3-H] Thymidine incorporation (DPM)
25000
20000
15000
10000
5000
0
none
anti-Ig
0.2
2
20
50
100
0.2
2
20
50
100
IBMX (μM)
Vehicle

figure 63

Figure 64. BCR-ligation suppresses PDE activity in immature B lymphocytes. Anti-Ig stimulation of WEHI-231 cells was shown to transiently downregulate basal PDE activity. In contrast, LPS stimulation, which induces cell proliferation, was shown to enhance PDE activity over the same time period.

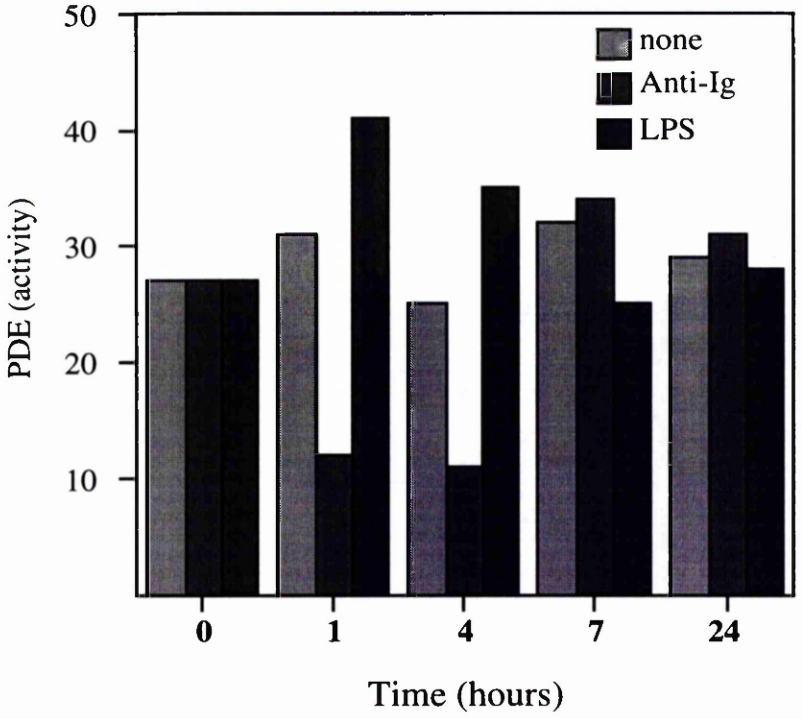
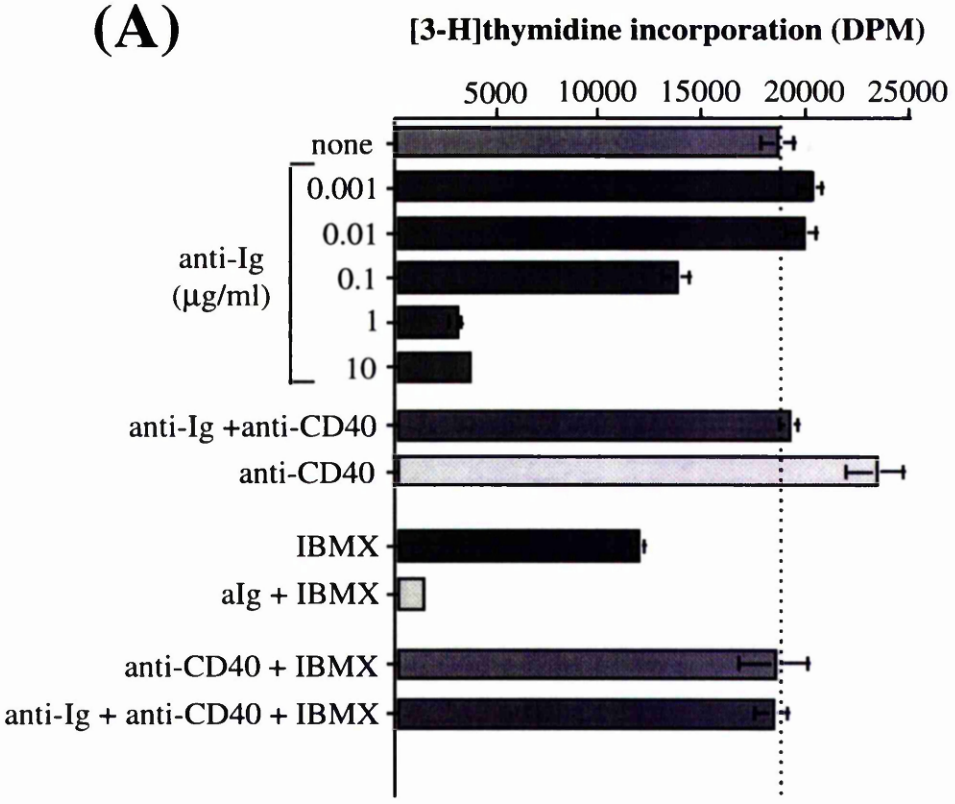


figure 64

Figure 65. Anti-CD40 mediated rescue from growth-arrest/apoptosis is independent of PDE activity, but can rescue from IBMX mediated growth arrest. A. WEHI-231 cells (10^4/well) were left untreated or treated with increasing concentrations of anti-Ig (0.001-10 μg/ml). In addition, cells were treated with IBMX (20 μM) with or without anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml). Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hours. Values shown are means ± SD of quadruplicate wells. **B.** WEHI-231 cells ($5x10^5$/ml) were left untreated, or treated with anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml), IBMX (20 μM) and anti-Ig plus anti-CD40 (both 10 μg/ml) with or without IBMX (20 μM). Levels of apoptosis were determined by PI staining and FACS analysis after 48 hours as described in Materials and Methods.

(A)



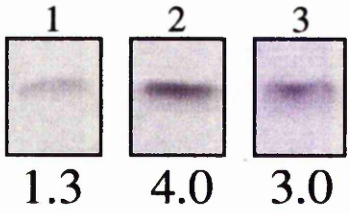
(B)

TREATMENT(S)	% APOPTOTIC CELLS
none	20.5
anti-Ig	33.5
anti-Ig + anti-CD40	25.2
anti-CD40	25.4
IBMX	28.4
anti-Ig + anti-CD40 + IBMX	24.4

figure 65

Figure 66. IBMX treatment reduces BCR-mediated Ras activation, but enhances BCR-mediated ERK-MAPKinase activity. **A.** WEHI-231 cells ($1x10^8$/ml) were stimulated as follows: **lane 1**, cells left untreated for 5 minutes, **lane 2**, cells stimulated with anti-Ig (10 μg/ml) for 5 minutes, **lane 3**, cells pre-treated with IBMX (10 μM) for 60 minutes and then stimulated with anti-Ig (10 μg/ml) for 5 minutes. GTP-bound Ras was then immunoprecipitated using Raf-1-RBD as bait. Ras activation was then determined by Western blotting using an anti-Ras antibody. Densitometry shows relative band density measured on Gel-Pro Analysis software. **B.** WEHI-231 cells (5 x 10^7/ml) were stimulated as follows: **lane 1**, cells left untreated for 30 minutes, **lane 2**, cells left untreated for 60 minutes and then stimulated with anti-Ig (10 μg/ml) for 30 minutes, **lane 3**, cells were pre-incubated with IBMX (10 μM) for 1 hour and then stimulated for 30 min with anti-Ig (10 μg/ml), **lane 4**, cells were pre-incubated with IBMX (20 μM) for 1 hour and then stimulated for 30 min with anti-Ig (10 μg/ml) were indicated. Levels of pERK/wERK expression were determined by Western blotting (15 μg/lane). Densitometry shows relative band density measured on Gel-Pro Analysis software.

(A)



(B)

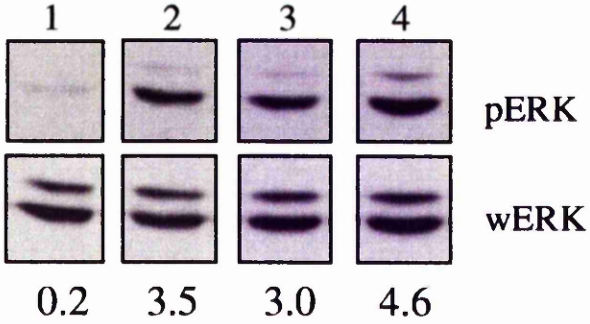


figure 66

Figure 67. Pharmacological inhibition of PKA by H-89 does not reverse BCR-mediated growth arrest. WEHI-231 cells (10^4/well) were left untreated or treated with anti-Ig (10 μg/ml) with or without H-89 (0.005-1 μM), anti-CD40 (10 μg/ml) with or without H-89 (0.005-1 μM) or anti-Ig plus anti-CD40 (both 10 μg/ml) with or without H-89 (0.005-1 μM). Proliferation was assessed by measuring DNA synthesis as indicated by [^{3}H]thymidine uptake at 48 hours. Values shown are means ± SD of quadruplicate wells.

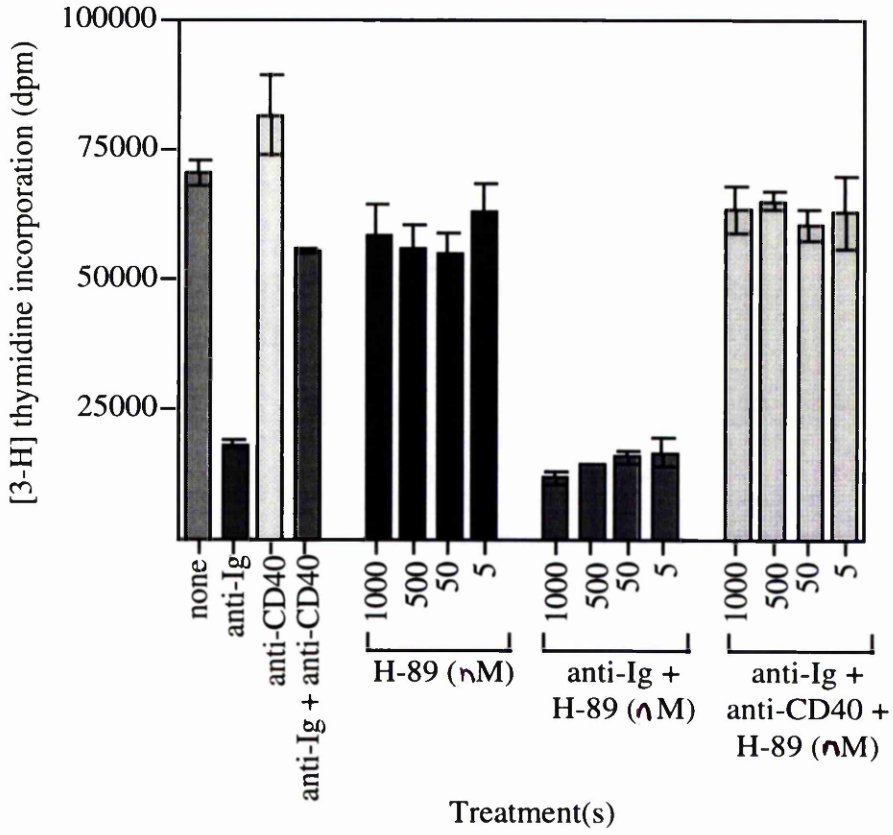
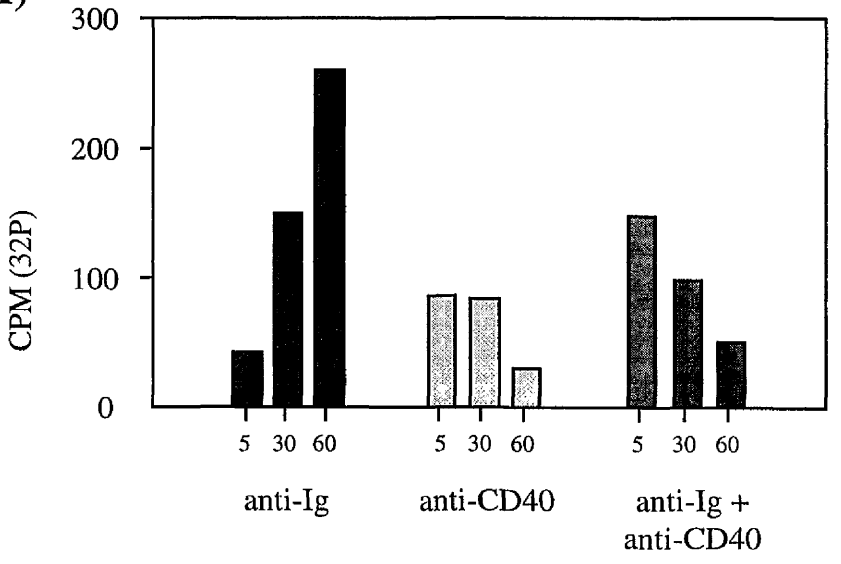

100000
75000
50000
25000
[3-H] thymidine incorporation (dpm)
none
anti-Ig
anti-CD40
anti-Ig + anti-CD40
1000
500
50
5
H-89 (nM)
1000
500
50
5
anti-Ig +
H-89 (nM)
1000
500
50
5
anti-Ig +
anti-CD40 +
H-89 (nM)
Treatment(s)

figure 67

Figure 68. BCR-ligation induces PtdIns(3,4,5)P_3 generation in WEHI-231 cells. A. WEHI-231 cells ($5x10^7$/ml) were left untreated or treated with anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml) for the times indicated. Reactions were stopped and lipids extracted as described in materials and methods. Lipid samples were dried under vacuum-centrifugation and subject to TLC analysis. The band corresponding to PtdIns(3,4,5)P_3 was scraped and ^{32}P levels determined by liquid scintillation counting. **B.** Repeat experiment of figure 57B. Values are % PtdIns(3,4,5)P_3 production compared to anti-Ig stimulated cells in anti-Ig plus anti-CD40 stimulated samples ± SD from triplicate samples.

(A)



(B)

Time (min)	anti-Ig + anti-CD40 (as a percentage of anti-Ig alone)
5	125 ± 43
30	94 ± 28
60	58 ± 4

figure 68

Figure 69. PI(3)K inhibitors wortmannin (Wm) and Ly294002 (Ly) inhibit BCR-mediated ERK-MAPKinase activation. WEHI-231 cells (5 x 10^7/ml) were stimulated as follows: **lane 1**, cells left untreated for 0 minutes, **lane 2**, cells left untreated for 210 minutes, **lane 3**, cells treated with anti-Ig (10 μg/ml) for 30 minutes, **lane 4**, cells treated for 1 hour with 0.5 nM Wm and then anti-Ig (10 μg/ml) for 30 minutes, **lane 5**, cells treated with 50 nM Wm for 1 hour and then anti-Ig (10 μg/ml) for 30 minutes, **lane 6**, cells treated with 5 nM Wm for 2 hours and then anti-Ig (10 μg/ml) for 30 minutes , **lane 7**, cells treated with 50 nM Wm for 2 hours and then anti-Ig (10 μg/ml) for 30 minutes, **lane 8**, cells treated with 1 μM Ly for 1 hour and then anti-Ig (10 μg/ml) for 30 minutes, **lane 9**, cells treated with 10 μM Ly for 1 hour and then anti-Ig (10 μg/ml) for 30 minutes, **lane 10**, cells treated with 1 μM Ly for 2 hours and then anti-Ig (10 μg/ml) for 30 minutes, **lane 11**, cells treated with 10 μM Ly for 2 hours and then anti-Ig (10 μg/ml) for 30 minutes. Levels of pERK/wERK expression were determined by Western blotting (15 μg/lane).

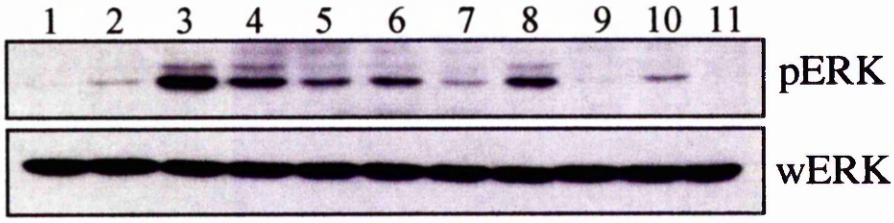


figure 69

Figure 70. PI(3)K plays no role in BCR-induced apoptosis. WEHI-231 cells ($5x10^5$/ml) were stimulated as indicated (anti-Ig and anti-CD40 both at 10 μg/ml). Cells treated with Wm or Ly294002 were pre-incubated in the presence of the indicated concentration of inhibitor for 75 minutes prior to further cell stimulation (if indicated). Levels of apoptosis were determined by PI staining and FACS analysis after 48 hours as described in materials and Methods.

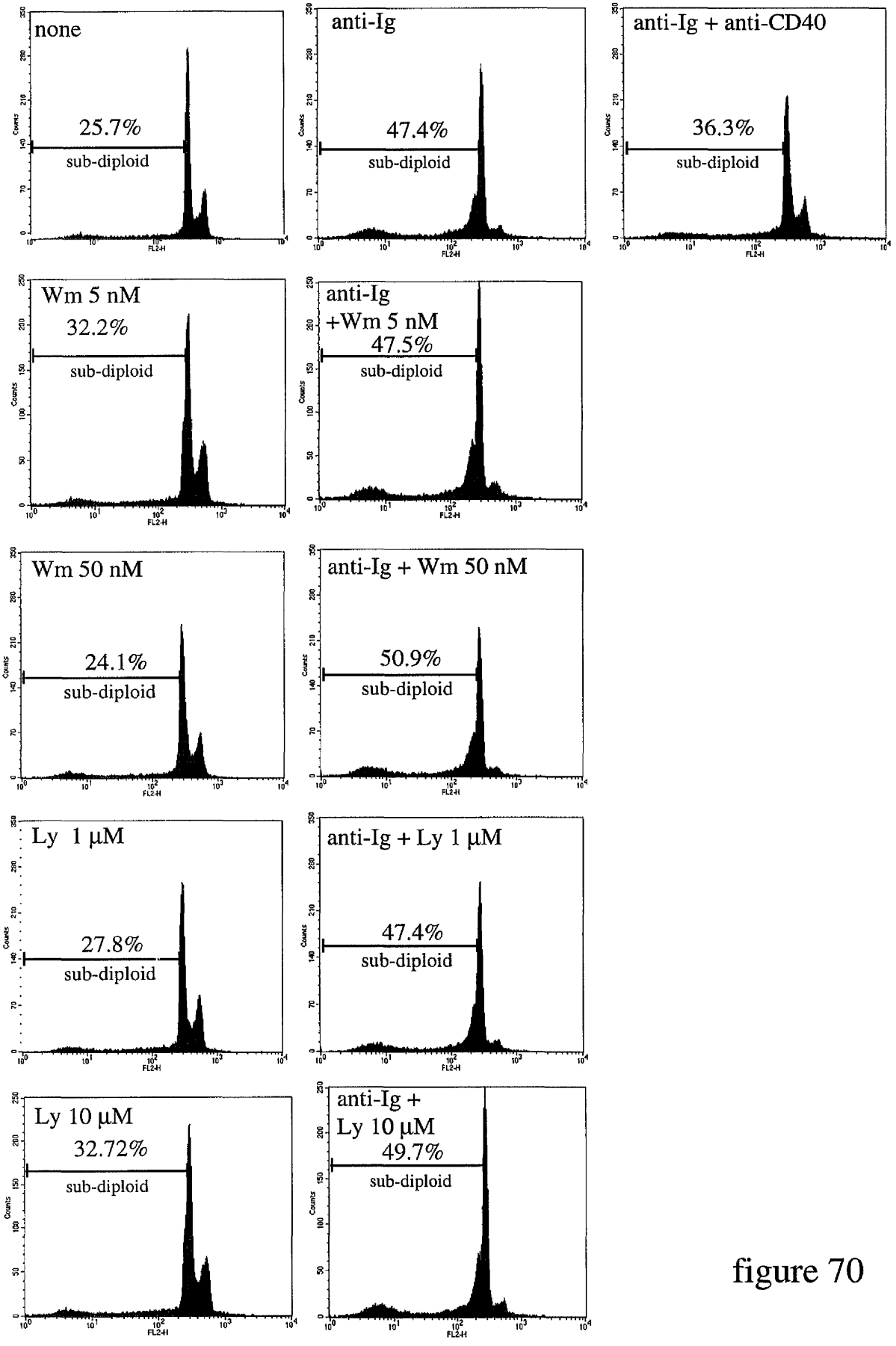

none
25.7%
sub-diploid
anti-Ig
47.4%
sub-diploid
anti-Ig + anti-CD40
36.3%
sub-diploid
Wm 5 nM
32.2%
sub-diploid
anti-Ig
+Wm 5 nM
47.5%
sub-diploid
Wm 50 nM
24.1%
sub-diploid
anti-Ig + Wm 50 nM
50.9%
sub-diploid
Ly 1 μM
27.8%
sub-diploid
anti-Ig + Ly 1 μM
47.4%
sub-diploid
Ly 10 μM
32.72%
sub-diploid
anti-Ig +
Ly 10 μM
49.7%
sub-diploid

figure 70

Figure 71. BCR-ligation downregulates p85α PI(3)K protein expression. WEHI-231 cells ($5x10^5$/ml) were stimulated as follows: lane 1, cells left unstimulated for 1 hour, **lane 2**, cells stimulated with anti-Ig (1 μg/ml) for 1 hour, **lane 3**, cells stimulated with anti-Ig (1 μg/ml) for 4 hours, **lane 4**, cells stimulated with anti-Ig (1 μg/ml) for 24 hours, **lane 5**, cells stimulated with anti-Ig (1 μg/ml) for 48 hours, **lane 6**, cells stimulated with anti-CD40 (10 μg/ml) for 1 hour, **lane 7**, cells stimulated with anti-CD40 (10 μg/ml) for 4 hours, **lane 8**, cells stimulated with anti-CD40 (10 μg/ml) for 24 hours, **lane 9**, cells stimulated with anti-CD40 (10 μg/ml) for 48 hours, **lane 10**, cells stimulated with anti-Ig plus anti-CD40 for 1 hour, **lane 11**, cells stimulated with anti-Ig plus anti-CD40 for 4 hours, **lane 12**, cells stimulated with anti-Ig plus anti-CD40 for 24 hours, **lane 13**, cells stimulated with anti-Ig plus anti-CD40 for 48 hours, **lane 14**, cells left unstimulated for 48 hours. Levels of PI(3)K p85α expression were determined by Western blotting (30 μg/lane).

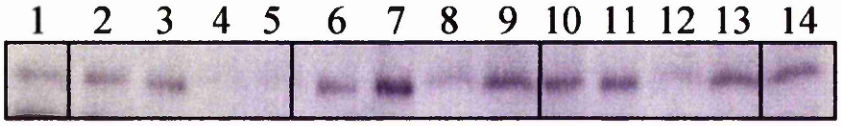
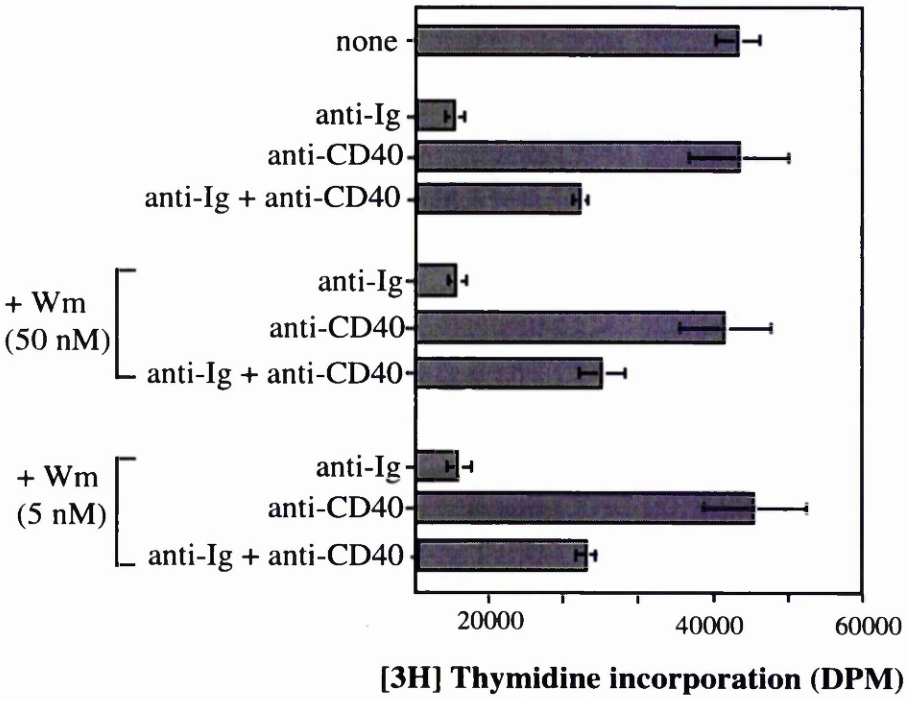


figure 71

Figure 72. PI(3)K activity is not involved in CD40-mediated rescue from growth arrest. **A.** WEHI-231 cells (10^4 cells/well) were left untreated or pre-incubated for 1 hour in wortmannin (5 or 50 nM) prior to stimulation with anti-Ig (10 μg/ml); anti-CD40 (10 μg/ml) or a combination of both. Proliferation was assessed by measuring DNA synthesis as indicated by [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells. **B.** WEHI-231 cells (10^4 cells/well) were left untreated or pre-incubated for 1 hour in Ly294002 (0.1 or 1 μM) prior to stimulation with anti-Ig (10 μg/ml); anti-CD40 (10 μg/ml) or a combination of both. Proliferation was assessed by measuring DNA synthesis as indicated by [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells.

(A)


none
anti-Ig
anti-CD40
anti-Ig + anti-CD40
+ Wm (50 nM)
anti-Ig
anti-CD40
anti-Ig + anti-CD40
+ Wm (5 nM)
anti-Ig
anti-CD40
anti-Ig + anti-CD40
20000
40000
60000
[3H] Thymidine incorporation (DPM)

(B)

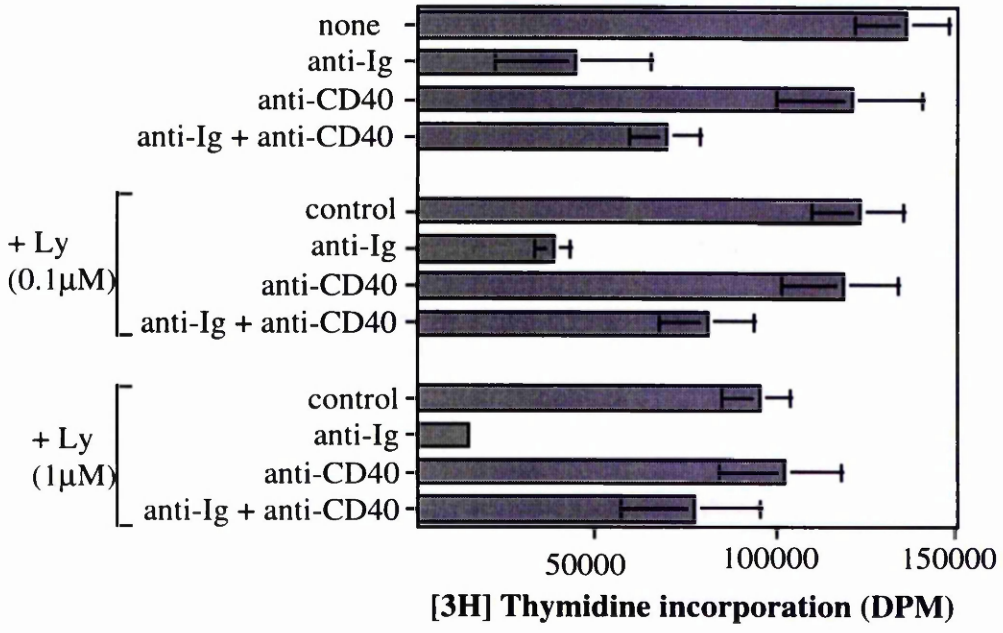

none
anti-Ig
anti-CD40
anti-Ig + anti-CD40
+ Ly (0.1μM)
control
anti-Ig
anti-CD40
anti-Ig + anti-CD40
+ Ly (1μM)
control
anti-Ig
anti-CD40
anti-Ig + anti-CD40
50000
100000
150000
[3H] Thymidine incorporation (DPM)

figure 72

Figure 73. Sustained PI(3)K inhibition does not prevent anti-CD40 mediated rescue from BCR-induced growth arrest in WEHI-231 cells. WEHI-231 cells (10^4 cells/well) were left untreated or pre-treated for 1 hour with either 5 or 50 nM wortmannin or 1 or 10 μM Ly294002. Cells were then left otherwise untreated or treated with anti-Ig (10 μg/ml) or anti-Ig plus anti-CD40 (both at 10 μg/ml) where indicated. All cell samples that had previously received either Wm or Ly294002 pre-incubation were treated with additional doses (similar concentrations) after 1, 2, 4, 8, 12, 16, 20, 24 and 28 hours. Proliferation was assessed by measuring DNA synthesis as indicated by [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells.

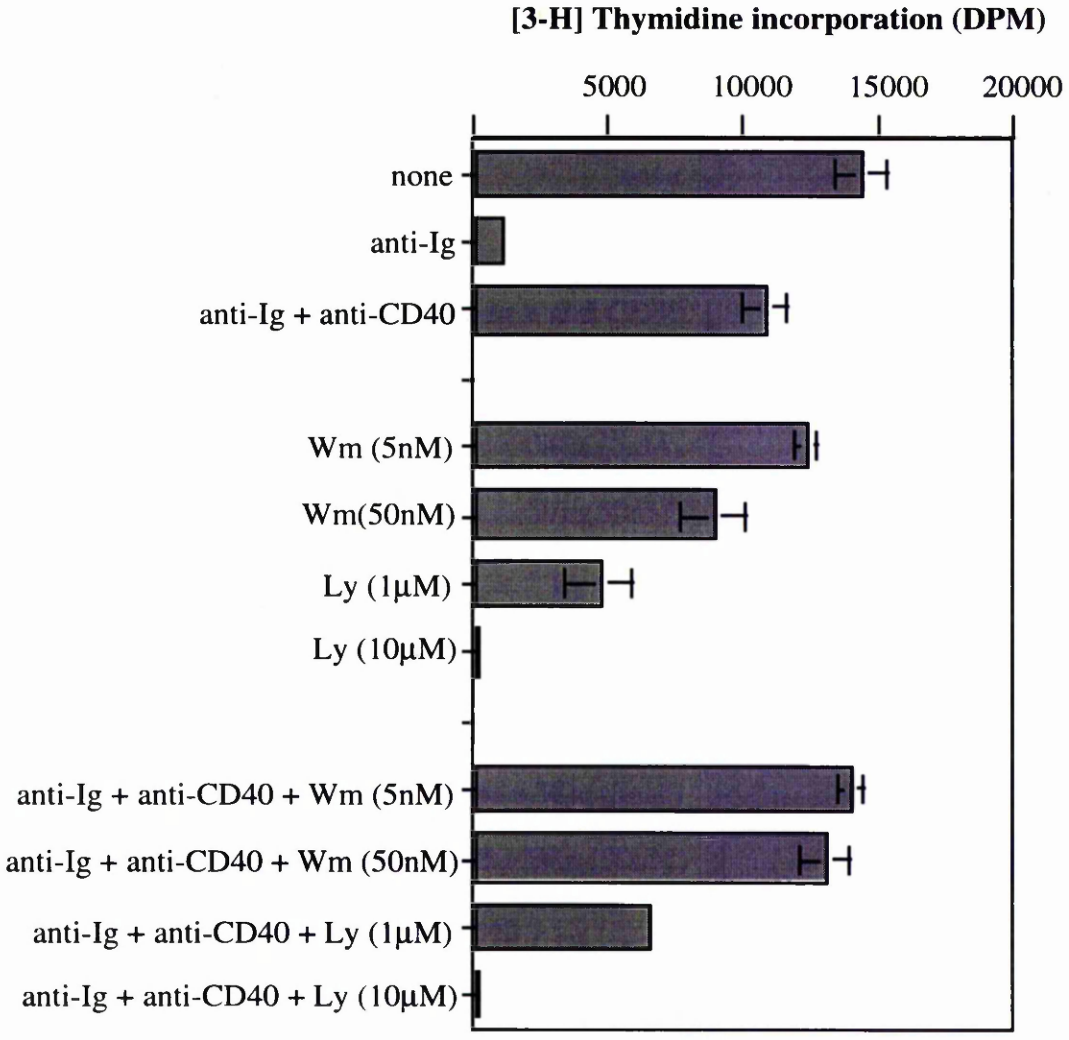

[3-H] Thymidine incorporation (DPM)
5000
10000
15000
20000
none
anti-Ig
anti-Ig + anti-CD40
Wm (5nM)
Wm(50nM)
Ly (1μM)
Ly (10μM)
anti-Ig + anti-CD40 + Wm (5nM)
anti-Ig + anti-CD40 + Wm (50nM)
anti-Ig + anti-CD40 + Ly (1μM)
anti-Ig + anti-CD40 + Ly (10μM)

figure 73

Figure 74. Long-term BCR-ligation, but not anti-CD40 treatment induces PtdIns(3,4,5)P_3 generation in WEHI-231 cells. WEHI-231 cells ($5x10^5$/ml) were left untreated or treated with anti-Ig (1 μg/ml), anti-CD40 (10 μg/ml) or anti-Ig plus anti-CD40 for the times indicated. Reactions were stopped and lipids extracted as described in Materials and Methods. Lipid samples were dried under vacuum-centrifugation and subject to TLC analysis. The band corresponding to PtdIns(3,4,5)P_3 was scrapped and ^{32}P levels determined by liquid scintillation counting. Values shown are the means ± SEM of triplicate samples.

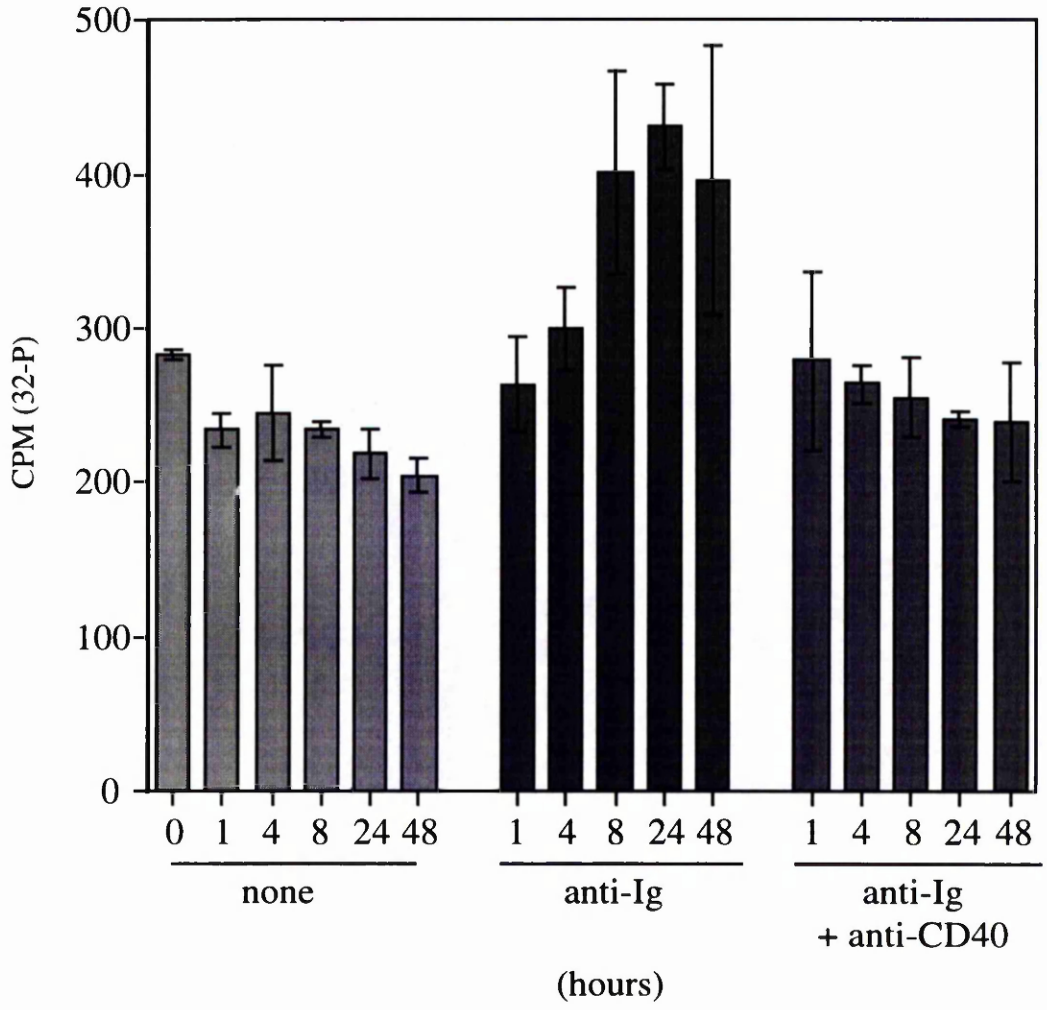
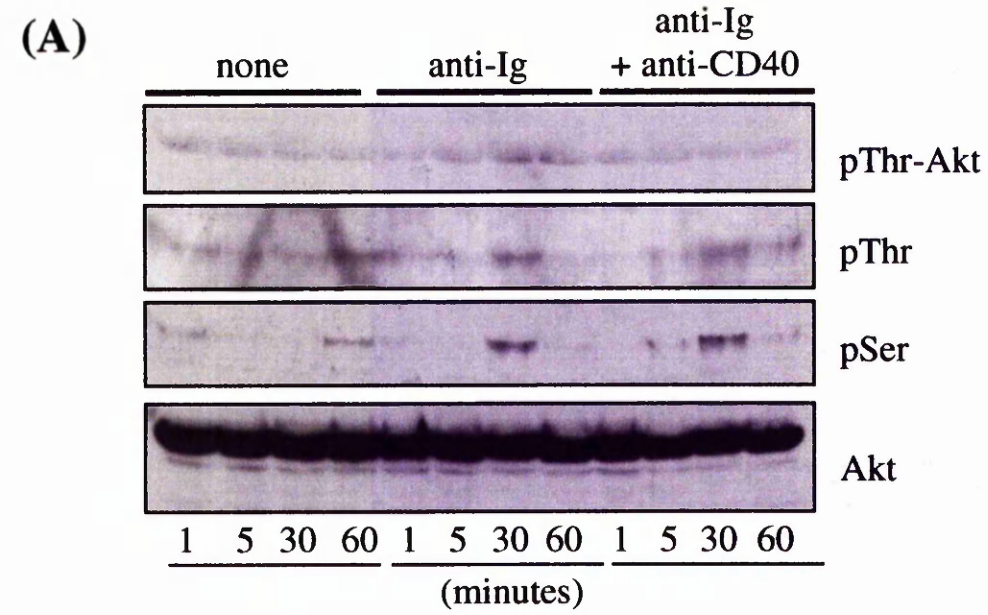

500
400
300
200
100
0
CPM (32-P)
0 1 4 8 24 48
1 4 8 24 48
1 4 8 24 48
none
anti-Ig
anti-Ig
+ anti-CD40
(hours)

figure 74

Figure 75. Akt is not activated during CD40-mediated rescue of WEHI-231 cells from BCR-induced growth-arrest/apoptosis. **A.** WEHI-231 cells (5×10^5/ml) were left untreated or stimulated with anti-Ig (1 μg/ml) or anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for the time periods indicated. Levels of $pThr^{308}$-Akt, wAkt, pThr or pSer expression were determined by Western blotting (70 μg/lane). **B.** WEHI-231 cells (5×10^5/ml) were stimulated as follows, **lane 1**, cells left unstimulated for 0 hours, **lane 2**, cells stimulated with anti-Ig (1 μg/ml) for 1 hour, **lane 3**, cells stimulated with anti-Ig (1 μg/ml) for 8 hours, **lane 4**, cells stimulated with anti-Ig (1 μg/ml) for 24 hours, **lane 5**, cells stimulated with anti-CD40 (10 μg/ml) for 1 hour, **lane 6**, cells stimulated with anti-CD40 (10 μg/ml) for 8 hours, **lane 7**, cells stimulated with anti-CD40 (10 μg/ml) for 24 hours, **lane 8**, cells stimulated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 1 hour, **lane 9**, cells stimulated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 8 hours, **lane 10**, cells stimulated with anti-Ig (1 μg/ml) and anti-CD40 (10 μg/ml) for 24 hours, **lane 11**, cells left unstimulated for 24 hours. Levels of $pThr^{308}$-Akt or wAkt expression were determined by Western blotting (15 μg/lane).


(A)
none
anti-Ig
anti-Ig
+ anti-CD40
pThr-Akt
pThr
pSer
Akt
1 5 30 60 1 5 30 60 1 5 30 60
(minutes)

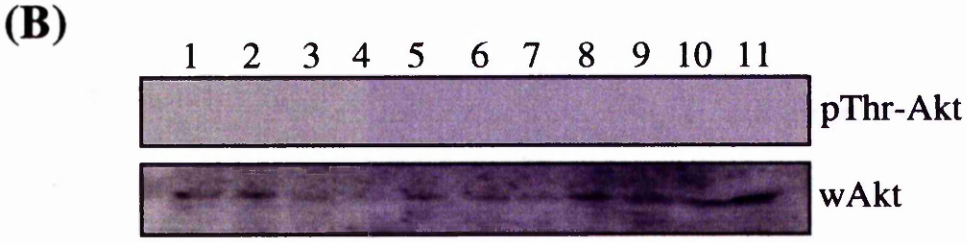

(B)
1 2 3 4 5 6 7 8 9 10 11
pThr-Akt
wAkt

figure 75

Figure 76. BCR-ligation is coupled to sphingosine-kinase in WEHI-231 B cells. WEHI-231 cells ($1x10^6$/ml) were left untreated or treated with anti-Ig (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml) for the time periods indicated. Reactions were stopped and lipids extracted as described in Materials and Methods. Lipid samples were dried under vacuum-centrifugation and subject to TLC analysis. The band corresponding to SPP was scraped and [^{3}H]serine levels determined by liquid scintillation counting.

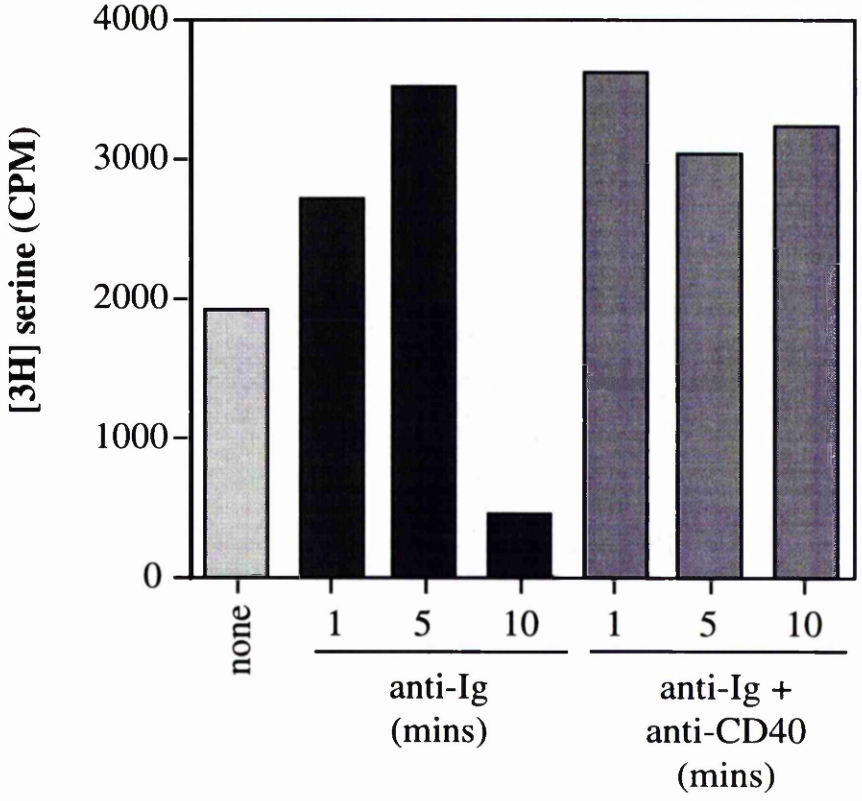
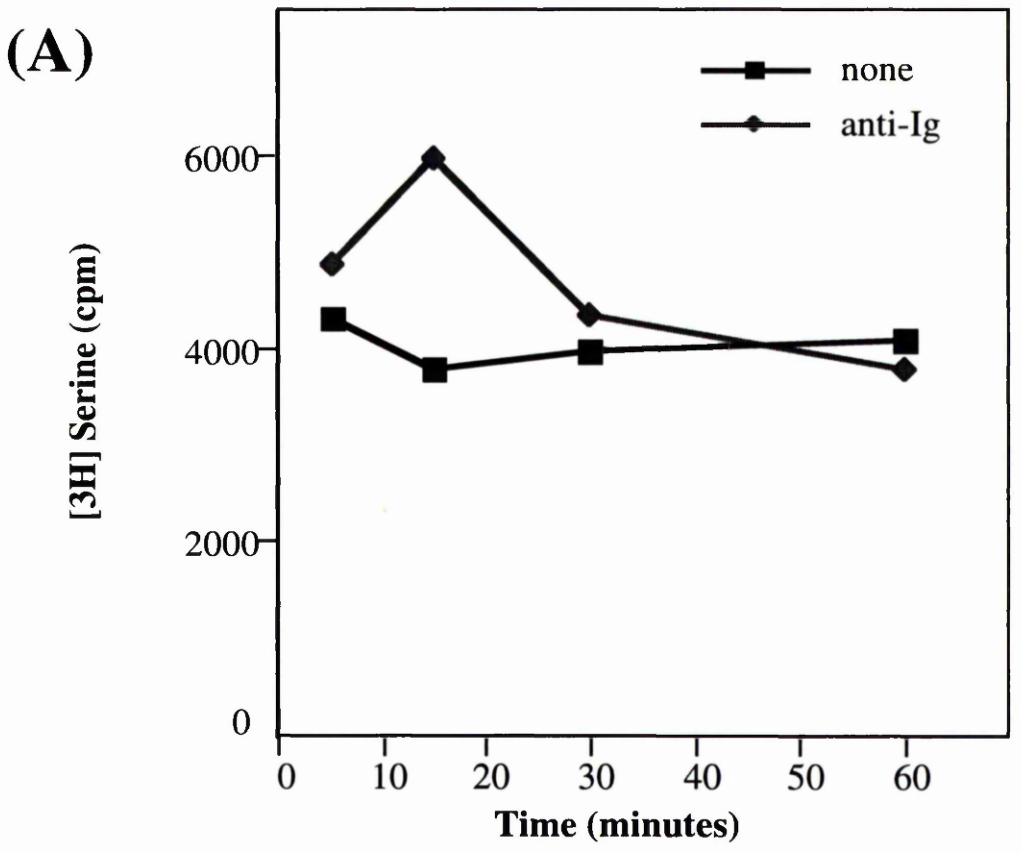

4000
3000
2000
1000
0
[3H] serine (CPM)
none
1
5
10
1
5
10
anti-Ig
(mins)
anti-Ig +
anti-CD40
(mins)

figure 76

Figure 77. BCR and CD40 are both coupled to sphingosine kinase in WEHI-231 cells. A. WEHI-231 cells ($1x10^6$/ml) were left untreated or treated with anti-Ig (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml) for the time periods indicated. Reactions were stopped and lipids extracted as described in Materials and Methods. Lipid samples were dried under vacuum-centrifugation and subject to TLC analysis. The band corresponding to SPP was scraped and [^{3}H]serine levels determined by liquid scintillation counting. **B.** WEHI-231 cells ($1x10^6$/ml) were left untreated, or treated with anti-Ig plus anti-CD40 (both 10 μg/ml) for the time periods indicated. Reactions were stopped and lipids extracted as described in Materials and Methods. Lipid samples were dried under vacuum-centrifugation and subject to TLC analysis. The band corresponding to SPP was scraped and [^{3}H]serine levels determined by liquid scintillation counting.


(A)
none
anti-Ig
6000
4000
2000
0
[3H] Serine (cpm)
0
10
20
30
40
50
60
Time (minutes)

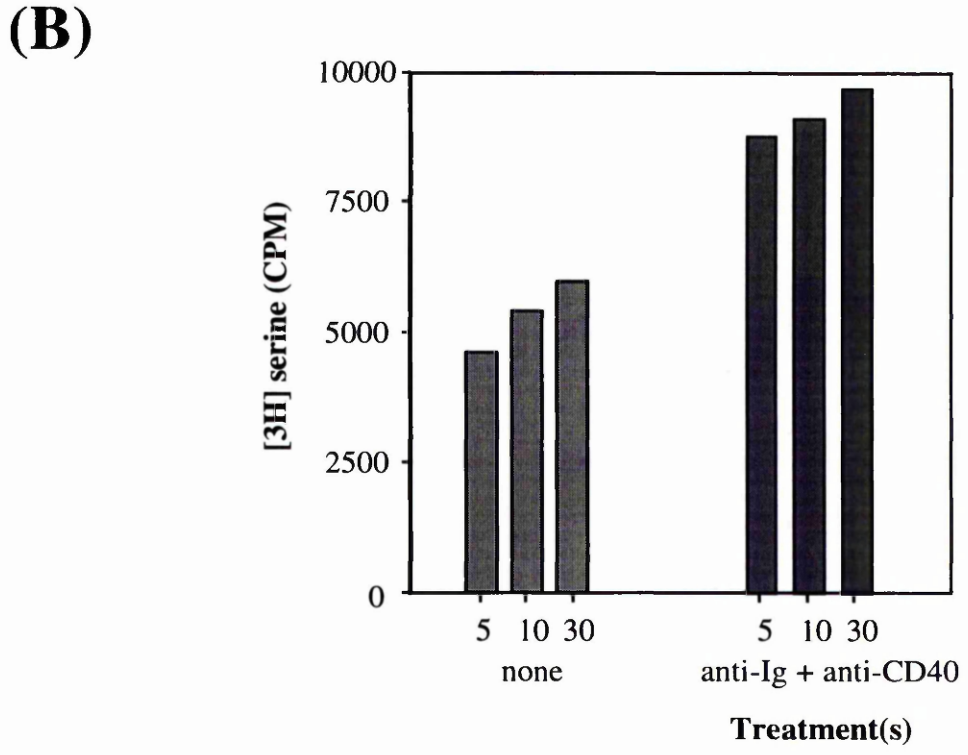

(B)
10000
7500
5000
2500
0
[3H] serine (CPM)
5
10
30
5
10
30
none
anti-Ig + anti-CD40
Treatment(s)

figure 77

Figure 78. The BCR of mature splenic B lymphocytes is coupled to sphingosine kinase activation. Freshly isolated mature splenic B lymphocytes ($2x10^6$/ml) were left untreated or treated with anti-Ig (50 μg/ml) for the time periods indicated. Reactions were stopped and lipids extracted as described in Materials and Methods. Lipid samples were dried under vacuum-centrifugation and subject to TLC analysis. The band corresponding to SPP was scraped and [^{3}H]serine levels determined by liquid scintillation counting.

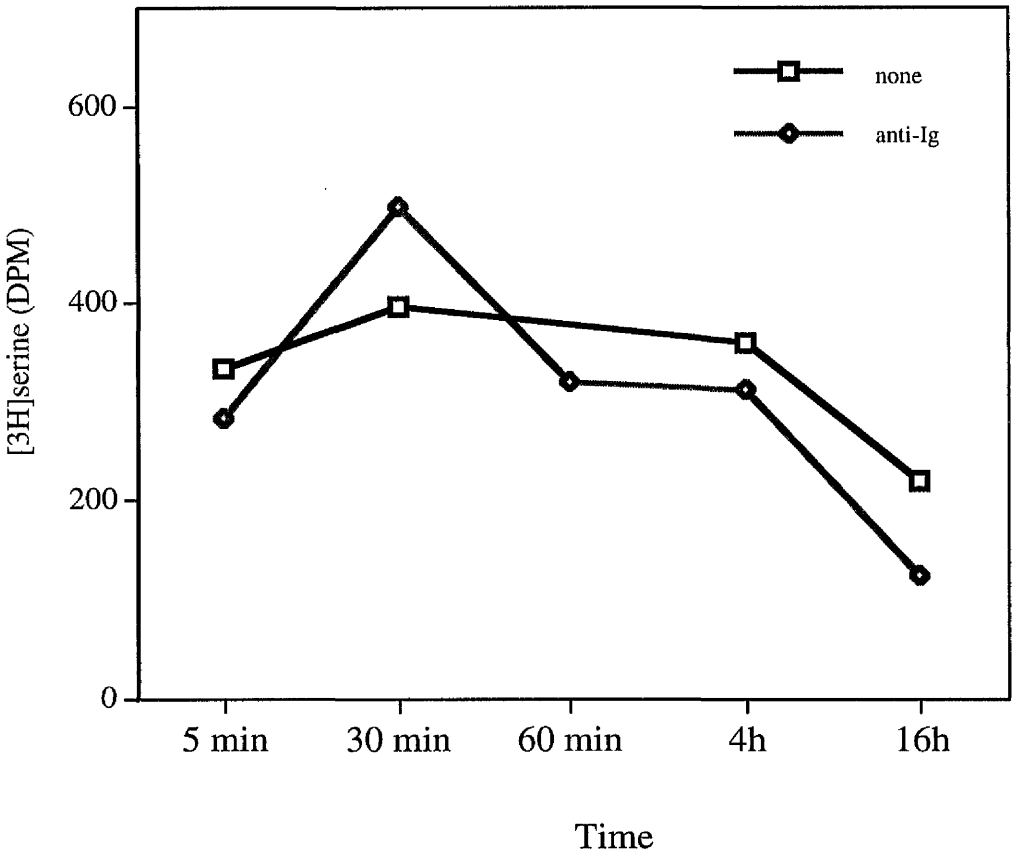
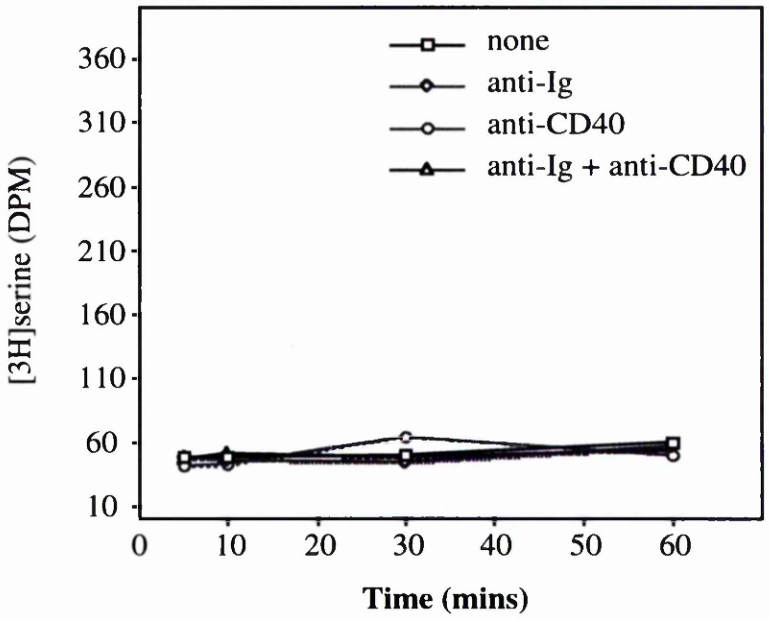

none
anti-Ig
600
400
200
0
[3H]serine (DPM)
5 min
30 min
60 min
4h
16h
Time

figure 78

Figure 79. SPP is not secreted by WEHI-231 cells under apoptotic or proliferative conditions and exogenous SPP treatment does not affect WEHI-231 cell growth. **A.** WEHI-231 cells ($1x10^6$/ml) were left untreated or treated with anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml) for the time periods indicated. Supernatants were collected and lipids extracted as described in Materials and Methods. Lipid samples were dried under vacuum-centrifugation and subject to TLC analysis. The band corresponding to SPP was scraped and [^{3}H]serine levels determined by liquid scintillation counting. **B.** WEHI-231 cells (10^4 cells/well) were left untreated or treated with anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml). Additional samples were treated with SPP (100 nM, 1 μM or 10 μM) with or without anti-Ig (10 μg/ml) or vehicle controls for SPP. Proliferation was assessed by measuring DNA synthesis as indicated by [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells.

(A)


none
anti-Ig
anti-CD40
anti-Ig + anti-CD40
[3H]serine (DPM)
360
310
260
210
160
110
60
10
0
10
20
30
40
50
60
Time (mins)

(B)

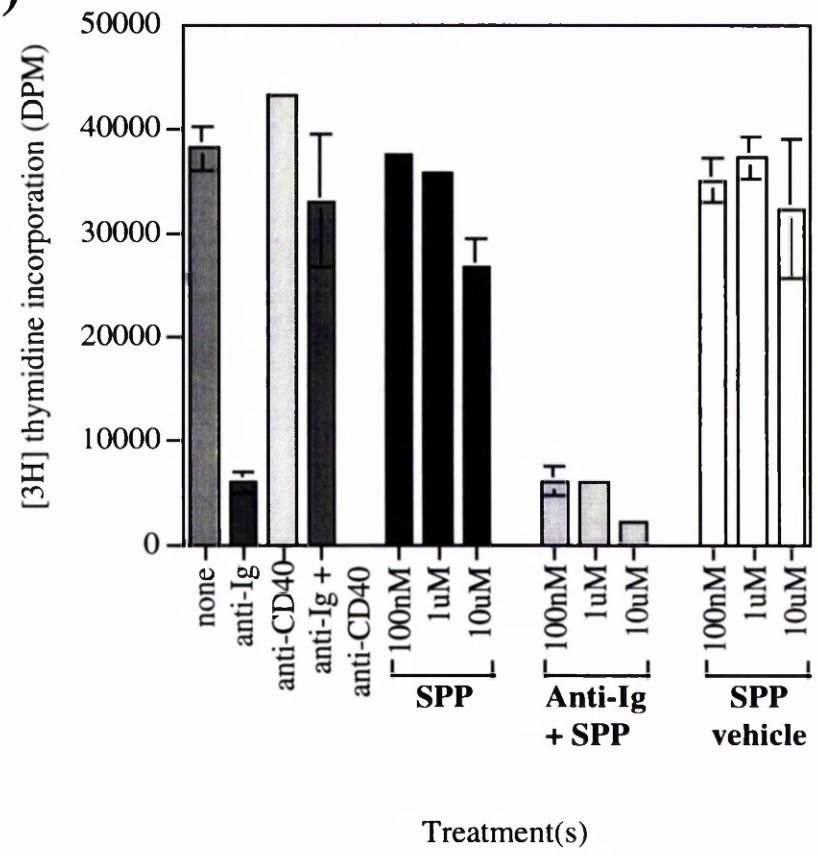

[3H] thymidine incorporation (DPM)
50000
40000
30000
20000
10000
0
none
anti-Ig
anti-CD40
anti-Ig + anti-CD40
100nM
1uM
10uM
SPP
100nM
1uM
10uM
Anti-Ig + SPP
100nM
1uM
10uM
SPP vehicle
Treatment(s)

figure 79

Figure 80. Long-term BCR-ligation suppresses basal SPP production which is restored with anti-CD40 co-treatment. WEHI-231 cells ($5x10^5$) were left untreated or treated with anti-Ig (1 μg/ml) or anti-Ig plus anti-CD40 (10 μg/ml) for the time periods indicated. Reactions were stopped and lipids extracted as described in Materials and Methods. Lipid samples were dried under vacuum-centrifugation and subject to TLC analysis. The band corresponding to SPP was scraped and [^{3}H]serine levels determined by liquid scintillation counting. Values shown are means ± SEM of triplicate samples.

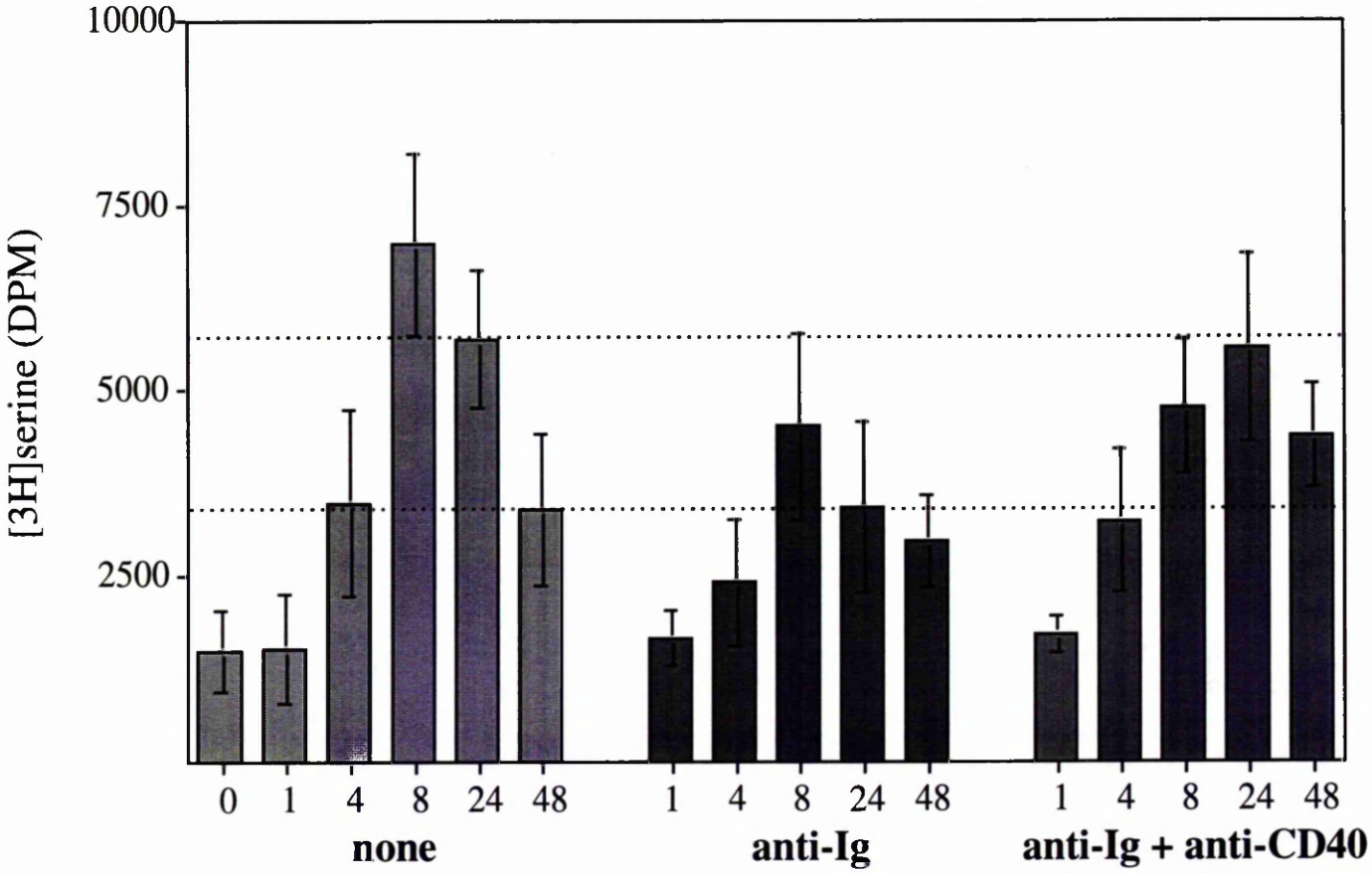

10000
7500
5000
2500
[3H]serine (DPM)
0 1 4 8 24 48
1 4 8 24 48
1 4 8 24 48
none
anti-Ig
anti-Ig + anti-CD40

figure 80

Figure 81. Inhibition of sphingosine kinase induces growth-arrest in WEHI-231 cells. WEHI-231 cells (10^4 cells/well) were left untreated or treated with anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or anti-Ig plus anti-CD40 (both 10 μg/ml). Additional samples were treated with SPP (0.1, 1, 5, 10, 25 μM) with or without anti-Ig (10μg/ml) or anti-Ig plus anti-CD40 (10μg/ml) or vehicle controls for SPP. Proliferation was assessed by measuring DNA synthesis as indicated by [^{3}H]thymidine uptake at 48 hours. Values shown are the means ± SD of quadruplicate wells.

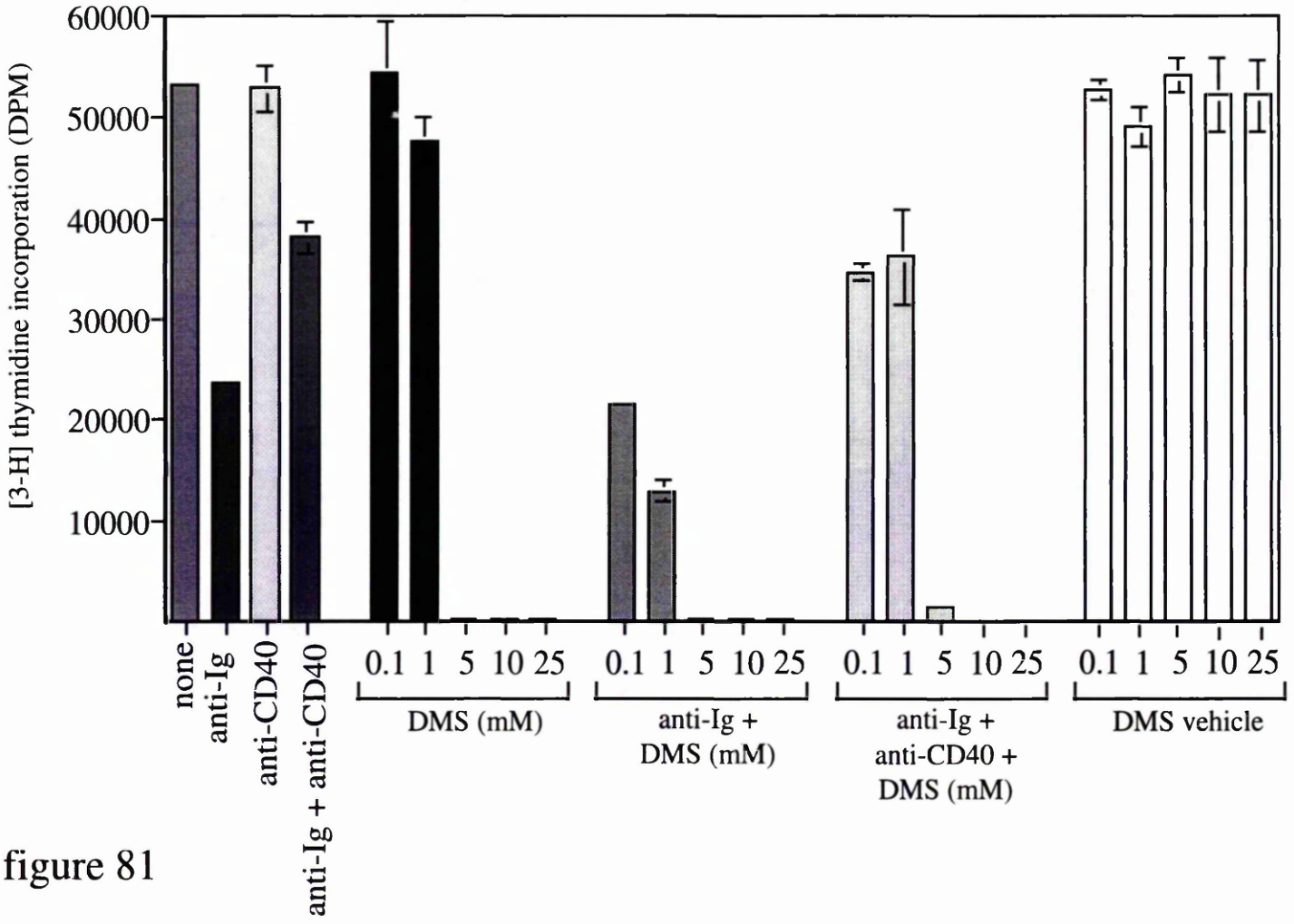

[3-H] thymidine incorporation (DPM)
60000
50000
40000
30000
20000
10000
none
anti-Ig
anti-CD40
anti-Ig + anti-CD40
0.1 1 5 10 25
DMS (mM)
0.1 1 5 10 25
anti-Ig +
DMS (mM)
0.1 1 5 10 25
anti-Ig +
anti-CD40 +
DMS (mM)
0.1 1 5 10 25
DMS vehicle

figure 81

Figure 82. Inhibition of sphingosine kinase induces apoptosis in WEHI-231 cells. WEHI-231 cells ($5x10^5$/ml) were stimulated as indicated (anti-Ig and anti-CD40 both at 10 μg/ml). Cells treated with DMS were pre-incubated in the presence of the indicated concentration of inhibitor for 60 minutes prior to further cell stimulation (if indicated). Levels of apoptosis were determined by PI staining and FACS analysis after 48 hours as described in Materials and Methods.

figure 82

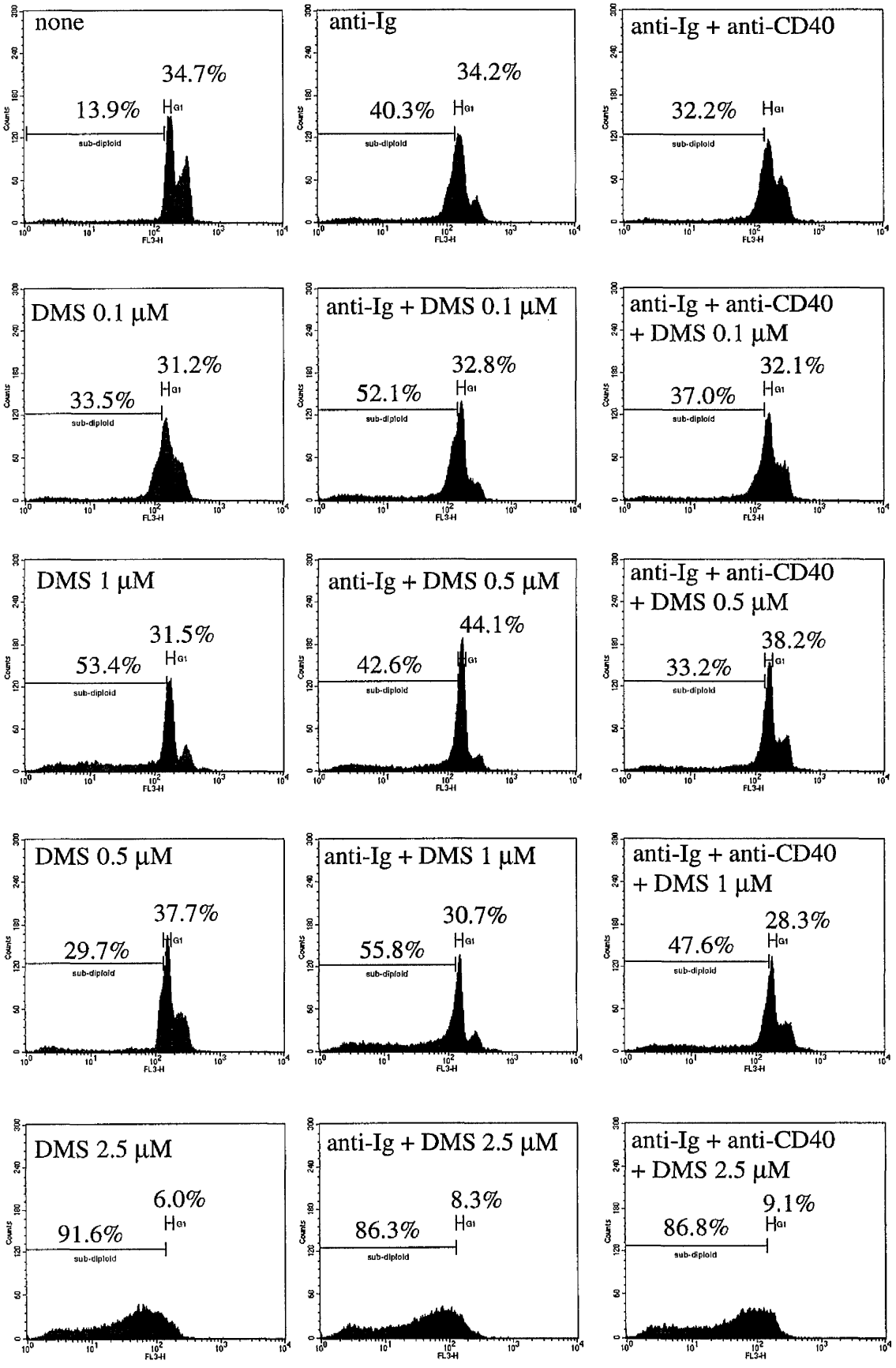

none
34.7%
13.9%
sub-diploid
anti-Ig
34.2%
40.3%
sub-diploid
anti-Ig + anti-CD40
32.2%
sub-diploid
DMS 0.1 µM
31.2%
33.5%
sub-diploid
anti-Ig + DMS 0.1 µM
32.8%
52.1%
sub-diploid
anti-Ig + anti-CD40
+ DMS 0.1 µM
32.1%
37.0%
sub-diploid
DMS 1 µM
31.5%
53.4%
sub-diploid
anti-Ig + DMS 0.5 µM
44.1%
42.6%
sub-diploid
anti-Ig + anti-CD40
+ DMS 0.5 µM
38.2%
33.2%
sub-diploid
DMS 0.5 µM
37.7%
29.7%
sub-diploid
anti-Ig + DMS 1 µM
30.7%
55.8%
sub-diploid
anti-Ig + anti-CD40
+ DMS 1 µM
28.3%
47.6%
sub-diploid
DMS 2.5 µM
6.0%
91.6%
sub-diploid
anti-Ig + DMS 2.5 µM
8.3%
86.3%
sub-diploid
anti-Ig + anti-CD40
+ DMS 2.5 µM
9.1%
86.8%
sub-diploid
Counts
FL3-H

Figure 83. Model for the differential activation of ERK-MAPKinase by BCR or CD40 ligation. Taken from Purkerson *et. al.* (97).

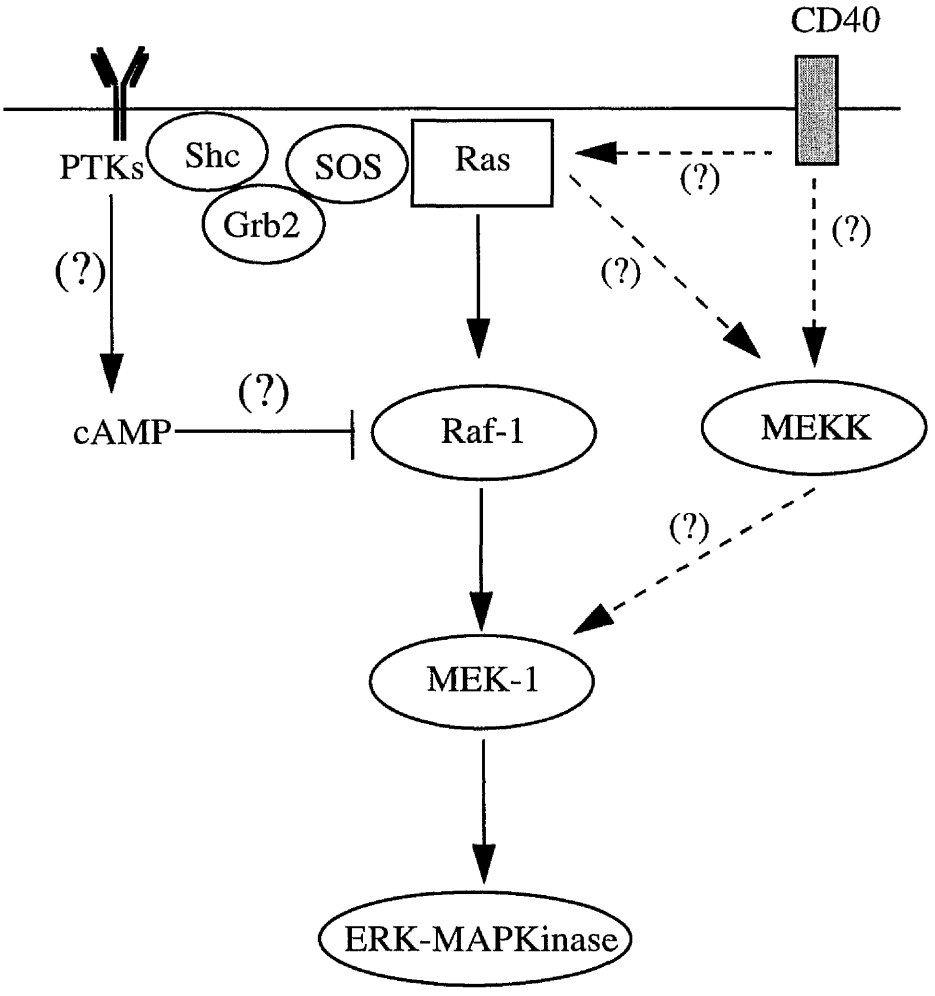


figure 83

Figure 84. Model for the role of PtdIns(3,4,5)P_3 in the induction of apoptosis in WEHI-231 cells.

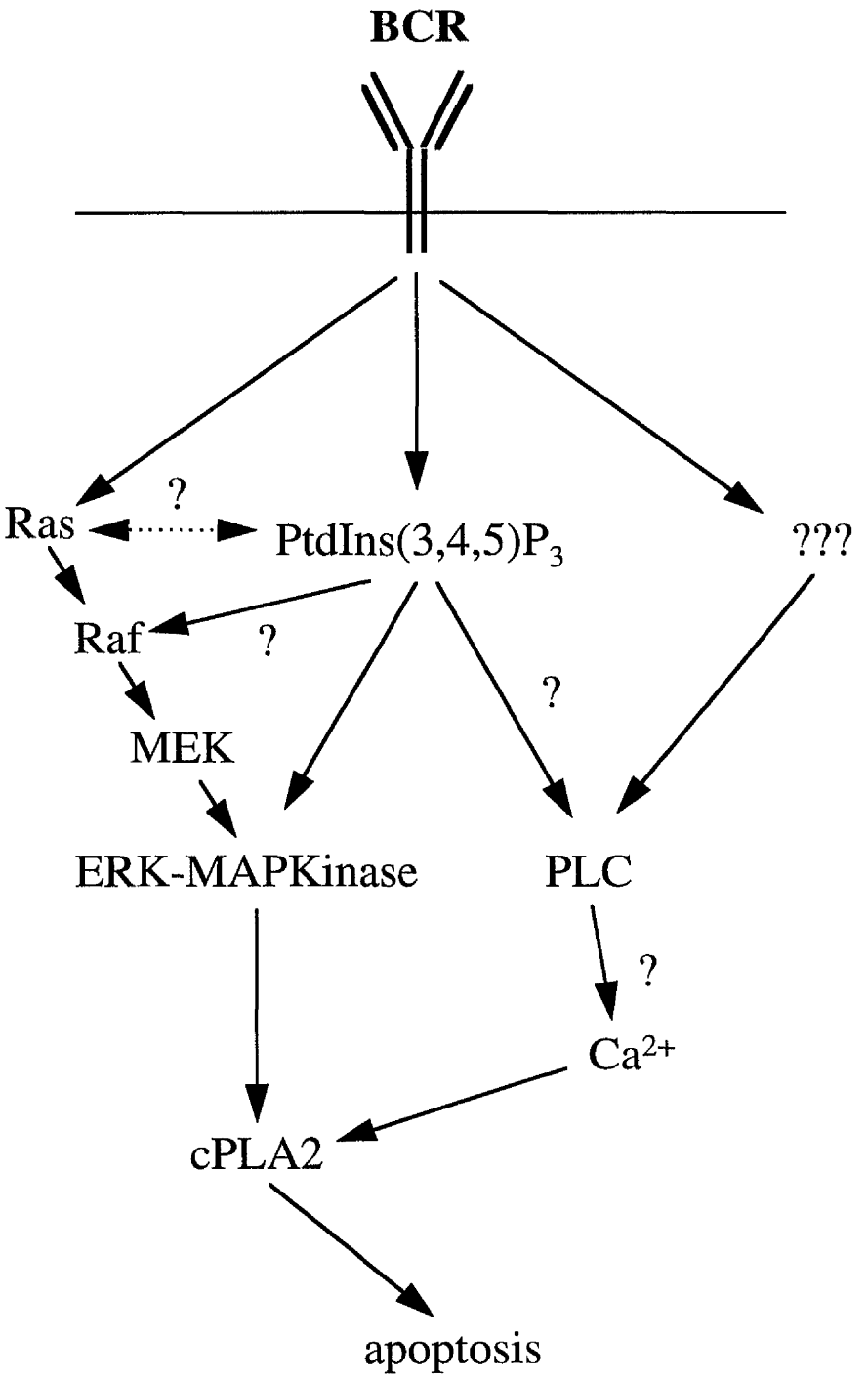

BCR
Ras
?
PtdIns(3,4,5)P3
???
Raf
?
?
MEK
ERK-MAPKinase
PLC
?
Ca2+
cPLA2
apoptosis

figure 84

CHAPTER 7 - GENERAL DISCUSSION.

The deletion of immature B lymphocytes with self-reactive BCRs is a recognised phenomenon of B lymphocyte maturation and development. However, the molecular and biochemical mechanisms responsible for this are still unclear. This investigation has focused on the signalling mechanisms activated during the induction of growth arrest and apoptosis following BCR ligation in the WEHI-231 cell line, which acts as a model for immature B lymphocytes. In addition, the signalling pathways activated by CD40 stimulation, which reverses BCR-induced apoptosis, were also examined to determine whether this rescue signal mediates its affect by inducing anti-apoptotic signals or modulating the pro-apoptotic signals generated via BCR ligation.

Previous studies within our lab (53) highlighted a role for the phospholipase, $cPLA_2$, in the maturation of B lymphocytes and the induction of apoptotic mechanisms in immature B lymphocytes. The investigation of the signalling mechanisms involved in the activation of this enzyme therefore represented a good starting point in the determination of BCR-induced signalling events.

Initial studies investigated the kinetics of BCR-induced growth arrest in order to determine whether short exposures to antigen (anti-Ig) were sufficient to induce the activation of growth inhibitory signalling cascades, or whether prolonged exposure to antigen was required. Interestingly, our data suggests that early BCR-mediated signalling events are capable of committing approximately 30% of cells to undergo growth arrest, whereas 100% commitment requires exposure to antigen for 8 or more hours. Our studies also showed that CD40 co-stimulation can reverse BCR-induced growth arrest even when added to culture after anti-Ig. However, our results also indicate that the ability of CD40 co-stimulation to rescue WEHI-231 cells from the negative signalling events activated via BCR stimulation is ineffectual once cells have fully committed to growth arrest. These results are in agreement with other studies, including work by John Monroe (8) who suggests that self-reactive immature B lymphocyte are "primed" for deletion during early BCR-mediated signalling events, but then enter a "temporal-window" in which external factors can redirect the intrinsic apoptotic signal allowing for cellular activation or receptor editing.

Due to the ability of short-term BCR-ligation to induce growth arrest we investigated the signalling molecules activated during early BCR-mediated responses. As $cPLA_2$ has previously been implicated in the induction of BCR-mediated negative signalling events, initial studies investigated whether BCR-ligation induced the activation of the MAPKinase family of enzymes, as these have been implicated, along with calcium, in the activation $cPLA_2$.

BCR-ligation induced the activation of an early ERK-MAPKinase signal, but not the other members of the MAPKinase family. Studies using the ERK-MAPKinase inhibitors confirmed that this early ERK-MAPKinase signal was coupled to BCR-mediated apoptotic signals. Preliminary data also suggests that this ERK-MAPKinase lies upstream of $cPLA_2$. Additionally, anti-CD40 stimulation, which has previously been shown to reverse BCR-induced cPLA2 activation was also shown to downregulate this early ERK-MAPKinase signal. These results are in agreement with another study (40) which together confirm a role for ERK-MAPKinase in the induction of BCR-induced apoptosis. These results are potentially very interesting because they suggest that ERK-MAPKinase, which is more often than not described as a mitogenic signal, can also initiate negative signalling events.

The mechanisms coupling the BCR to ERK-MAPKinase activation were also examined in order to determine if early ERK-MAPKinase signals were activated through classical or cross-talk signalling pathways. Results indicated that Ras and PI(3)K activities were activated by BCR-ligation with similar kinetics to ERK-MAPKinase. Indeed, inhibition of PI(3)K was shown to abolish BCR-induced ERK-MAPKinase activation. This is suggestive of an upstream role for PI(3)K in the activation of ERK-MAPKinase. Interestingly, a recent study has highlighted an important role for PI(3)K and Grb2 in the activation of ERK-MAPKinase in airway smooth muscle cells following stimulation with SPP. Coupled to our preliminary data which shows that the BCR is coupled to an early sphingosine kinase signal (with kinetics of activation which precede that of both PI(3)K and ERK-MAPKinase, it is tempting to speculate that the BCR may activate a SPP/PI(3)K/Ras/ERK-MAPKinase signal cascade (**figure 85**). Further evidence to support this hypothesis comes from our studies examining the effect of the sphingosine kinase inhibitor, DMS, on BCR-induced Ras and ERK-MAPKinase activation. These studies indicated that DMS was effective in downregulating BCR-mediated Ras activation, and at higher concentrations, downregulation of ERK-MAPKinase. Inhibition of this early BCR-induced sphingosine kinase signal with DMS did not prevent BCR-induced apoptosis. This is probably due to our suggested role for sphingosine kinase and SPP generation in the basal proliferation of WEHI-231 cells and the induction of apoptosis should we remove this signal. However, if SPP and PI(3)K form part of a signalling pathway which is coupled to ERK-MAPKinase activation following BCR-ligation why does our data suggest that PI(3)K inhibition, unlike ERK-MAPKinase inhibition, is insufficient to block BCR-induced apoptosis? This is suggestive of the activation of multiple ERK-MAPKinase activating pathways following BCR-ligation, such as the classical Ras/Raf/ERK-MAPKinase pathway or activation via PKC (**figure 85**). Indeed, studies have already indicated that PKC inhibition can block BCR-induced ERK-MAPKinase activation in WEHI-231 cells (38). It would be interesting to further

investigate the potential role of multiple ERK-MAPKinase activating pathways following BCR-ligation. In particular it would be interesting to examine whether inhibition of sphingosine kinase blocks the early, BCR-induced, generation of PtdIns(3,4,5)P_3 to examine whether sphingosine kinase lies upstream of PI(3)K to provide further evidence to confirm the above hypothesis.

The ability of CD40 co-stimulation to almost completely block BCR-induced apoptosis suggests that CD40 recruits signalling elements which block BCR-mediated ERK-MAPKinase activation either at ERK-MAPKinase itself or immediately upstream, probably at the level of Ras, the latter of which we have confirmed in earlier studies in this investigation. However, what signalling elements are involved in this process are still unclear. Early studies (results not shown) indicated that the Rap1 protein, a competitor for Raf from Ras is not involved. However, the role of other GTPases have not been examined and warrant further study. In addition, CD40 is known to rescue WEHI-231 cells from BCR-induced apoptosis by increasing the expression of the anti-apoptotic protein Bcl-x_L suggesting that in addition to uncoupling the BCR from the induction of apoptotic signalling pathways, CD40 co-stimulation also directly activates anti-apoptotic signals (83). Indeed, work by our group and others (54) have shown that overexpression of Bcl-x_L in WEHI-231 cells renders these cells resistant to BCR-induced apoptosis. Another recognised anti-apoptotic signal is PKB or Akt, a protein kinase originally discovered as a product of a retrovirus-encoded oncogene that transforms lymphoid cells that is known to lie downstream of PI(3)K activity. Unfortunately, our studies have been unable to either confirm or deny a role for this protein in the rescue of WEHI-231 cells from BCR-induced apoptosis. Although our results suggest CD40 co-stimulation does not induce PtdIns(3,4,5)P_3 generation, Akt is known to bind with higher affinity to another product of PI(3)K activity, namely PtdIns(3,4)P_2 (172). It would therefore be interesting to assay for the generation of this phosphoinositide following CD40 stimulation. In addition, the commercial antibodies Akt, both in our hands and others, are still very non-specific. Therefore the use of new, Akt specific substrates such as cross-tide, may be useful to measure the activity of Akt and help resolve some of the issues surrounding its role in the rescue of immature B lymphocyte from BCR-induced apoptosis.

In addition to examining the early signalling mechanisms initiated following BCR-ligation, we were interested in studying the long-term effects of BCR-ligation, especially with respect to the induction of growth arrest. Early studies indicated that although we could abolish BCR-induced apoptosis by inhibiting the early, BCR-induced ERK-MAPKinase signal, BCR-induced growth arrest remained. This is suggestive of the BCR signalling to two distinct signalling cascades, one linked to the induction of apoptotic programmes and the other linked to the induction of growth arrest (**figure 86**).

Our first observation from long-term studies was the appearance of a basal ERK-MAPKinase activity, which appeared cyclic in nature suggesting that it was intrinsically linked to cell cycle progression. Further studies confirmed that the inhibition of this signal via prolonged exposure to inhibitors of ERK-MAPKinase induced apoptosis. Interestingly, we also observed that BCR-ligation was also effective in the downregulation of this proliferative ERK-MAPKinase signal. Therefore, it became apparent that BCR ligation induces apoptosis via an early, ERK-MAPKinase dependent signal, but also promotes the inhibition of proliferative, basal, ERK-MAPKinase signals. Although this is interesting in its own right, suggesting a differential role for ERK-MAPKinase in immature B lymphocyte biology, the ability of CD40 co-stimulation to modulate both of these responses confirmed this hypothesis. Thus, our studies have shown that CD40 co-stimulation can downregulate the early, BCR-induced ERK-MAPKinase signal (and hence abolish apoptosis) and also re-establish basal ERK-MAPKinase signals downregulated by BCR-ligation (and hence rescue from growth arrest).

The molecular and biochemical mechanisms which regulate the downregulation of ERK-MAPKinase following BCR-ligation were examined. In addition, the pathways which regulate basal ERK-MAPKinase activity were also examined as these signals may give clues as to the mechanisms utilised by BCR-ligation in the induction of growth arrest.

The observation of Ras activity in untreated cells suggested that ERK-MAPKinase activity may be under the control of conventional Ras/Raf/ERK-MAPKinase pathways. However, when compared to the strength of Ras signal generated following CD40 stimulation and also the kinetics of basal ERK-MAPKinase activity, it would appear that other Ras-independent signals are generated which induce ERK-MAPKinase activation.

Our results suggest that sphingosine kinase may play a key role in the basal proliferation of WEHI-231 cells. Not only does sphingosine kinase inhibition induce apoptosis in these cells, but studies have shown that SPP levels, like ERK-MAPKinase, are regulated in a cyclic nature. Indeed, we have shown that the induction of growth arrest following BCR-ligation suppresses long-term basal sphingosine kinase signals. The ability of SPP to activate ERK-MAPKinase is well documented. However, the signalling mechanisms induced by this process are not. Further work to characterise the activation of ERK-MAPKinase by sphingosine kinase must therefore be addressed, especially to determine whether this mechanism is Ras dependent.

We have additional evidence to suggest that PI(3)K may also be involved in the basal proliferation of WEHI-231 cells. Although studies have shown that PtdIns(3,4,5)P_3 generation in untreated WEHI-231 cells does not vary with time, our studies indicate that inhibition of PI(3)K does block basal proliferation. The role of

PI(3)K in the basal proliferation of these cells may therefore result from the generation of PtdIns(3,4)P_2. Generation of this phosphoinositide is known to activate ERK-MAPKinase (150) in a PKC/Raf dependent manner. In addition, recent reports (149, 155) suggest a protein kinase only function for PI(3)K with the ability to activate ERK-MAPKinase. Our results therefore suggest a role for PI(3)K in both BCR-mediated responses and in the basal proliferation of WEHI-231 cells. Subsequently, it appears that BCR stimulated PI(3)K activity is anti-proliferative, whilst basal PI(3)K activity is proliferative in nature. This may reflect differential activation of PI(3)K isoforms. Previously, we have described how PI(3)K may couple the BCR to an early ERK-MAPKinase, apoptotic, signal. As the BCR has previously been shown to be G-protein coupled, it is tempting to speculate that BCR-induced PtdIns(3,4,5)P_3 generation could be coupled to the G-protein sensitive p110γ PI(3)K isoform. Basal PI(3)K activity resulting in PtdIns(3,4,5)P_3 generation may therefore result from p85α dependent PI(3)K activity, as we have shown that BCR-ligation and the induction of growth arrest downregulates p85α protein expression, which is restored with co-stimulation with anti-CD40. Although basal WEHI-231 cells maintained constant levels of PtdIns(3,4,5)P_3, ERK-MAPKinase activity was shown to cycle. This may be coupled the to generation of PtdIns(3,4)P_2 which is known to activate ERK-MAPKinase. This would explain the ability of PI(3)K to inhibit the basal proliferation of WEHI-231 cells. it would therefore be of interest to measure PtdIns(3,4)P_2 generation in basal and anti-Ig stimulated WEHI-231 cells.

Our studies have also indicated that the GTPase, Rap1, was extremely active in basal WEHI-231 cells. Although this would appear to be contradictory, as Rap1 is a known suppressor of Raf-1 activation, recent reports suggest that Rap1 is involved in the long-term activation of ERK-MAPKinase by a B-Raf dependent manner (44, 141). This pathway may therefore play a key role in the proliferative nature of WEHI-231 cells.

In conclusion, our studies suggest that basal ERK-MAPKinase activity in WEHI-231 cells, which is vital for cellular proliferation, may result from multiple signalling cascades, which appear to be both Ras dependent and Ras independent.

Having discovered that both PI(3)K and sphingosine kinase may play key roles in the activation of proliferative, basal, ERK-MAPKinase signals we investigated possible routes employed by BCR-ligation in the suppression of these signals. Firstly, initial studies examining Ras activity confirmed that whilst BCR-ligation abolished ERK-MAPKinase activity, Ras activity was unaffected. Indeed, other studies (**results not shown**) have shown that Grb2 phosphorylation is also unaffected by BCR-ligation. We therefore wanted to examine pathways which inhibit ERK-MAPKinase activity at, or below Raf.

Initial studies ruled out the involvement of Rap1, so we investigated other known suppressors of ERK-MAPKinase activities. However, because evidence provided by ourselves and others (108) have confirmed that ERK-MAPKinase activity can be both cytoplasmic or nuclear in origin in WEHI-231, we have to be aware that these different pools may be coupled to different biological responses. We examined whether the nuclear phosphatase, PAC-1, was involved in the suppression of ERK-MAPKinase by BCR-signalling. Our results suggested that BCR-ligation increased the association of PAC-1 with ERK-MAPKinase. Further studies to confirm that both these molecules were nuclear in location would confirm a role for PAC-1 in BCR-mediated responses. This suggests that basal ERK-MAPKinase activity may be involved the activation of transcription factors and it would therefore be interesting to examine which transcription factors are activated during the basal proliferation of WEHI-231 cells with the previously described ERK-MAPKinase targets of Elk-1 and c-Myc (93) being possible candidates.

Our studies have indicated that BCR-ligation leads to the inhibition of PDE activity. In addition, pharmacological inhibition of PDE activity and elevation of cAMP levels with forskolin appeared to induce moderate levels of growth arrest in WEHI-231 cells. These studies appeared to suggest a role for cAMP and PDE activity in the induction of growth arrest. Additionally, these results appeared to confirm earlier studies which indicated that Ras activity was unaffected by BCR-ligation. This is because cAMP mediated activation of PKA is known to inhibit Raf-1 activity. However, subsequent studies using the PKA inhibitor, H-89, were unsuccessful in reversing BCR-induced growth arrest suggesting that cAMP/PKA pathways were not utilised following BCR-ligation in the suppression of basal ERK-MAPKinase activity. However, in order to rule out the involvement of this pathway it would be interesting to measure cAMP generation and PKA activity following BCR-ligation.

Interestingly, sphingosine kinase activity is known to downregulate cAMP generation (165). Our studies indicate that CD40 co-stimulation can moderately restore long-term decreases in SPP generation (24-48 hours). This could be suggestive of CD40 co-stimulation downregulating cAMP signals through the re-establishment of sphingosine kinase activity, although this requires further study. However, a study by McKenzie *et. al.* (173) has suggested that cAMP mediated growth arrest in fibroblasts is due to the modulation of an intracellular target other than ERK-MAPKinase which results in reduced entry of cells into the S phase of the cell cycle. It would therefore be interesting to examine whether a similar model occurred in WEHI-231 cells.

In addition to examining the signalling events activated by BCR-ligation, studies in this investigation have also explored the role of AA in the induction of apoptosis. AA is generated by the hydrolysis of phospholipids by $cPLA_2$ and previous studies within our group have shown this enzyme to play a role in the induction of apoptosis.

Additionally, we have previously shown that CD40 co-stimulation can reduce BCR-induced AA generation, which has been shown to be due to the uncoupling of the BCR from $cPLA_2$ activity. However, studies in this investigation also suggest that CD40 mediated suppression of AA may also be due to the conversion of AA into PGE_2 which may play a role in the induction of proliferative responses in WEHI-231 cells.

Previous studies within our group have measured AA-induced apoptosis by DNA fragmentation. However, experiments within this investigation have suggested that AA treatment, unlike BCR-ligation, is relatively ineffective in downregulating basal ERK-MAPKinase activity. Subsequent studies therefore examined the DNA content of WEHI-231 cells to examine the affect of AA stimulation on cell cycle progression. These studies indicated increased numbers of cell in both sub-diploid and S-phase pools, results which would appear to confirm earlier observations involving ERK-MAPKinase. It is therefore possible that whilst some of the AA is utilised in the induction of apoptosis, some of the AA may also be metabolised into proliferative lipid second messengers such as PGE_2 by basal cyclooxygenase (COX) activity (**figure 87**). It would therefore be interesting to examine whether cyclooxygenase inhibitors such as indomethacin enhance AA-mediated apoptosis.

The role of PGE_2 in the biology of WEHI-231 cells was also examined. PGE_2 has previously been shown to prevent apoptosis and increase the expression of anti-apoptotic proteins such as Bcl-x_L (122) in human colon cancer cells. In addition, CD40 stimulation has been shown increase COX-2 expression and activity (125). Subsequent studies within this investigation have shown that PGE_2 production is suppressed following BCR-ligation in WEHI-231 cells, but is recovered by co-stimulation with anti-CD40 (**figure 87**). These results suggest a role for COXs in regulating WEHI-231 cell fate and indicate that in addition to suppressing BCR-induced signalling cascades, CD40 co-stimulation mediates some of its protective effects by utilising products of BCR-ligation.

This investigation has highlighted a number of interesting observations in the regulation of immature B lymphocyte cell fate. In particular, it has highlighted the role of the BCR in the induction of apoptosis and growth arrest as quite distinct signalling programmes (**figure 88**). It has also highlighted the role of ERK-MAPKinase as both an inducer of apoptosis and mediator of cell growth which reflects both differences in its mode of activation, but also its intracellular location.

Additionally this study is this first to describe the coupling of the BCR to sphingosine kinase activation, highlighting a role for this lipid kinase in the modulation of immature B lymphocyte cell fate.

Figure 85. BCR-induced apoptosis may involve the activation of ERK-MAPKinase by different routes including a SPP/PI(3)K/Ras/ERK-MAPKinase signal cascade, a PKC-dependent mechanism and perhaps the classical Shc(BLNK)/Ras/Raf/ERK-MAPKinase pathway.

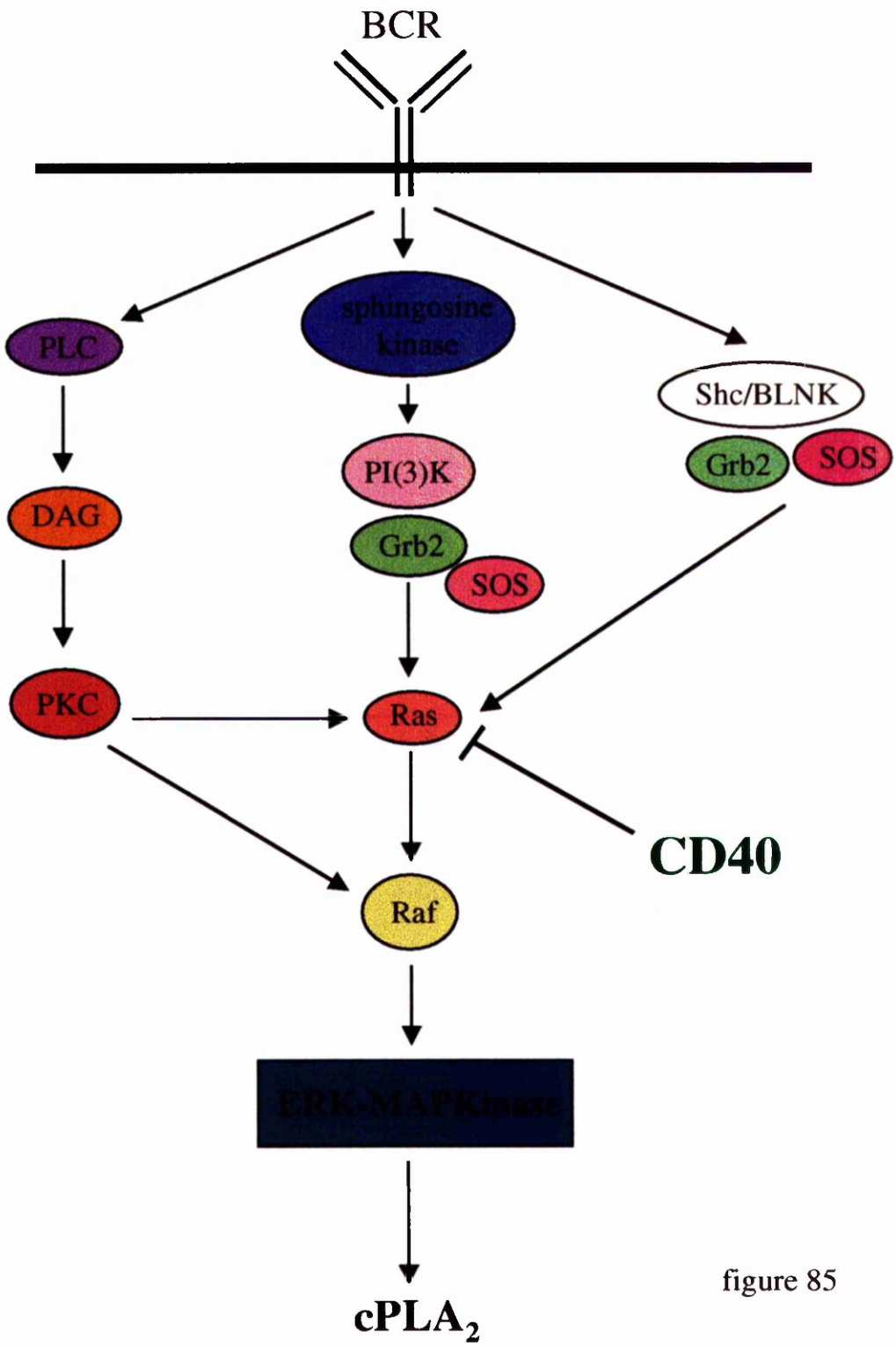
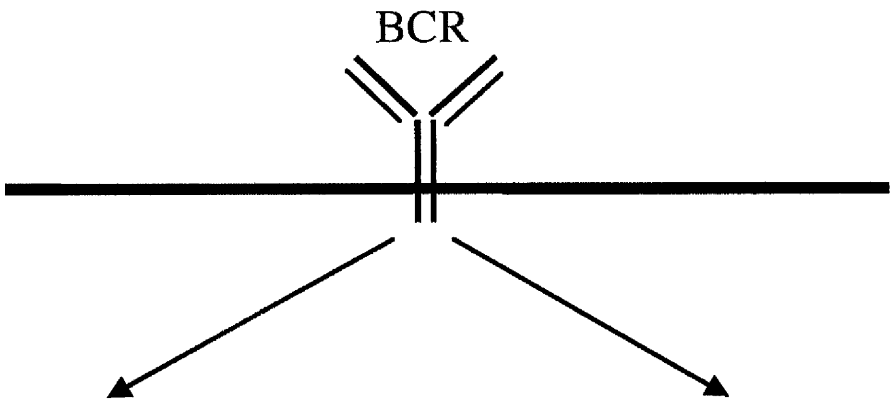

BCR
PLC
sphingosine kinase
Shc/BLNK
PI(3)K
Grb2
SOS
DAG
Grb2
SOS
PKC
Ras
CD40
Raf
ERK-MAPKinase
cPLA2

figure 85

Figure 86. BCR-ligation activates distinct signalling cascades involved in growth arrest and apoptosis.



APOPTOSIS

- cPLA2
- AA generation

GROWTH ARREST

- Inhibition of basal:

ERK-MAPKinase.
PI(3)K.
Sphingosine kinase.
PGE2 production.
PDE activity.

- Activation of:

cAMP generation.

figure 86

Figure 87. Role of AA and PGE2 in the induction of apoptosis and proliferative responses in WEHI-231 cells.

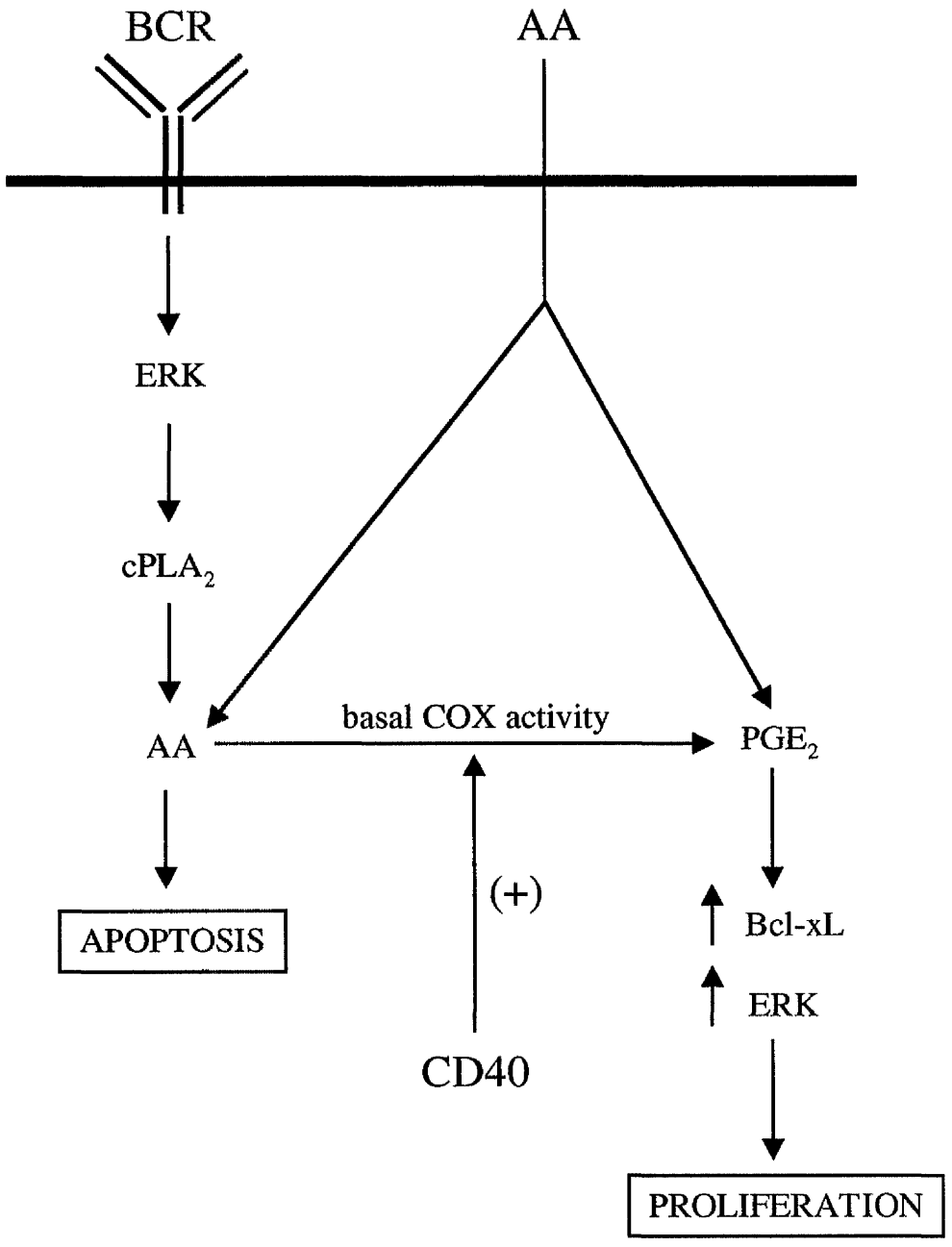

BCR
AA
ERK
cPLA2
AA
basal COX activity
PGE2
APOPTOSIS
(+)
Bcl-xL
ERK
CD40
PROLIFERATION

figure 87

Figure 88. Integrated signalling mechanisms responsible for the induction of apoptosis and growth arrest in WEHI-231 cells.

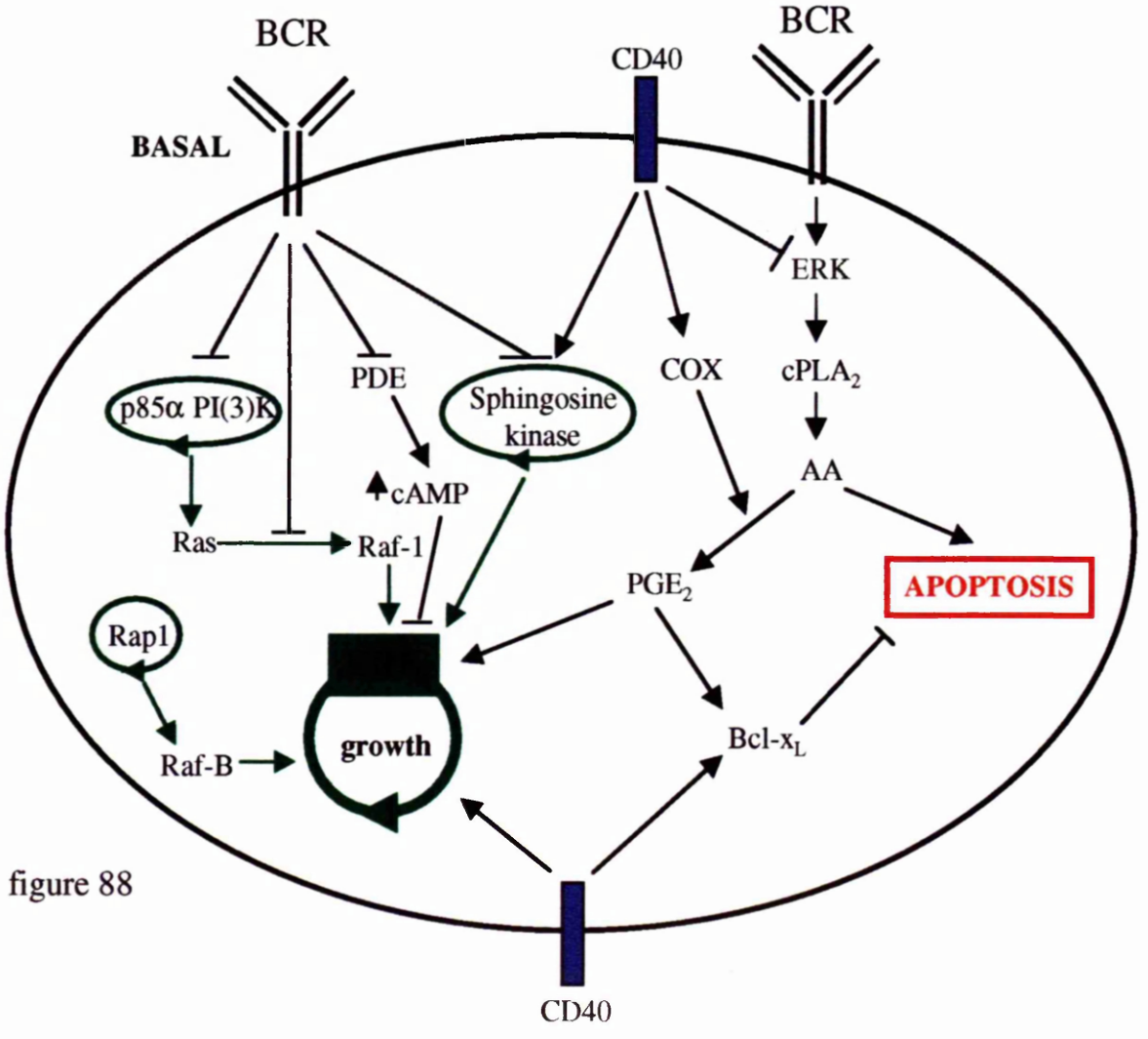

BCR
BASAL
CD40
BCR
ERK
COX
cPLA2
PDE
Sphingosine kinase
p85α PI(3)K
cAMP
AA
Ras
Raf-1
PGE2
APOPTOSIS
Rap1
growth
Raf-B
Bcl-xL
CD40

figure 88

BIBLIOGRAPHY

1. Lydyard, P., and C. Grossi. 1996. Development of the Immune System. In *Immunology*. I. Roitt, J. Brostoff, and D. Male, eds. Dianne Zack, p. 10.1.

2. Cory, S. 1999. Immunology. Wavering on commitment [news; comment]. *nature 401:538.*

3. Tarlinton, D. 1994. B-cell differentiation in the bone marrow and the periphery. *Immunol Rev 137:203.*

4. Cushley, W., and M. M. Harnett. 1993. Cellular signalling mechanisms in B lymphocytes. *Biochem J 292 (Pt 2):313.*

5. Schatz, D. G. 1999. Developing B-cell theories [news; comment]. *Nature 400:614.*

6. Nagata, K., T. Nakamura, F. Kitamura, S. Kuramochi, S. Taki, K. S. Campbell, and H. Karasuyama. 1997. The Ig alpha/Igbeta heterodimer on mu-negative proB cells is competent for transducing signals to induce early B cell differentiation. *Immunity 7:559.*

7. Kurosaki, T. 1999. Genetic analysis of B cell antigen receptor signaling. *Annual Review Of Immunology 17:555.*

8. Monroe, J. G. 2000. Balancing signals for negative selection and activation of developing B lymphocytes. *clin immunol 95:S8.*

9. Cronin, F. E., M. Jiang, A. K. Abbas, and S. A. Grupp. 1998. Role of mu heavy chain in B cell development. I. Blocked B cell maturation but complete allelic exclusion in the absence of Ig alpha/beta. *Journal Of Immunology 161:252.*

10. Benschop, R. J., and J. C. Cambier. 1999. B cell development: signal transduction by antigen receptors and their surrogates. *Current Opinion in Immunology 11:143.*

11. Neuberger, M. S. 1997. Antigen receptor signaling gives lymphocytes a long life [comment]. *Cell 90:971.*

12. LeBien, T. W. 1998. B-cell lymphopoiesis in mouse and man [comment]. *Current Opinion In Immunology 10:188.*

13. Hertz, M., and D. Nemazee. 1998. Receptor editing and commitment in B lymphocytes. *Current Opinion In Immunology 10:208.*

14. Sandel, P. C., and J. G. Monroe. 1999. Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter. *Immunity 10:289.*

15. Loder, F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers, and R. Carsetti. 1999. B cell development in the spleen takes place in discrete

steps and is determined by the quality of B cell receptor-derived signals. *Journal Of Experimental Medicine 190:75.*

16. Brauweiler, A., I. Tamir, J. Dal Porto, R. J. Benschop, C. D. Helgason, R. K. Humphries, J. H. Freed, and J. C. Cambier. 2000. Differential Regulation of B Cell Development, Activation and Death by the Src Homology 2 Domain-containing 5' Inositol Phosphatase (SHIP). *Journal of Experimental Medicine 191:1545.*

17. Craxton, A., K. L. Otipoby, A. Jiang, and E. A. Clark. 1999. Signal transduction pathways that regulate the fate of B lymphocytes. *advances in immunology 73:79.*

18. MacLennan, I. C. 1998. B-cell receptor regulation of peripheral B cells. *Current Opinion In Immunology 10:220.*

19. Wheeler, K., and J. Gordon. 1996. Co-ligation of surface IgM and CD40 on naive B lymphocytes generates a blast population with an ambiguous extrafollicular/germinal centre cell phenotype. *International Immunology 8:815.*

20. O'Connor, L., D. C. S. Huang, L. A. O'Reilly, and A. Strasser. 2000. Apoptosis and cell division. *Current Opinion in Cell Biology 12:257.*

21. Marshall, C. 1999. How do small GTPase signal transduction pathways regulate cell cycle entry? *Current Opinion in Cell Biology 11:732.*

22. Pomerantz, J., N. Schreiber-Agus, N. J. LiÈgeois, A. Silverman, L. Alland, L. Chin, J. Potes, K. Chen, I. Orlow, H. W. Lee, C. Cordon-Cardo, and R. A. DePinho. 1998. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell 92:713.*

23. Wu, M., R. E. Bellas, J. Shen, and G. E. Sonenshein. 1998. Roles of the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p21WAF1/CIP1 in receptor-mediated apoptosis of WEHI 231 B lymphoma cells. *J Exp Med 187:1671.*

24. Guo, M., and B. A. Hay. 1999. Cell proliferation and apoptosis. *Current Opinion in Cell Biology 11:745.*

25. Cosulich, S., and P. Clarke. 1996. Apoptosis: does stress kill? *CURRENT BIOLOGY 6:1586.*

26. Wallach, D., E. E. Varfolomeev, N. L. Malinin, Y. V. Goltsev, A. V. Kovalenko, and M. P. Boldin. 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annual Review Of Immunology 17:331.*

27. Villa, P., S. H. Kaufmann, and W. C. Earnshaw. 1997. Caspases and caspase inhibitors. *TRENDS IN BIOCHEMICAL SCIENCES 22:388.*

28. Zhivotovsky, B., S. Orrenius, O. T. Brustugun, and S. O. Doskeland. 1997. Injected cytchrome c induces apoptosis. *Nature 391:449.*

29. Hengartner, M. O. 1998. Apoptosis. Death cycle and Swiss army knives [news; comment]. *Nature 391:441.*

30. Porter, A. G. 1999. Protein translocation in apoptosis. *trends cell biol 9:394.*

31. Jacobson, M. D. 1997. Apoptosis: Bcl-2-related proteins get connected. *CURRENT BIOLOGY 7:R277.*

32. Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M. E. Greenberg. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell 91:231.*

33. Franke, T. F., and L. C. Cantley. 1997. Apoptosis. A Bad kinase makes good [news]. In *Nature*, Vol. 390, ENGLAND, p. 116.

34. Pao, L., A. M. Carbone, and J. C. Cambier. 1997. Antigen Receptor Structure and Signaling in B cells. In *Lymphocyte signalling*. H. M. M., and K. P. Rigley, eds. John Wiley & Sons.

35. Reth, M. 1995. The B-cell antigen receptor complex and co-receptors. *Immunol Today 16:310.*

36. Campbell, K. S. 1999. Signal transduction from the B cell antigen-receptor. *Current Opinion In Immunology 11:256.*

37. Kurosaki, T. 1997. Molecular mechanisms in B cell antigen receptor signaling. *Curr Opin Immunol 9:309.*

38. DeFranco, A. L. 1997. The complexity of signaling pathways activated by the BCR. *Curr Opin Immunol 9:296.*

39. Deehan, M. R., G. G. Klaus, M. J. Holman, W. Harnett, and M. M. Harnett. 1998. MAPkinase: a second site of G-protein regulation of B-cell activation via the antigen receptors. *Immunology 95:169.*

40. Lee, J. R., and G. A. Koretzky. 1998. Extracellular signal-regulated kinase-2, but not c-Jun NH2-terminal kinase, activation correlates with surface IgM-mediated apoptosis in the WEHI 231 B cell line. *J Immunol 161:1637.*

41. Kelly, M. E., and A. C. Chan. 2000. Regulation of B cell function by linker proteins. *Current Opinion in Immunology 12:267.*

42. York, R. D., H. Yao, T. Dillon, C. L. Ellig, S. P. Eckert, E. W. McCleskey, and P. J. Stork. 1998. Rap1 mediates sustained MAP kinase activation induced by nerve growth factor [see comments]. *Nature 392:622.*

43. Blaukat, A., I. Ivankovic-Dikic, E. Grönroos, F. Dolfi, G. Tokiwa, K. Vuori, and I. Dikic. 1999. Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. *Journal Of Biological Chemistry 274:14893.*

44. McLeod, S. J., R. J. Ingham, J. L. Bos, T. Kurosaki, and M. R. Gold. 1998. Activation of the Rap1 GTPase by the B cell antigen receptor. *Journal of Biological Chemistry 273:29218.*

45. Berridge, M. J., and R. F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature 341:197.*

46. Ishiai, M., M. Kurosaki, R. Pappu, K. Okawa, I. Ronko, C. Fu, M. Shibata, A. Iwamatsu, A. C. Chan, and T. Kurosaki. 1999. BLNK required for coupling Syk to PLC gamma 2 and Rac1-JNK in B cells. *Immunity 10:117.*

47. Benschop, R. J., D. Melamed, D. Nemazee, and J. C. Cambier. 1999. Distinct signal thresholds for the unique antigen receptor-linked gene expression programs in mature and immature B cells. *Journal of Experimental Medicine 190:749.*

48. Mellor, H., and P. P.J. 1998. The extended protein kinase C superfamily. *Biochemical Journal 332:281.*

49. King, L. B., A. Norvell, and J. G. Monroe. 1999. Antigen Receptor-Induced Signal Transduction Imbalances Associated with the Negative Selection of Immature B Cells. *Journal of Immunology 162:2655.*

50. Monroe, J. G. 1997. Molecular mechanisms regulating B-cell negative selection. *Biochemical Society Transactions 25:643.*

51. Carman, J. A., R. J. Wechsler-Reya, and J. G. Monroe. 1996. Immature stage B cells enter but do not progress beyond the early G1 phase of the cell cycle in response to antigen receptor signaling. *J Immunol 156:4562.*

52. Gilbert, J. J., T. R. Pettitt, S. D. Seatter, S. D. Reid, M. J. Wakelam, and M. M. Harnett. 1998. Antagonistic roles for phospholipase D activities in B cell signaling: while the antigen receptors transduce mitogenic signals via a novel phospholipase D activity, phosphatidylcholine-phospholipase D mediates antiproliferative signals. *Journal Of Immunology 161:6575.*

53. Gilbert, J. J., A. Stewart, C. A. Courtney, M. C. Fleming, P. Reid, C. G. Jackson, A. Wise, M. J. Wakelam, and M. M. Harnett. 1996. Antigen receptors on immature, but not mature, B and T cells are coupled to cytosolic phospholipase A2 activation: expression and activation of cytosolic phospholipase A2 correlate with lymphocyte maturation. *J Immunol 156:2054.*

54. Wiesner, D. A., J. P. Kilkus, A. R. Gottschalk, J. Quintáns, and G. Dawson. 1997. Anti-immunoglobulin-induced apoptosis in WEHI 231 cells involves the slow formation of ceramide from sphingomyelin and is blocked by bcl-XL. *Journal Of Biological Chemistry 272:9868.*

55. Seatter, S. D., J. J. Gilbert, and M. M. Harnett. 1997. Lipid signalling in lymphocyte activation and cell death. *Biochemical Society Transactions 25:300S.*

56. Ballou, L. R., S. J. Laulederkind, E. F. Rosloniec, and R. Raghow. 1996. Ceramide signalling and the immune response. *Biochim Biophys Acta 1301:273.*

57. Healy, J. I., and C. C. Goodnow. 1998. Positive versus negative signaling by lymphocyte antigen receptors. *Annu Rev Immunol 16:645.*

58. Norvell, A., and J. G. Monroe. 1996. Acquisition of surface IgD fails to protect from tolerance-induction. Both surface IgM- and surface IgD-mediated signals induce apoptosis of immature murine B lymphocytes. *Journal Of Immunology 156:1328.*

59. Cheng, P. C., M. L. Dykstra, R. N. Mitchell, and S. K. Pierce. 1999. A Role for Lipid Rafts in B Cell Antigen Receptor Signaling and Antigen Targeting. *Journal of Experimental Medicine 190:1549.*

60. O'Rourke, L., R. Tooze, and D. T. Fearon. 1997. Co-receptors of B lymphocytes. *CURRENT OPINION IN IMMUNOLOGY 9:324.*

61. Fujimoto, M., J. C. Poe, P. J. Jansen, S. Sato, and T. F. Tedder. 1999. CD19 amplifies B lymphocyte signal transduction by regulating Src-family protein tyrosine kinase activation. *Journal Of Immunology 162:7088.*

62. Li, X., and R. H. Carter. 1998. Convergence of CD19 and B cell antigen receptor signals at MEK1 in the ERK2 activation cascade. *Journal Of Immunology 161:5901.*

63. Venkataraman, C., P. J. Lu, A. M. Buhl, C. S. Chen, J. C. Cambier, and S. Bondada. 1998. CD72-mediated B cell activation involves recruitment of CD19 and activation of phosphatidylinositol 3-kinase. *European Journal Of Immunology 28:3003.*

64. Pezzuto, A., P. S. Rabinovitch, B. Dorken, G. Moldenhauer, and E. A. Clark. 1988. Role of the CD22 human B cell antigen in B cell triggering by anti-immunoglobulin. *Journal of Immunology 140:1791.*

65. Tooze, R. M., G. M. Doody, and D. T. Fearon. 1997. Counterregulation by the coreceptors CD19 and CD22 of MAP kinase activation by membrane immunoglobulin. *Immunity 7:59.*

66. Greer, S. F., and L. B. Justement. 1999. CD45 regulates tyrosine phosphorylation of CD22 and its association with the protein tyrosine phosphatase SHP-1. *Journal Of Immunology 162:5278.*

67. Bolland, S., R. N. Pearse, T. Kurosaki, and J. V. Ravetch. 1998. SHIP modulates immune receptor responses by regulating membrane association of Btk. *Immunity 8:509.*

68. Tamir, I., J. C. Stolpa, C. D. Helgason, K. Nakamura, P. Bruhns, M. Daeron, and J. C. Cambier. 2000. The RasGAP-binding protein p62dok is a mediator of inhibitory FcgammaRIIB signals in B cells. *immunity 12:347.*

69. Kiener, P. A., M. N. Lioubin, L. R. Rohrschneider, J. A. Ledbetter, S. G. Nadler, and M. L. Diegel. 1997. Co-ligation of the antigen and Fc receptors gives rise to the selective modulation of intracellular signaling in B cells. Regulation of the association of phosphatidylinositol 3-kinase and inositol 5'-phosphatase with the antigen receptor complex. *J Biol Chem 272:3838.*

70. Durie, F. H., T. M. Foy, S. R. Masters, J. D. Laman, and R. J. Noelle. 1994. The role of CD40 in the regulation of humoral and cell-mediated immunity. *IMMUNOLOGY TODAY 15:406.*

71. Costello, R. T., J.-A. Gaustaut, and D. Olive. 1999. What is the real role of CD40 in cancer immunotherapy ? *Immunology Today.*

72. van Kooten, C., and J. Banchereau. 1997. Functions of CD40 on B cells, dendritic cells and other cells. *Curr Opin Immunol 9:330.*

73. Kehry, M. R. 1996. CD40-mediated signaling in B cells. Balancing cell survival, growth, and death. *J Immunol 156:2345.*

74. Gray, D. 1997. CD40 Signalling in T-dependent B Cell Responses. In *Lymphocyte Signalling: Mechansims, Subversion and Manipulation.* M. M. Harnett, and K. P. Rigley, eds. John Wiley & Sons Ltd., p. 77.

75. Grewal, I. S., and R. A. Flavell. 1998. CD40 and CD154 in cell-mediated immunity. *annual review of immunology 16:111.*

76. Hasbold, J., C. Johnson-Léger, C. J. Atkins, E. A. Clark, and G. G. Klaus. 1994. Properties of mouse CD40: cellular distribution of CD40 and B cell activation by monoclonal anti-mouse CD40 antibodies. *EUROPEAN JOURNAL OF IMMUNOLOGY 24:1835.*

77. Faris, M., F. Gaskin, J. T. Parsons, and S. Man Fu. 1994. CD40 Signaling Pathway: Anti-CD40 Monoclonal Antibody Induces Rapid Dephosphorylation and Phosphorylation of tyrosine-phosphorylated Proteins Including Protein tyrosine Kinase Lyn, Fyn, and Syk and the Appearance of a 28-kD tyrosine Phosphorylated Protein. *Journal of Experimental Medicine 179:1923.*

78. Santos-Argumedo, L., J. Gordon, A. W. Heath, and M. Howard. 1994. Antibodies to murine CD40 protect normal and malignant B cells from induced growth arrest. *cellular immunology 156:272.*

79. Wong, B., J. Arron, and Y. Choi. 1997. T cell receptor signals enhance susceptibility to Fas-mediated apoptosis. *J Exp Med 186:1939.*

80. Scott, D. W., T. Grdina, and Y. Shi. 1996. T cells commit suicide, but B cells are murdered! *Journal Of Immunology 156:2352.*

81. Nossal, G. J. 1992. Cellular and molecular mechanisms of B lymphocyte tolerance. *advances in immunology 52:283.*

82. Benhamou, L. E., T. Watanabe, D. Kitamura, P. A. Cazenave, and P. Sarthou. 1994. Signaling properties of anti-immunoglobulin--resistant variants of WEHI-231 B lymphoma cells. *EUROPEAN JOURNAL OF IMMUNOLOGY 24:1993.*

83. Choi, M. S., L. H. Boise, A. R. Gottschalk, J. Quintans, C. B. Thompson, and G. G. Klaus. 1995. The role of bcl-XL in CD40-mediated rescue from anti-mu-induced apoptosis in WEHI-231 B lymphoma cells. *Eur J Immunol 25:1352.*

84. Schauer, S. L., Z. Wang, G. E. Sonenshein, and T. L. Rothstein. 1996. Maintenance of nuclear factor-kappa B/Rel and c-myc expression during CD40 ligand rescue of WEHI 231 early B cells from receptor-mediated apoptosis through modulation of I kappa B proteins. *JOURNAL OF IMMUNOLOGY 157:81.*

85. Baichwal, V. R., and P. A. Baeuerle. 1997. Activate NF-kappa B or die? *CURRENT BIOLOGY 7:R94.*

86. Bras, A., A. Ruiz-vela, G. Gonlalez de Buitrago, and C. Martinez-A. 1999. Caspase activation by BCR cross-linking in immature B cells: differential effects on growth arrest and apoptosis. *The FASEB Journal 13:931.*

87. Marshall-Clarke, S., G. Owen, and L. Tasker. 1996. Ligation of CD40 with soluble CD40 ligand reverses anti-immunoglobulin-mediated negative signalling in murine B lymphoma cell lines but not in immature B cells from neonatal mice. *Immunology 87:624.*

88. Hasbold, J., and G. G. B. Klaus. 1990. Anti-immunoglobulin antibodies induce apoptosis in immature B cell lymphomas. *European Journal of Immunology 20:1685.*

89. Carsetti, R., G. Kohler, and M. C. Lamers. 1995. Transitional B cells are the target of negative selection in the B cell compartment. *Journal of Experimental Medicine 181:2129.*

90. Sumimoto, S., T. Heike, S. Kanazashi, N. Shintaku, E. Y. Jung, D. Hata, K. Katamura, and M. Mayumi. 1994. Involvement of LFA-1/intracellular adhesion molecule-1-dependent cell adhesion in CD40-mediated inhibition of human B lymphoma cell death induced by surface IgM crosslinking. *Journal Of Immunology 153:2488.*

91. Dhanasekaran, N., and E. Premkumar Reddy. 1998. Signaling by dual specificity kinases. *Oncogene 17:1447.*

92. Madhani, H. D., and G. R. Fink. 1998. The riddle of MAP kinase signaling specificity. *Trends Genet 14:151.*

93. Sutherland, C. L., A. W. Heath, S. L. Pelech, P. R. Young, and M. R. Gold. 1996. Differential activation of the ERK, JNK, and p38 mitogen-activated protein kinases by CD40 and the B cell antigen receptor. *JOURNAL OF IMMUNOLOGY 157:3381.*

94. Jarvis, W. D., J. r. Fornari FA, K. L. Auer, A. J. Freemerman, E. Szabo, M. J. Birrer, C. R. Johnson, S. E. Barbour, P. Dent, and S. Grant. 1997. Coordinate regulation of stress- and mitogen-activated protein kinases in the apoptotic actions of ceramide and sphingosine. *Mol Pharmacol 52:935.*

95. Elion, E. A. 1998. Routing MAP kinase cascades [comment]. *SCIENCE 281:1625.*

96. Henning, S. W., and D. A. Cantrell. 1998. GTPases in antigen receptor signalling. *Current Opinion in Immunology 10:322.*

97. Purkerson, J. M., and D. C. Parker. 1998. Differential coupling of membrane Ig and CD40 to the extracellularly regulated kinase signaling pathway. *J Immunol 160:2121.*

98. Jiang, A., A. Craxton, T. Kurosaki, and E. A. Clark. 1998. Different protein tyrosine kinases are required for B cell antigen receptor-mediated activation of extracellular signal-regulated kinase, c-Jun NH2-terminal kinase 1, and p38 mitogen-activated protein kinase. *Journal Of Experimental Medicine 188:1297.*

99. Graves, J. D., K. E. Draves, A. Craxton, J. Saklatvala, E. G. Krebs, and E. A. Clark. 1996. Involvement of stress-activated protein kinase and p38 mitogen-activated protein kinase in mIgM-induced apoptosis of human B lymphocytes. *Proc Natl Acad Sci U S A 93:13814.*

100. Berberich, I., G. Shu, F. Siebelt, J. R. Woodgett, J. M. Kyriakis, and E. A. Clark. 1996. Cross-linking CD40 on B cells preferentially induces stress-activated protein kinases rather than mitogen-activated protein kinases. *EMBO JOURNAL 15:92.*

101. Kashiwada, M., Y. Kaneko, H. Yagita, K. Okumura, and T. Takemori. 1996. Activation of mitogen-activated protein kinases via CD40 is distinct from that stimulated by surface IgM on B cells. *Eur J Immunol 26:1451.*

102. Salmon, R. A., I. N. Foltz, P. R. Young, and J. W. Schrader. 1997. The p38 mitogen-activated protein kinase is activated by ligation of the T or B lymphocyte antigen receptors, Fas or CD40, but suppression of kinase activity does not inhibit apoptosis induced by antigen receptors. *JOURNAL OF IMMUNOLOGY 159:5309.*

103. van den Brink, M. R., R. Kapeller, J. C. Pratt, J. H. Chang, and S. J. Burakoff. 1999. The extracellular signal-regulated kinase pathway is required for activation-induced cell death of T cells. *Journal Of Biological Chemistry 274:11178.*

104. Zhu, L., X. Yu, Y. Akatsuka, J. A. Cooper, and C. Anasetti. 1999. Role of mitogen-activated protein kinases in activation-induced apoptosis of T cells. *immunology 97:26.*

105. Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *SCIENCE 270:1326.*

106. Jäättelä, M., M. Benedict, M. Tewari, J. A. Shayman, and V. M. Dixit. 1995. Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *ONCOGENE 10:2297.*

107. Craxton, A., P. I. Chuang, G. Shu, J. M. Harlan, and E. A. Clark. 2000. The CD40-Inducible Bcl-2 Family Member A1 Protects B Cells from Antigen receptor-Mediated Apoptosis. *Cellular Immunology 200:56.*

108. Shirakata, Y., K. Ishii, H. Yagita, K. Okumura, M. Taniguchi, and T. Takemori. 1999. Distinct Subcellular Localisation and Substrate Specificity of Extracellular Signal-regulated Kinase in B cells upon Stimulation with IgM and CD40. *Journal of Immunology 163:6589.*

109. Murakami, M., S. Shimbara, T. Kambe, H. Kuwata, M. V. Winstead, J. A. Tischfield, and I. Kudo. 1998. The functions of five distinct mammalian phospholipase A2S in regulating arachidonic acid release. Type IIa and type V secretory phospholipase A2S are functionally redundant and act in concert with cytosolic phospholipase A2. *Journal Of Biological Chemistry 273:14411.*

110. Leslie, C. C. 1997. Properties and regulation of cytosolic phospholipase A2. *Journal Of Biological Chemistry 272:16709.*

111. Heller, R., and M. Kronke. 1994. Tumor Necrosis Factor Receptor-mediated Signaling Pathways. *Journal of Cell Biology 126:5.*

112. Hirabayashi, T., K. Kume, K. Hirose, T. Yokomizo, M. Iino, H. Itoh, and T. Shimizu. 1999. Critical duration of intracellular Ca2+ response required for continuous translocation and activation of cytosolic phospholipase A2. *journal of biological chemistry 274:5163.*

113. Hernandez, M., S. L. Burillo, M. S. Crespo, and M. L. Nieto. 1998. Secretory phospholipase A2 activates the cascade of mitogen-activated protein kinases and cytosolic phospholipase A2 in the human astrocytoma cell line 1321N1. *J Biol Chem 273:606.*

114. Pinelli, E., N. Poux, L. Garren, B. Pipy, M. Castegnaro, D. J. Miller, and A. Pfohl-Leszkowicz. 1999. Activation of mitogen-activated protein kinase by fumonisin B(1) stimulates cPLA(2) phosphorylation, the arachidonic acid cascade and cAMP production. *Carcinogenesis 20:1683.*

115. Palombella, V. J., and J. Vilek. 1989. Mitogenic and cytotoxic actions of tumor necrosis factor in BALB/c 3T3 cells. Role of phospholipase activation. *JOURNAL OF BIOLOGICAL CHEMISTRY 264:18128.*

116. Zhang, Y., B. Yao, S. Delikat, S. Bayoumy, X. H. Lin, S. Basu, M. McGinley, P. Y. Chan-Hui, H. Lichenstein, and R. Kolesnick. 1997. Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell 89:63.*

117. Yao, B., Y. Zhang, S. Delikat, S. Mathias, S. Basu, and R. Kolesnick. 1995. Phosphorylation of Raf by ceramide-activated protein kinase. *Nature 378:307.*

118. Atsumi, G., M. Tajima, A. Hadano, Y. Nakatani, M. Murakami, and I. Kudo. 1998. Fas-induced arachidonic acid release is mediated by Ca2+-independent phospholipase A2 but not cytosolic phospholipase A2, which undergoes proteolytic inactivation. *J Biol Chem 273:13870.*

119. Beyaert, R., and W. Fiers. 1994. Molecular mechanisms of tumor necrosis factor-induced cytotoxicity. What we understand and what we do not. *FEBS Letters 340:9.*

120. Marnett, L. J., S. W. Rowlinson, D. C. Goodwin, A. S. Kalgutkar, and C. A. Lanzo. 1999. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *Journal Of Biological Chemistry 274:22903.*

121. Morteau, O. 1999. COX-2: promoting tolerance [news; comment]. *Nature Medicine 5:867.*

122. Sheng, H., J. Shao, J. D. Morrow, R. D. Beauchamp, and R. N. DuBois. 1998. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *CANCER RESEARCH 58:362.*

123. Vane, J. R., Y. S. Bakhle, and R. M. Botting. 1998. Cyclooxygenases 1 and 2. *Annual Review Of Pharmacology And Toxicology 38:97.*

124. Barry, O. P., M. G. Kazanietz, D. Pratico, and G. A. FitzGerald. 1999. Arachidonic acid in platelet microparticles up-regulates cyclooxygenase-2-dependent prostaglandin formation via a protein kinase C/mitogen-activated protein kinase-dependent pathway. *journal of biological chemistry 274:7545.*

125. Zhang, Y., H. J. Cao, B. Graf, H. Meekins, T. J. Smith, and R. P. Phipps. 1998. CD40 engagement up-regulates cyclooxygenase-2 expression and prostaglandin E2 production in human lung fibroblasts. *J Immunol 160:1053.*

126. Garrone, P., L. Galibert, F. Rousset, S. M. Fu, and J. Banchereau. 1994. Regulatory effects of prostaglandin E2 on the growth and differentiation of human B lymphocytes activated through their CD40 antigen. *Journal Of Immunology 152:4282.*

127. Rang, H. P., and M. M. Dale. 1991. Local Hormones, Inflammation and Allergy. Churchill Livingston, London, p. 243.

128. Yeung, K., T. Seitz, S. Li, P. Janosch, B. McFerran, C. Kaiser, F. Fee, K. D. Katsanakis, D. W. Rose, H. Mischak, J. M. Sedivy, and W. Kolch. 1999. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature 401:173.*

129. Neel, B. G. 1997. Role of phosphatases in lymphocyte activation. *CURRENT OPINION IN IMMUNOLOGY 9:405.*

130. Yakura, H. 1998. Phosphatases and kinases in lymphocyte signaling. *Immunol Today 19:198.*

131. Alexander, D. R. 2000. The CD45 tyrosine phosphatase: a positive and negative regulator of immune cell function. *Seminars in Immunology 12:349.*

132. Pani, G., K. A. Siminovitch, and C. J. Paige. 1997. The motheaten mutation rescues B cell signaling and development in CD45-deficient mice. *J Exp Med 186:581.*

133. Dustin, L. B., D. R. Plas, J. Wong, Y. T. Hu, C. Soto, A. C. Chan, and M. L. Thomas. 1999. Expression of dominant-negative src-homology domain 2-containing protein tyrosine phosphatase-1 results in increased Syk tyrosine kinase activity and B cell activation. *Journal Of Immunology 162:2717.*

134. Fujimoto, M., A. P. Bradney, J. C. Poe, D. A. Steeber, and T. F. Tedder. 1999. Modulation of B lymphocyte antigen receptor signal transduction by a CD19/CD22 regulatory loop. *immunity 11:191.*

135. Cook, S. J., J. Beltman, K. A. Cadwallader, M. McMahon, and F. McCormick. 1997. Regulation of mitogen-activated protein kinase phosphatase-1 expression by extracellular signal-related kinase-dependent and Ca2+-dependent signal pathways in Rat-1 cells. *Journal Of Biological Chemistry 272:13309.*

136. Rohan, P. J., P. Davis, C. A. Moskaluk, M. Kearns, H. Krutzsch, U. Siebenlist, and K. Kelly. 1993. PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. *Science 259:1763.*

137. Ward, Y., S. Gupta, P. Jensen, M. Wartmann, R. J. Davis, and K. Kelly. 1994. Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. *Nature 367:651.*

138. Grumont, R. J., J. E. J. Rasko, A. Strasser, and S. Gerondakis. 1996. Activation of the Mitogen-Activated Protein Kinase Pathway Induces Transcription of the PAC-1 Phosphatase Gene. *Molecular and Cellular Biology 16:2913.*

139. Chu, Y., P. A. Solski, R. Khosravi-Far, C. J. Der, and K. Kelly. 1996. The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. *Journal Of Biological Chemistry 271:6497.*

140. Bos, J. L. 1997. Ras-like GTPases. *Biochim Biophys Acta 1333:M19.*

141. Marshall, C. J. 1998. Signal transduction. Taking the Rap [news; comment]. In *Nature*, Vol. 392, ENGLAND, p. 553.

142. Vossler, M. R., H. Yao, R. D. York, M. G. Pan, C. S. Rim, and P. J. Stork. 1997. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell 89:73.*

143. Michie, A. M., M. M. Harnett, and M. D. Houslay. 1997. Cyclic Nucleotide Signalling Throughout T Cell Maturation. In *Lymphocyte Signalling: Mechanisms, Subversion and Manipulation.* M. M. Harnett, and K. P. Rigley, eds. John Wiley and Sons., p. 247.

144. Kelly, K., and K. A. Knox. 1995. Differential regulatory effects of cAMP-elevating agents on human normal and neoplastic B cell functional response following ligation of surface immunoglobulin and CD40. *Cellular Immunology 166:93.*

145. Goldstein, M. D., A. Cochrane, and T. H. Watts. 1997. Cyclic-AMP modulates downstream events in CD40-mediated signal transduction, but inhibition of protein kinase A has no direct effect on CD40 signaling. *J Immunol 159:5871.*

146. Liu, H., and D. H. Maurice. 1999. Phosphorylation-mediated activation and translocation of the cyclic AMP-specific phosphodiesterase PDE4D3 by cyclic AMP-dependent protein kinase and mitogen-activated protein kinases. A potential mechanism allowing for the coordinated regulation of PDE4D activity and targeting. *Journal Of Biological Chemistry 274:10557.*

147. Spiegel, S., D. Foster, and R. Kolesnick. 1996. Signal transduction through lipid second messengers. *Curr Opin Cell Biol 8:159.*

148. Alberola-Ila, J., S. Takaki, J. D. Kerner, and R. M. Perlmutter. 1997. Differential signaling by lymphocyte antigen receptors. *Annual Review Of Immunology 15:125.*

149. Wymann, M. P., and L. Pirola. 1998. Structure and function of phosphoinositide 3-kinases. *Biochimica Et Biophysica Acta 1436:127.*

150. Toker, A., and L. C. Cantley. 1997. Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature 387:673.*

151. Walker, E. H., O. Perisic, C. Ried, L. Stephens, and R. L. Williams. 1999. Structural insights into phosphoinostide 3-kinase catalysis ans signalling. *Nature 402:313.*

152. Marte, B. M., and J. Downward. 1997. PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem Sci 22:355.*

153. Burow, M. E., C. B. Weldon, B. M. Collins-Burow, N. Ramsey, A. McKee, A. Klippel, J. A. McLachlan, S. Clejan, and B. S. Beckman. 2000. Cross-talk between Phosphatidylinositol 3-Kinase and Sphingomyelinase Pathways as a mechanism for Cell Survival/Death Decisions. *Journal of Biological Chemistry 275:9628.*

154. Lopez-Ilasaca, M., P. Crespo, P. G. Pellici, J. S. Gutkind, and R. Wetzker. 1997. Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science 275:394.*

155. Bondeva, T., L. Pirola, G. Bulgarelli-Leva, I. Rubio, R. Wetzker, and M. P. Wymann. 1998. Bifurcation of lipid and protein kinase signals of PI3Kgamma to the protein kinases PKB and MAPK. *Science 282:293.*

156. Aagaard-Tillery, K. M., and D. F. Jelinek. 1996. Phosphatidylinositol 3-kinase activation in normal human B lymphocytes. *J Immunol 156:4543.*

157. Suzuki, H., Y. Terauchi, M. Fujiwara, S. Aizawa, Y. Yazaki, T. Kadowaki, and S. Koyasu. 1999. Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. *Science 283:390.*

158. Ariga, T., W. D. Jarvis, and R. K. Yu. 1998. Role of sphingolipid-mediated cell death in neurodegenerative diseases. *JOURNAL OF LIPID RESEARCH 39:1.*

159. Pyne, S., D. G. Tolan, A. M. Conway, and N. Pyne. 1997. Sphingolipids as differential regulators of cellular signalling processes. *Biochemical Society Transactions 25:549.*

160. Kohama, T., A. Olivera, L. Edsall, M. M. Nagiec, R. Dickson, and S. Spiegel. 1998. Molecular cloning and functional characterization of murine sphingosine kinase. *Journal Of Biological Chemistry 273:23722.*

161. Gómez-Muñoz, A., D. W. Waggoner, L. O'Brien, and D. N. Brindley. 1995. Interaction of ceramides, sphingosine, and sphingosine 1-phosphate in regulating DNA synthesis and phospholipase D activity. *Journal Of Biological Chemistry 270:26318.*

162. Cuvillier, O., G. Pirianov, B. Kleuser, P. G. Vanek, O. A. Coso, S. Gutkind, and S. Spiegel. 1996. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature 381:800.*

163. Waggoner, D. W., J. Xu, I. Singh, R. Jasinska, Q. X. Zhang, and D. N. Brindley. 1999. Structural organization of mammalian lipid phosphate phosphatases: implications for signal transduction. *biochimica et biophysica acta 1439:299.*

164. Rakhit, S., A. M. Conway, R. Tate, T. Bower, N. J. Pyne, and S. Pyne. 1999. Sphingosine 1-phosphate stimulation of the p42/p44 mitogen-activated protein

kinase pathway in airway smooth muscle. Role of endothelial differentiation gene 1, c-Src tyrosine kinase and phosphoinositide 3-kinase. *Biochemical Journal 338 (Pt 3):643.*

165. Pyne, S., and N. Pyne. 2000. Sphingosine 1-phosphate signalling in mammalian cells. *Biochemical Journal 349:385.*

166. Goetzl, E. J., Y. Kong, and B. Mei. 1999. Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. *Journal Of Immunology 162:2049.*

167. Duckworth, B. C., and L. C. Cantley. 1997. Conditional inhibition of the mitogen-activated protein kinase cascade by wortmannin. Dependence on signal strength. *Journal Of Biological Chemistry 272:27665.*

168. Meier, R., D. R. Alessi, P. Cron, M. Andjelkovic, and B. A. Hemmings. 1997. Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta. *journal of biological chemistry 272:30491.*

169. Melendez, A., R. A. Floto, D. J. Gillooly, M. M. Harnett, and J. M. Allen. 1998. FcgammaRI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. *JOURNAL OF BIOLOGICAL CHEMISTRY 273:9393.*

170. Edsall, L. C., J. R. Van Brocklyn, O. Cuvillier, B. Kleuser, and S. Spiegel. 1998. N,N-Dimethylsphingosine is a potent competitive inhibitor of sphingosine kinase but not of protein kinase C: modulation of cellular levels of sphingosine 1-phosphate and ceramide. *Biochemistry 37:12892.*

171. Knaus, U. G., and G. M. Bokoch. 1998. The p21Rac/Cdc42-activated kinases (PAKs). *International Journal Of Biochemistry And Cell Biology 30:857.*

172. Cantley, L. C., and B. G. Neel. 1999. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proceedings Of The National Academy Of Sciences Of The United States Of America 96:4240.*

173. McKenzie, F. R., and J. Pouysségur. 1996. cAMP-mediated growth inhibition in fibroblasts is not mediated via mitogen-activated protein (MAP) kinase (ERK) inhibition. cAMP-dependent protein kinase induces a temporal shift in growth factor-stimulated MAP kinases. *Journal Of Biological Chemistry 271:13476.*

1. Su, L., and M. David. 1999. Inhibition of B cell receptor-mediated apoptosis by IFN. *Journal Of Immunology 162:6317.*
2. Gold, M. R., M. P. Scheid, L. Santos, M. Dang-Lawson, R. A. Roth, L. Matsuuchi, V. Duronio, and D. L. Krebs. 1999. The B cell antigen receptor activates the Akt (protein kinase B)/glycogen synthase kinase-3 signaling pathway via phosphatidylinositol 3-kinase. *Journal ofImmunology 163:1894.*