FcγRIIb: Signalling Aspects and Implications for Autoimmune Disease

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To my parents;

for proving there is such a thing as a happy ending.

For yesterday is but a dream,
And tomorrow is only a vision;
But today well lived
Makes every yesterday a dream of happiness,
And every tomorrow a vision of hope.
Look well, therefore, to this day.

SANSKRIT PROVERB

Declaration

I declare that the following thesis embodies the results of my own special work, that it has been composed by myself and that it does not include work forming part of a thesis presented successfully for a degree in this or another University.

Kirsty Stevenson Brown
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Abbreviations

Ab	Antibody
ADCC	Antibody dependent cell mediated cytotoxicity
Ag	Antigen
APC	Antigen-presenting cell
ATP	Adenosine 5'-triphosphate
BAP	BCR-associated protein
BCR	B cell receptor
BLNK	B cell LiNKer protein
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
cDNA	Complementary DNA
CDK	Cyclin dependent kinase
CFSE	Carboxy-fluorescein diacetate succinimidyl ester
Con A	Concanavalin A
COX	Cyclooxygenase
cPLA$_2$	Cytosolic phospholipase A_2
CRP	C-reactive protein
CTLA	Cytotoxic T-lymphocyte antigen
DAG	Diacylglycerol
dATP	2'-deoxyadenosine 5'-triphosphate
dbcAMP	Dibutyryl cyclic adenosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dH$_2$O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
DOK	Downstream of kinase
dTTP	2'-deoxythymidine 5'-triphosphate
DTT	1,4-dithiothreitol
dUTP	2'-deoxyuridine 5'-triphosphate
ECL	Enhanced chemiluminescence

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(β–aminoethylether)tetraaceticacid
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ErkMAPKinase	Extracellular signal-regulated protein kinase
ES	Excretory-secretory
F(ab')$_2$	Fragment antigen binding
FAM	6'carboxy-fluorescin
Fc	Fragment crystallisable
FcαR	Receptor for the constant region of IgA
FcδR	Receptor for the constant region of IgD
FcεR	Receptor for the constant region of IgE
FcγR	Receptor for the constant region of IgG
FcμR	Receptor for the constant region of IgM
FcRn	Neonatal Fc receptor
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FITC	Fluoroscein isothiocyanate
G-protein	GTP-binding protein
GAP	GTPase activating protein
GC	Germinal centre
GDP	Guanine 5'-diphosphate
GEF	Guanine exchange factor
GPI	Glycosyl phophatidyl inositol
Grb2	Growth factor Receptor Binding protein 2
GSK-3	Glycogen synthase kinase 3
HEL	Hen-egg lysosyme
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horse radish peroxidase
HSC	Haematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IgA/D/E/G/M	Immunoglobulin A/D/E/G/M

IgSF	Immunoglobulin super family
I-κβ	Inhibitor-κB
IKK	I-κB kinase
IL	Interleukin
$InsP_3$	Inositol trisphosphate
$InsP_4$	Inositol tetrakisphosphate
IP	Immune precipitate
IPTG	Isopropylthiogalactoside
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	c-Jun N terminal kinase
KIR	Killer inhibitory receptor
LAT	Linker for activation of T cells
LIR	Leukocyte inhibitory receptor
LY	LY294002
LPR	Lymphoproliferative
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MEK or MKK	MAPKinase kinase
MEKK or MKKK	MAPKinase kinase kinase
MHC	Major Histocompatibility Complex
$\Delta\psi m$	Mitochondrial transmembrane potential
mIgM	Membrane immunoglobulin M
MLB	Magnesium lysis buffer
mRNA	Messenger ribonucleic acid
NADPH	β-Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear Factor -κB
NIK	NF-κB-inducing kinase
NK	Natural killer
OD	Optical density

PA	Phosphatidic acid
PAC	Phosphatase of activated cells
PAK	p21 activated kinase
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PC-PLD	Phosphatidylcholine-phospholipase D
PCR	Polymerase chain reaction
PDK	PIP_3 dependent kinase
PHA	Phytohemagglutinin
PH domain	Pleckstrin homology domain
PI-3-K	Phosphatidyl inositol-3 kinase
PKA/PKB/PKC	Protein kinase A/B/C
PLA/PLC/PLD	Phospholipase A/C/D
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride
PTB domain	Phosphotyrosine binding domain
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-bisphosphate
PTK	Protein tyrosine kinase
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
Rb	Retinoblastoma protein
RBD	Ras binding domain
RF	Rheumatoid factor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAPK	Stress-activated protein kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH2 domain	Src homology 2 domain
SH3 domain	Src homology 3 domain

SHIP	SH2 domain containing inositol phosphatase
SHP	SH2 domain containing protein tyrosine phosphatase
sIg	Surface immunoglobulin
SLP-65	SH2 domain containing linker protein – 65 kDa
SOS	Son of sevenless
T1	Immature-transitional B cell
TAMRA	6-carboxy-tetramethylrhodamine
TBE	Tris-Borate/EDTA buffer
TBS	Tris buffered saline
TBS-T	Tris buffered saline containing Tween-20
T_C	Cytotoxic T lymphocyte
TCR	T cell receptor
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
T_H	Helper T lymphocyte
TNF	Tumour necrosis factor
TRAF	TNF-Receptor-associated protein
Tris	Tris (hydroxymethyl) methylamine
U	Units
XLA	X-linked agammaglobulinaemia

Summary

Fc receptors (FcRs) provide a critical link between the humoral and cellular arms of the immune system through the targeting of antigen-antibody complexes to effector cells and modulation of an immune response. B cells express a low affinity IgG Fc receptor, FcγRIIb, which negatively regulates antigen receptor-mediated proliferative signalling through the binding of IgG-containing immune complexes. Co-ligation of the B cell antigen receptor (BCR) complex with FcγRIIb promotes the induction of B cell growth arrest in the G_1 phase of the cell cycle and this ultimately results in commitment to apoptosis. Furthermore, FcγRIIb co-ligation also acts as an important negative feedback mechanism to switch off ongoing B cell responses once the pathogen has been cleared. Thus, on resting B cells, FcγRIIb acts to prevent aberrant B cell activation by immune complexes and suppresses the potential induction of autoimmunity.

It was therefore the aim of this study to identify and characterise the key signalling pathways responsible for FcγRIIb-mediated negative regulation of BCR-mediated proliferation. During the course of this study, it emerged that in normal splenic B cells, FcγRIIb signalling appears to act by modulating the BCR signalling threshold, via the rapid recruitment and phosphorylation of a variety of phosphatases that ultimately result in the uncoupling of the BCR from MAPKinase activation. We have corroborated these published findings that the tyrosine kinases SHP-1 and SHP-2 and the inositol 5'-phosphatase, SHIP-1, are recruited during FcγRIIb signalling and we have extended these studies in that we have generated novel data regarding their mechanism of action. We now also show that FcγRIIb co-ligation, in addition to preventing the initiation of MAPKinase signalling, induces the rapid recruitment and activation of the MAPKinase phosphatase, Pac-1, resulting in the abrogation of ongoing ErkMAPK signalling. Furthermore, we show for the first time that FcγRIIb co-ligation results in the activation of the inositol 3'-phosphatase, PTEN, with kinetics which suggest that recruitment of this tumour suppressor element antagonises BCR-coupling to the PI-3-K/Akt pathway and hence abrogates pro-

survival mechanisms in B cells. Taken together, this dual pronged mechanism of FcγRIIb-mediated abrogation of the ErkMAPKinase and Akt pathways provides a molecular rationale for the biological consequences of BCR-FcγRIIb co-ligation, namely commitment to growth arrest and apoptosis. Finally, analysis of potential downstream molecular targets of ErkMAPKinase and Akt revealed that FcγRIIb-signalling not only inhibits the phosphorylation and activation of the tumour suppressor protein, retinoblastoma (Rb), but also induces the phosphorylation and activation of the pro-apoptotic tumour suppressor protein, p53 and disruption of mitochondrial potential. Inhibition of Rb activation and consequent induction of genes required for the transition to S phase, is consistent with the observed FcγRIIb-mediated arrest in the G_1 phase of the cell cycle. Similarly induction of p53 and collapse of mitochondrial integrity provide insight into the effector mechanisms underlying FcγRIIb-driven commitment to B cell apoptosis.

To maintain homeostasis and tolerance to self-antigens, B cells require a balance of signals via activatory and inhibitory co-receptors. Thus, aberrant signalling through FcγRIIb during B cell development could lead to the induction of autoimmunity and/or promote the progression of certain autoimmune diseases. Consistent with this, recently published studies in FcγRIIb-deficient mice suggested that this lesion could result in collagen-induced arthritis (CIA) in normally resistant strains of mice. Similarly, FcγRIIb-deficient mice have been reported to display lupus-like symptoms of autoimmune disease suggesting that dysfunction of FcγRIIb-mediated negative regulation of B cell activation plays a causative role in onset and/or progression of these diseases. We have investigated this role of FcγRIIb in regulating the development of autoimmunity by analysing the functionality of FcγRIIb signalling during onset and progression of CIA in susceptible (DBA/1, H-2^q) strains and also in the spontaneous murine systemic lupus erythematosus model, MRL-*lpr/lpr*. In contrast to the knockout studies, we have demonstrated that FcγRIIb-mediated B cell inhibition appears to be intact throughout development of CIA in naturally susceptible strains of mice, suggesting that this regulatory pathway is not important in the induction or progression of this disease in H-2^q susceptible strains of mice. Clearly,

although, dysfunctional FcγRIIb signalling could play a role in predisposing mice of different genetic backgrounds to CIA, these findings make it unlikely that this molecular lesion will provide a uniform risk factor for this disease. In contrast, FcγRIIb-mediated B cell inhibition was found to be dysregulated in the later stages of lupus-like disease in MRL-*lpr/lpr* mice. Suggesting that FcγRIIb may be a susceptibility factor, contributing to the genetic and environmental background that is required for the emergence of lupus-like disease in mice.

To determine whether the differential findings regarding dysfunctional FcγRIIb signalling, as a susceptibility factor in murine models of arthritis and systemic lupus erythematosus (SLE), reflected the molecular mechanisms underlying onset and progression of human disease, the functionality of FcγRIIb signalling in peripheral blood cells from autoimmune patients was investigated. B cells encode two isoforms of FcγRIIb, FcγRIIb1 and FcγRIIb2. While both isoforms of FcγRIIb are able to mediate negative regulation of B cell activation, FcγRIIb2 can also internalise IgG-containing immune complexes. A sequence insertion in the cytoplasmic tail of FcγRIIb1 disrupts its ability to modulate receptor endocytosis. Therefore, it is possible that differences in the relative expression levels of the two isoforms of FcγRIIb may influence the regulation of B cell responses by immune-complexes and may be related to, or indicative of, disease susceptibility and progression. An investigation of the mRNA message levels of FcγRIIb in peripheral blood cells from autoimmune patients, revealed that FcγRIIb1 expression levels were lower in rheumatoid arthritis patients compared to normal controls. In contrast to the murine lupus-like model, message levels of the two isoforms in patients with systemic lupus erythematosus were similar. These preliminary results indicate that progression of rheumatoid arthritis in humans may be perpetuated by a down-regulation of the inhibitory receptor, thus resulting in a dysregulation in the balance of normal B cell signalling.

Chapter 1 - General Introduction

1.1 The Immune Response

The immune response is the defence mechanism initiated by the host following an encounter with a foreign substance or pathogen. The cells and molecules that make up the immune response collaborate through a finely balanced network of interactions. This network enables the host to detect the invasive agent, recruit and co-ordinate an attack and finally, once the agent has been successfully eliminated, suppress any further response. Host immunity can be broadly divided into two functionally distinct but interacting systems, the innate and the adaptive immune responses.

Both the innate and adaptive immune responses depend upon the activities of white blood cells derived from common pluripotent haematopoieitc stem cells in the bone marrow. These stems cells divide to produce specialised myeloid and lymphoid progenitors cells. The myeloid precursor cell gives rise to erythrocytes (red blood cells that transport oxygen), platelets (important in blood clotting), monocytes and granulocytes. The granulocytes are further comprised of eosinophils, neutrophils and basophils. Mast cells, which are similar to basophils, are also derived from the myeloid precursor cell but complete their maturation in the tissues. The lymphoid progenitor gives rise to two major types of lymphocytes, B lymphocytes (B cells) and T lymphocytes (T cells). These cells are involved in the adaptive immune response and will be discussed in more detail in later sections. Other cells derived from the common lymphoid progenitor include natural killer (NK) cells and dendritic cells (DCs), however some DCs are myeloid derived. NK cells are large granular lymphocytes that release lytic granules upon stimulation and play an important role in innate immunity and clearance of viral infection. Dendritic cells are able to trap antigen on their cell surface and present it to other cells of the immune system. These antigen-presenting cells (APCs) are also able to deliver cell activating signals and therefore play a central role in both innate and adaptive immunity.

The innate immune system is non-specific and functions regardless of the antigenic nature of the foreign substance or pathogen. Yet the innate response is an integrated effort by the host to combat microbial invasion, decrease tissue injury and cell death, promote recovery of the host and reduce the likelihood of secondary or opportunistic infections (Fearon and Locksley, 1996). The innate response relies primarily on cell surface receptors and secreted proteins of myeloid cells to recognise conserved pathogen structures such as carbohydrate, lipid, protein and DNA components associated with infection. These cellular structures can trigger phagocytic cells such as macrophages (derived from monocytes) and neutrophils to engulf foreign particles and destroy the ingested material by the release of potent intracellular enzymes and chemicals. Further members of the myeloid family are induced to release pro-inflammatory substances (cytokines) that promote an immune response and aid clearance of the pathogen. However, the innate system is only effective in eliminating infectious agents with common surface molecules that can be recognised by the neutrophils, macrophages and dendritic cells. Moreover, many infectious agents have evolved mechanisms to avoid detection by the innate immune cells. Thus, the adaptive immune system evolved to provide an improved recognition mechanism and a more versatile means of defence. In addition, the adaptive system possesses the ability to fine-tune its response with successive antigenic encounters and generate immunological memory to prevent against subsequent re-infection by the same pathogen.

The adaptive immune system relies upon lymphocytes to recognise antigens and react in a highly specific and selective manner. B cells are associated with the production and secretion of antigen-specific antibodies (immunoglobulin, Ig). Antibodies circulate in the bloodstream and permeate other body fluids, where they bind and react specifically to an antigen. Once bound to the antigen, antibodies can protect the host from the antigen-bearing pathogen by processes of neutralisation, opsonisation and complement activation. For example, formation of antigen-antibody immune complexes can prevent antigen binding to certain target host receptors thus neutralising its effects. Moreover, the antibody coating can also enable the antigen to be recognised as foreign by

phagocytic cells, a process known as opsonisation, and thus promote its destruction. Immune complexes also provide a receptor for a system of plasma proteins, known as complement. Activation of this cascade of proteins enhances opsonisation of the antigen and can directly lyse some bacteria.

T cells can be divided into $CD8^+$, cytotoxic (T_C) cells and $CD4^+$, helper (T_H) cells. T_C cells direct the destruction of host cells that have become infected by viruses or other pathogens whilst T_H cells can modulate the activities of other cells, such as B cells, macrophages and T_C. Following an antigenic encounter, T_H cells can differentiate into either T_H1 or T_H2 effector T cells. The overall balance of cytokines present early in the immune response determines the class of effector cell produced. Thus, for example, by producing different repertoires of cytokines effector T cells can bias the adaptive immune response towards a cellular or a humoral (antibody) response. This choice can determine whether the pathogen survives or is eliminated and is therefore one of the most important events in the initiation of an adaptive immune response. Recently, a small subpopulation (~10%) of $CD4^+$ T_H cells have been shown to express the CD25 marker and are associated with the suppression of harmful immunopathological responses to self or foreign antigens (reviewed by Maloy and Powrie, 2001). These naturally occurring suppresser T cells, now regarded as regulatory T cells (T_R), possess the ability to inhibit the proliferation of other T cell populations both *in vitro* and *in vivo*. Further investigations into the functional role of these cells could have widespread implications in the field of autoimmunity and maintenance of self-tolerance.

As the innate system only represents the first line of defence there is considerable interplay between it and the adaptive response. Communication between the two systems produces a diverse repertoire of immunological responses. Antigen-presenting cells of the innate system, such as dendritic cells and macrophages, appear to play a central role in this aspect. These potent cells can present antigen in a form recognisable by T cells thus resulting in a specific cell-mediated response. Whilst the opsonisation process, described

above, is a classic example of how antibodies produced by B cells can aid the innate system in the recognition of potential pathogens.

The cells responsible for mediating the humoral and cellular responses have become major targets of biomedical research. In the case of B and T cells there is growing recognition that inappropriate stimulation and activation of these cells can result in a variety of chronic inflammatory processes, including autoimmune disease. Much of this research has concentrated on the co-operation between T and B cells in mediating a normal adaptive immune response. In particular, investigating how communication between these cells can lead to a dysfunctional immune response. Therefore studies into the molecular and biochemical mechanisms underlying lymphocyte responses are important in an attempt to understand how these diseases arise and to aid in the decision of the best course of therapy.

1.2 The role of B cells in the adaptive immune response

1.2.1 Generation and selection of B cells

B cells are the principal cellular mediators of the specific humoral response to infection by bacteria, viruses and parasites. The developmental processes prior to antigen recognition need to be tightly regulated to ensure a continual production of B cells bearing antigen receptors of distinct specificity. However, whilst the system must produce antibodies that are able to identify any encountered antigen, at the same time it must avoid the generation of autoreactive B cells that recognise 'self' antigens. The complex process requires the integration of signals at each stage, not only from antigen but also from a structured microenvironment made up of a vast array of extracellular, soluble mediators and accessory cells. The following section will discuss the different stages of B cell development and the generation and selection of the diverse B cell receptor repertoire. This developmental process is summarised in **Figure 1.1**.

In mammals the earliest B cells (pro-B cells) develop from pluripotential hematopoietic stem cells (HSC) in the foetal liver. Commitment of HSCs to the

B cell lineage occurs early on in the developmental process and is shown to be dependent on the expression of Pax5, a paired box transcription factor (Cory, 1999). Pax5 acts to promote the expression of B lineage genes whilst suppressing those genes concerned with T cell, erythroid or myeloid cell development. In later embryonic stages, the site of B cell production moves to the bone marrow where it continues during adult life (Alt, *et al.*, 1997). Once committed, the precursor B cells pass through a number of developmental stages marked by a series of changes in location and in the expression of genes, intracellular proteins, and surface markers. Once the immature B cell stage is reached the B cells emerge into the periphery and migrate to the secondary lymphoid organs, such as the spleen and lymph nodes. Here, in association with specialised antigen-presenting cells and stromal cells, B cell recognition of antigen can lead to one of several developmental pathways: (1) cellular anergy (unresponsiveness) and/or programmed cell death, (2) activation, proliferation and differentiation into high rate antibody secreting plasma cells, or (3) differentiation into memory B cells (Tarlinton, 1994).

The different B cell developmental events can be broadly divided into two main stages; antigen independent and antigen dependent (reviewed by Rolink and Melchers, 1991). The antigen independent stage occurs in the bone marrow and is concerned with producing a repertoire of immature B cells with a functional antigen receptor (B cell receptor, BCR). Importantly, in accordance with Burnet's Clonal Selection Theory, each B cell that is produced by this process should express a receptor that is specific for a single antigen (Burnet, 1959). Thus, the mature antigen receptors are clonally distributed. The processes involved in the formation of BCR complexes have been studied in detail in mouse models and have been shown to be essential in directing the development and survival of the immature cell.

The production of a BCR relies on a complex pattern of immunoglobulin gene rearrangements to produce one functional heavy chain, followed by one functional light chain (Coleclough *et al.*, 1981). This rearrangement is dependent on the expression of recombination-activating genes, RAG-1 and

RAG-2, at the pro- and pre-B cell stages of differentiation (Spanopoulou, 1994). Rearrangement of the D (diversity), J (joining) and V (variable) heavy chain locus genes begins in the early pro-B stage with the joining of D_H to J_H. Cells are allowed to progress to the next stage if a productive rearrangement has been achieved. Progression to the late pro-B cell stage is accompanied by the joining of a V_H gene to the pre-formed DJ_H complex. Although no functional immunoglobulin is expressed in late pro-B cells, recent studies have shown the expression of BCR accessory Ig-α/Ig-βheterodimers on the surface in association with calnexin (Nagata *et al.*, 1997) (**Figure 1.2**). These accessory molecules, in particular Ig-β, have been shown to be essential for the continuing development of the pro-B cells to the pre-B cell stage (Gong and Nussenzweig, 1996, Nagata *et al.*, 1997). Indeed, these studies have shown that mice deficient in Ig-β 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 Ig-α/Ig-β-calnexin receptor on pro-B cells may be required for successful initiation of V_H to D_HJ_H gene rearrangement (Nagata *et al.*, 1997).

A successful first rearrangement means that intact μ heavy chains are produced and expressed in an immunoglobulin-like "pre-BCR" complex with surrogate light chains, λ5 and VpreB (Karasuyama *et al.*, 1994). λ5 bears close similarity to the known constant (C) λ light-chain domains (see below), whilst VpreB resembles a variable (V) domain but bears an extra N-terminal protein sequence. If this first rearrangement is unsuccessful, a second rearrangement is undertaken. Pro-B cells in which both rearrangements are unsuccessful are unable to produce a pre-B cell receptor. Surface expression of a pre-BCR is known to be important for instructing the cell to stop further V_H gene rearrangements (Neuberger, 1997) by inhibiting recombination at the heavy chain locus, a process described as allelic exclusion (reviewed by Benschop and Cambier, 1999). Pre-B cell receptor expression also drives the transition to the large pre-B cell stage and induces proliferation whilst signalling to the cell that gene rearrangements to the immunoglobulin light chain should begin. Thus, a lack of pre-B cell receptor expression results in a block in B cell development at the pre-B cell stage.

Once a light chain gene (κ or λ) has been rearranged successfully a BCR consisting of μ heavy chain, conventional light chains and accessory Ig-α/Ig-β molecules is expressed by the immature B cells. This intact surface IgM is the first BCR to exhibit antigen specificity (Reth, 1992) and thus the B cell enters the antigen-dependent stage of development. It has been estimated that about 10^8 B lineage precursors are generated every day in the murine bone marrow, which in turn give rise to about 2×10^7 sIgM-expressing immature B cells (Osmond, 1986). Thus, it is clear that the majority of B cells (80%) maturing in the bone marrow undergo a process of negative selection. Indeed, ligation of the antigen receptors on the vast majority of immature B cells leads ultimately to anergy (Goodnow *et al.,* 1988) or deletion via apoptosis (programmed cell death) (Nemazee and Burki, 1989; Hasbold and Klaus, 1990; Hartley *et al.*, 1991).

The deletion or silencing of self-reactive B cell clones is important for the maintenance of self-tolerance by the immune system. Immature B cells which are capable of recognising self-antigens are eliminated or inactivated, preventing them from developing further and secreting antibodies that bind to self-cells or tissues. Self-reactive immature B cells may also be rescued from deletion by undergoing receptor editing, where the autoreactive receptor is replaced with the product of a further rearrangement event (Radic *et al.*, 1993). This editing process is aided by the reactivation of the RAG gene system, which is believed to allow further light-chain rearrangements (Hertz and Nemazee, 1998). However, most of the immature B cells do not make new rearrangements and are subsequently deleted.

By maintaining high level expression of mIgM, transitional-immature (T1) B cells entering the periphery remain sensitive to antigen deletion for a number of days (Allman *et al.,* 1993). This is especially important for the development of tolerance, since not all self-antigens are expressed within the bone marrow (Sandel and Monroe, 1999; Monroe, 2000). To promote their survival and migration to the spleen, these cells require T cell-dependent help. Typically, of the 2×10^7 sIgM B cells that develop daily in murine bone marrow only 10% will

reach the spleen and only 1-3% will survive and develop to the next stage of maturation (Allman *et al.,* 1993). The development into a transitional (T2) B cell is accompanied by the surface expression of IgD and requires constant BCR-derived signals for progression (Carsetti *et al.,* 1995; Neuberger, 1997; Sandel and Monroe, 1999). In addition, stimulation via cytokines or co-receptor ligation helps to shape the BCR repertoire and signalling thresholds (Craxton *et al.,* 1999). As the B cells migrate into the primary follicles of the spleen they are finally regarded as "mature" IgM^{low} IgD^{high} B cells.

In contrast to the immature cell, the mature B cell responds positively to antigen-receptor ligation. In the presence of T cell-derived cytokines and cell-cell contact, the activated B cell can develop into an IgM antibody-secreting plasma cell or undergo isotype switching and V region somatic mutation to become a memory B cell (reviewed in Cushley and Harnett, 1993; Liu *et al.,* 1992). Following re-exposure to the same antigen and affinity maturation, the memory B cell can evolve into an IgG secreting plasma cell. The formation of memory is critical for mounting a rapid, specific secondary immune response and the ability of the immune system to generate these memory B cells forms the basis of effective vaccination.

The generation of memory B cells in the germinal centres (GCs) of secondary lymphoid organs has been actively researched for many years (Loder *et al.,* 1999) and is briefly outlined in **Figure 1.3**. T-cell dependent antigen-activated B cells enter the primary lymphoid follicles where they are driven to proliferate and form germinal centres. In the primary immune response, GCs first appear around 4-5 days after antigenic encounter. The proliferating B cells (lymphoblasts) enter the dark zone of the germinal centre where they continue to proliferate. The rapidly dividing centroblasts lose expression of sIg and undergo somatic mutation of the immunoglobulin variable-domain genes. Centroblasts give rise to small, non-dividing centrocytes, which express the mutated antigen-receptors. Centrocytes with high affinity antigen-receptors are selected for survival in the light zone of the germinal centre. The follicles consist of follicular dendritic cells (FDCs) that are able to fix unprocessed antigen on

their cell surface through the expression of complement receptors and receptors for the Fc portion of immunoglobulin. Since centrocytes are programmed to die unless actively rescued, high levels of apoptosis accompany their migration into the light zone. *In vitro* studies have shown that two signals enable centrocyte survival. The first is generated by FDC-displayed antigen resulting in ligation of the high-affinity surface immunoglobulin, the second involves ligation of CD40 on the surface of centrocytes, suggesting a role for helper T-cell interactions in promoting centrocyte rescue (Craxton *et al.*, 1999). This process results in the rescue and selection of high affinity B cell clones, as redundant and low affinity specificites are selected against. The positively selected centrocytes then go on to establish the memory B cell pool in the apical light zone, providing the precursors for plasma cell generation.

The developmental stages described in this section give rise to a population of "conventional" B cells. However a second population of B cells exists that appear to arise from distinct stem cells early on in development (reviewed by Hayakawa and Hardy, 2000). These latter cells are known as B-1 B cells, to distinguish them from the conventional B-2 B cells described above. This subset of B cells was first distinguished from conventional B cells by the surface expression of CD5. Stem cells arising from the foetal liver give rise to B cell progenitors that have little or no terminal deoxynucleotidyl transferase (TdT), which in turn give rise to B-1 cells. A lack of TdT activity in these B-1 cells results in limited immunoglobulin heavy chain rearrangements and the expression of low-affinity antigen receptors for multivalent self-antigens. The restricted diversity of B-1 cell antigen-receptors includes broad specificities in antigen recognition, particularly to common bacterial polysaccharides such as lipopolysaccharide (LPS) and phosphorylcholine (PC). Thus B-1 B cells are particularly important in newborns, as they are presumed to be responsible for natural immunity prior to the production of conventional B-2 B cells in adult life.

Predominantly located in the peritoneal cavity, the self-renewing population of mature B-1 cells express sIgM with little or no surface expression of IgD. Little is known about the function of B-1 cells. However, the propensity of B-1 cells to

produce low-affinity autoreactive antibodies implicates a role for these cells in autoimmune disease. Indeed, B-1 B cell populations that produce autoantibodies are expanded in autoimmune disease models such as SHP-1 (motheaten) and Lyn deficient, mice (Hayakawa and Hardy, 2000). Whilst elevated levels of B-1a ($CD5^+$) cells have been found in a proportion of patients with rheumatoid arthritis and systemic lupus erythematosus (Porakishvili *et al.*, 2001).

1.2.2 B Cell Receptor (BCR) structure

From the above description of B cell generation and development, it can be seen that components of the B cell receptor (BCR) for antigen recognition play important roles in determining the fate of B cells even before they encounter antigen. The mature BCR is functionally divided into two main units; the membrane-bound, ligand binding, immunoglobulin (Ig) molecule and the accessory, signal transducing molecules, Ig-α (CD79a) and Ig-β (CD79b) (reviewed in Reth, 1992, 1994; Pao *et al.*, 1997) (**Figure 1.4**). In contrast to the T cell receptor for antigen, which only recognises processed antigen in association with MHC molecules, the BCR recognises antigens in solution or on cell surfaces in a native conformation. B cell activation by antigen leads to antigen uptake by the BCR, processing and presentation to T cells.

Antibodies of all heavy-chain isotypes namely, IgM, IgD, IgG, IgA and IgE, can be produced either in secreted or membrane-bound receptor form. However, only IgM and IgD are expressed on the surface of mature naive B cells. The ligand-binding unit consists of a tetrameric complex of Ig heavy chain homodimers; each linked by disulphide bonds to an Ig light chain. A transmembrane region of 25 amino acids anchors it to the cell surface. The sIg subunit lacks any intrinsic enzymatic activity within its cytoplasmic domains; thus it associates with accessory signal transducing molecules. Encoded by the *mb1* and *b29* genes respectively (Reth, 1995), Ig-α (34 kDa) and Ig-β (38 kDa) possess single, immunoglobulin extracellular domains and exist as disulphide linked heterodimers.

Cellular signalling is mediated by a common mechanism in many immunoreceptors. In the case of the BCR, the associated accessory molecules possess consensus sequence motifs responsible for the initiation of signal transduction. This motif originally designated as TAM, ARAM or RETH, is now termed the immunoreceptor tyrosine-based activation motif (ITAM) (Cambier *et al.*, 1995; Cambier, 1995). This motif, spaced over 18 amino acids, is composed of a twice-repeated YxxL sequence flanking seven variable residues (**Table 1.1**). Antigen-ligation of the BCR promotes the phosphorylation of the two tyrosine residues within each ITAM (four tandem motifs per BCR) by non-receptor protein tyrosine kinases (PTKs) (**Figure 1.4**). Phosphorylation of the tyrosine residues is essential to the function of the motif and is deemed as being necessary and sufficient to initiate signal transduction (Cambier, 1995).

Other ITAM containing membrane proteins include the CD3 and ζ-chains of the T cell receptor (TCR) and several receptors for the Fc domain of immunoglobulin (FcRs). The discovery of this critical motif and the subsequent studies into the initiation of ITAM mediated signal transduction has revolutionised immunoreceptor biology.

1.2.3 BCR Signalling

The downstream signalling pathways that couple sIg ligation to B cell activation and differentiation, have become a major area of research in the field of immunology, in the quest to understand and ultimately 'control' the immune response. The current model of sIg mediated B cell activation demonstrates that the earliest event detected following BCR ligation is the activation of protein tyrosine phosphorylation (reviewed in Kurosaki, 1997, 1999; Campbell, 1999). Recruitment and activation of three distinct types of non-receptor protein tyrosine kinases (PTKs) are known, these include the Src- (including Lyn, Blk and Fyn), Syk- and Tec- (Bruton's tyrosine kinase; Btk) PTKs (Cambier, 1995; Kurosaki, 1999) (**Figure 1.4**). The domain structures of these PTKs are shown in **Figure 1.5A**. In resting B cells a small amount of the Src-family PTKs are associated with the BCR as folded, inactive molecules via hinged N-terminal regions. Following BCR ligation, the Src-family PTKs become activated, unfold,

and phosphorylate the tyrosines within the BCR accessory molecules (**Figure 1.5B**). The phosphorylated ITAMs create new binding sites for the tandem SH2 domains of Syk. Once bound, Syk becomes tyrosine phosphorylated, increasing its activity by at least 10-fold. Btk is proposed to be activated in a similar Src-PTK dependent manner (Takata and Kurosaki, 1996). Together these activated kinases phosphorylate downstream targets in the signalling cascade. This simple model, however, does not explain recent findings suggesting that both $Lyn^{-/-}$ and $Syk^{-/-}$ B cells can still signal through their BCR (Craxton *et al.*, 1999). This suggests that the BCR can activate both Lyn- and Syk-independent pathways and that differential activation of these PTKs could play a role in directing BCR-mediated signalling cascades down distinct paths, producing different physiological outcomes.

Indeed, Lyn has been shown to be involved in the tyrosine phosphorylation of the B cell inhibitory co-receptors, FcγRIIb (section **1.4**) and CD22 (reviewed by Doody *et al.*, 1995; DeFranco *et al.*, 1998; Tsubata, 1999). Following activation by Lyn, both receptors recruit and activate a SH2-domain containing protein tyrosine phosphatase (SHP-1) which is thought to inhibit the association of Syk and Src-family PTKs with the BCR ITAMs by dephosphorylating Ig-β. Inhibition of Src-family PTK activity may also be achieved by the phosphorylation of the inhibitory site within the PTK, by C-terminal Src kinase (Csk). Csk acts to keep the PTKs in a folded, inactive conformation until dephosphorylation of the site by the transmembrane protein tyrosine phosphatase CD45 (reviewed by Thomas, 1999). Since in resting cells both CD45 and Csk are constitutively active enzymes, this suggests that their opposing activities keep the Src-family PTKs in a net dephosphorylated state. The role of co-receptors in modulating BCR signal is discussed further in section **1.2.5**.

In addition to the initiation of signal transduction events, the BCR serves to target bound antigen for processing and presentation by MHC class II molecules. Recent advances in the understanding of BCR functions have suggested a role for sphingolipid- and cholesterol-rich plasma membrane lipid micro-domains, termed lipid rafts, in the initiation of both signalling and antigen

targeting functions of the BCR (Cheng, *et al.*, 1999). Upon antigen binding, the BCR complex is rapidly recruited into these lipid rafts bringing the BCR into close contact with downstream signalling molecules and adaptor proteins. Subsequently, BCR bound antigen is targeted to the endocytic MHC class II peptide-loading compartments. Importantly, these rafts were shown to contain the Src-family kinase Lyn, but were deficient in the membrane phosphatase, CD45 (Cheng, *et al.*, 1999). Thus, these lipid rafts have been proposed to function as platforms for both receptor signalling and antigen internalisation.

The early BCR-mediated signalling events result in the aggregation and activation of a number of important downstream enzymes and adapter proteins that in turn result in the initiation of three independent, yet potentially interactive, signalling pathways (**Figure 1.6**) (reviewed by Cushley and Harnett, 1993). The first pathway involves the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (Ins(1,4,5)P_3) by poly-phosphoinositide-specific phospholipase C-γ (PLC-γ). The second results in the generation of phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P_3) by phosphatidylinositol 3-kinase (PI-3-kinase) and finally the classical Ras/MAPKinase cascade can be activated. These pathways all converge at the nucleus where they regulate transcription factors and ultimately dictate the fate of the cell.

1.2.3.1 Activation of PLC-γ

The PLC-γ pathway was one of the first downstream pathways shown to be activated by the BCR (DeFranco, 1997). The generation of second messengers, DAG and Ins(1,4,5)P_3, has been shown to be important for the activation of protein kinase C (PKC) isoforms, the release of intracellular calcium stores and the nuclear translocation of NF-κB (Berridge and Irvine, 1989; Berridge, 1993; Lee and Rhee, 1995). DAG activates certain serine/threonine protein kinase C (PKC) isoforms at the plasma membrane (reviewed by Parekh *et al.*, 2000) whilst Ins(1,4,5)P_3 binds to receptors ($InsP_3R$) in the endoplasmic reticulum. $InsP_3R$ activation leads to a process of capacitative calcium entry, by which the

depletion of intracellular stores activates calcium influx across the plasma membrane (Parekh and Penner, 1997).

PKC isoforms are divided into three groups depending on their structure and cofactor regulation (Dekker and Parker, 1994). Conventional PKCs (α, β1,β2 and γ) require both calcium and DAG for activation, novel (δ, ε, η, μ and θ) are calcium independent but require DAG whilst, atypical PKCs (ξ, λ/ι) require neither. Several studies have highlighted the role for PKCs in modulating the BCR-mediated response in a maturation stage-dependent manner. Studies in knockout or depletion models of PKC isoforms have demonstrated that a lack of DAG-responsive PKC isoform activation, in particular PKCβ, may be responsible for BCR-induced apoptosis in immature B cells (Leitges *et al.,* 1996, King *et al.,* 1999). Thus, apoptosis can be overcome by stimulating immature B cells with phorbol esters (e.g. PMA), which bypass the BCR and activate the conventional and novel PKC isoenzymes directly (Nishizuka, 1992; King *et al.,* 1999).

The PLC family contain three homologous groups of enzymes (PLC-β, γ and δ) all of which contain pleckstrin homology (PH) domains. In tyrosine kinase dependent signalling, including most immune receptors, one or both of the two isoforms of PLC-γ, PLC-γ1 and PLC-γ2, are activated. In addition to the PH domain, PLC-γ isoforms also contain two SH2 domains and an SH3 domain (Lee and Rhee, 1995). The SH2 domain aids the recruitment and phosphorylation of PLC-γ by the PTKs Btk and Syk, whilst the PH domain permits docking to the inner plasma membrane via a PtdIns(3,4,5)P_3 dependent mechanism (Campbell, 1999). The translocation of PLC-γ to the cell membrane and its subsequent PTK-mediated tyrosine phosphorylation is essential for its activation. Indeed, a deficiency in either Btk or Syk results in ablation of PLC-γ activation and Ins(1,4,5)P_3 production. PtdIns(3,4,5)P_3 can bind or activate Btk which, in turn, phosphorylates and activates PLC-γ. In addition, PtdIns(3,4,5)P_3 may directly activate PLC-γ via PH domain binding interactions. Although the SH2 domain of PLC-γ can bind to Syk *in vitro*, reconstitution experiments indicated that additional factors were required to initiate a BCR-mediated

calcium response (Kelly and Chan, 2000). More recently, the B cell specific adapter protein, BLNK (B cell linker protein), also termed SLP-65, has been shown to be required for coupling Btk and Syk activation to the activation of PLC-γ (Fu *et al.*, 1998; Ishiai *et al.*, 1999). Indeed, studies using the chicken DT40 cell line have shown that BLNK gene disruption abolishes PLC-γ phosphorylation (Ishiai *et al.*, 1999). BLNK also has an involvement in the recruitment and activation of Vav, Grb2 and Nck, serving to focus these signalling effectors at the plasma membrane for phosphorylation by Syk.

PLC-γ1 deficient mice are embryonic lethal and fail to mobilise calcium (Ji *et al.*, 1997). Whilst PLC-γ2 insufficiency is not lethal (Wang *et al.*, 2000), PLC-γ2 deficient B cells have signalling defects similar to that seen in Btk knockouts (Khan *et al.*, 1995) or the naturally occurring *xid* immunodeficient mice (Rawlings *et al.*, 1993) both of which possess a mutation in the PH domain of Btk.

1.2.3.2 Activation of PI-3-K

Phosphatidylinositol 3-kinase (PI-3-Kinase) catalyses the phosphorylation of the inositol phospholipids PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 on the 3' position of the inositol ring to produce PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively (Fry, 1994) (**Figure 1.7**). The three classes of PI-3-K are determined by their *in vitro* substrate specificities. Class I PI-3-kinases phosphorylate all three forms of inositol phospholipid and can be further subdivided into Class IA and Class IB. Class IA comprise a p110 catalytic subunit (α, β or δ) and a regulatory SH2/SH3-domain containing p85 family adapter subunit. The Class IA isoforms are normally activated by PTK coupled receptors. Class IB isoforms consist of a p110γ catalytic subunit and are activated following signalling via G-protein coupled receptors. Class II isoforms phosphorylate PI and PtdIns(4)P whilst Class III phosphorylates PI only.

PI-3-Kinase activity and the generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, are known to be important for BCR-mediated B cell proliferation, differentiation (Aagaard-Tillery and Jelinek, 1996) and survival (Campbell, 1999). Such that

inhibition of PI-3-Kinase activity in actively cycling B cells results in apoptosis (Craxton *et al.*, 1999). However, a negative role for PI-3-Kinase has been shown in a human RL B cell line where BCR-induced growth-arrest can be blocked by inhibiting PI-3-Kinase activity (Craxton *et al.*, 1999). The interaction of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with SH2 and PH domain containing proteins, such as PLC-γ, Btk, Vav, PDK1/2 and Akt/PKB permits their recruitment to the plasma membrane (Aagaard-Tillery and Jelinek, 1996; Alessi *et al.*, 1997; Falasca *et al.*, 1998; Franke *et al.*, 1997). PI-3-Kinase activation following BCR ligation was thought to be solely dependent on PTK activity. However, studies have demonstrated the essential requirement for upstream activation of the GTPase Ras (Kodaki *et al.*, 1994) which then binds the p110 catalytic sub-unit of the p85-dependent PI-3-Kinase (Genot and Cantrell, 2000). This mechanism establishes a link between the PI-3-Kinase pathway and the pathways driven by GTP binding proteins, such as the Ras MAPK pathway. PI-3-Kinase activation may also result from recruitment and phosphorylation by CD19 following BCR ligation. (Aagaard-Tillery and Jelinek, 1996; Kurosaki, 1999; DeFranco, 1997). Upon BCR-ligation, the cytoplasmic tail of CD19 becomes tyrosine-phosphorylated by the Src-family PTK Lyn and provides binding sites for PI-3-Kinase, the GEF Vav, Shc and the Src-family PTKs. The binding of PI-3-Kinase to CD19 following BCR-ligation serves to localise PI-3-Kinase to the plasma membrane where its phosphoinositide substrates are located.

1.2.3.3 Activation of the Ras/MAPKinase pathway

The mitogen-activated protein (MAP) kinases are a family of serine-threonine protein kinases. They are activated by a wide range of extracellular stimuli and are able to mediate a wide range of cellular functions from proliferation and activation to growth arrest and cell death (**Figure 1.8**). The MAPKinase family is further classified into three sub-groups; the classical extracellular signal-regulated kinases (ErkMAPKinase), the c-Jun N-terminal kinases, also known as the stress activated protein kinases (JNK/SAPK) and the p38 MAPKinases (Dhanasekaran and Premkumar-Reddy, 1998; Elion, 1998). Activation of each sub-group is determined by distinct upstream MAPKinase kinases (MEKs).

MAPKs are activated by dual phosphorylation on tyrosine and threonine residues, located in a T-x-Y motif. Each sub-group of MAPKinase is independently activated and regulated by a specific MEKK (MAPKinase kinase kinase) and MEK following cell stimulation. However, significant pathway cross talk exists. Following MAPKinase activation, independent activation of downstream transcription factors occurs thus, ErkMAPKinase activates Elk-1 and c-myc, JNK activates c-Jun and ATF-2 and p38 MAPKinase activates ATF-2 and MAX. The phosphorylation of these transcriptional regulators enables the MAPKinase families to dictate gene expression and hence, cellular responses.

In B cells, coupling of the BCR to the MAPKinase pathway relies on the formation of adapter protein scaffolds, which are recruited by activated protein tyrosine kinases. These complexes then facilitate the recruitment of downstream guanine nucleotide exchange factors (GEFs) and kinase cassettes of the MAPKinase pathway. Following BCR ligation, the adapter protein Shc binds to the phosphorylated BCR ITAMs and is in turn phosphorylated by the PTK Syk. Activated Shc then recruits Grb2-SoS complexes to the phosphorylated ITAMs (Li *et al.*, 1993; Harmer and DeFranco, 1997). In conjunction with a GTPase activating protein (RasGAP), the GEF SoS (son of sevenless) regulates the activities of the guanine nucleotide binding protein Ras.

Small GTPases, such as Ras, cycle between inactive GDP-bound and active GTP-bound states and thus function as signal relays linking membrane receptors to signal transduction pathways. GEFs promote the exchange of GDP for GTP resulting in the production of an active GTPase to turn on downstream signalling effectors, whilst GAPs accelerate the normally slow intrinsic GTPase activity, thereby inactivating it (Henning and Cantrell, 1998). Thus, following BCR ligation Grb2-SoS complexes acts to displace GDP from the GTPase Ras to generate an active GTP-bound form (**Figure 1.9**). Ras is then able to regulate a wide range of downstream cellular processes, from cytoskeletal reorganisation through to modulation of transcription (reviewed by Vojtek and Der, 1998). In particular, active Ras can bind and derepress Raf, a

serine/threonine kinase, resulting in the activation of MAPKinase kinases (MEK) and the subsequent downstream activation of the ErkMAPKinases (ErkMAPK) (Kolch, 2000) (**Figure 1.8**). Active Ras also promotes cell survival by direct interactions with PI-3-Kinase, resulting in the production of the second messengers, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3.

It is important to note that Ras activation can also occur through an alternative route due to the utilisation of the linker protein BLNK (Kelly and Chan, 2000). Whilst already implicated as an intermediate adapter in the membrane recruitment and activation of PLC-γ by Syk, BLNK is known to associate with Grb2 and SoS to form a BLNK/Grb2/SoS complex. Thus, bypassing the requirement for the adapter protein, Shc, in Ras activation. In addition, co-recruitment of the GEF Vav by BLNK (Fu *et al.*, 1998) can in turn activate the Rho-family of GTPases (Rac, RhoA and Cdc42) (Crespo *et al.*, 1997). These GTPases provide a critical linkage for the activation of the other MAPKs, JNK/SAPK and p38 MAPK, by specific MEKKs (MEKK1-4). (**Figure 1.9**). Rho-GTPases are also able to activate PI-5-Kinase, leading to the activation of PLC-γ and the generation of Ins(3,4,5)P_3 and calcium mobilisation (Campbell, 1999). Alternative pathways following GEF activation may also occur due to the structural similarities between the effector binding domains of the GTPases Ras and Rap-1. The Rap-1 GEF, C3G, interacts with Crk, a Grb2 like adapter molecule, to form a C3G-Crk complex that associates with phosphorylated receptor tyrosine kinases. Activation of Rap-1 by C3G may lead to the sequestering of the downstream effector Raf, the down-regulation of MAPKinase activation and modulation of the cellular response (**Figure 1.9**). However, in some cases, Rap-1 activation may actually occur in a Crk/C3G independent manner. Studies by York *et. al.*, 1998 have shown that formation of a stable complex between Rap-1 and β-Raf promotes the activation of ERK-MAPKinase in nerve growth factor treated PC12 cells, suggesting that Rap-1 activity can positively regulate receptor-mediated responses. Indeed, transient Ras-dependent MAPKinase activation in PC12 cells results in proliferation, whilst sustained Rap-1-dependent MAPKinase activation induces differentiation (York *et a l.*, 1998). Utilisation of this alternative pathway process may be

facilitated by the differential cellular localisation of these two GTPases; Ras is predominantly membrane bound whilst Rap-1 is located in endocytic and lysosomal vesicles.

MAPKinase pathways are able to regulate cell cycle machinery thus controlling the fate of a cell and dictating whether it progress through to rounds of proliferation or enters into growth arrest or apoptosis. In B cells, the ErkMAPKinase pathway has been shown to promote proliferation by inducing the expression of cyclin D1 (Lavoie *et al.*, 1996) (section **1.2.4**). The cyclins are pivotal in controlling successful passage of cells through the cell cycle. Phosphorylation of certain Ribosomal S6 Kinase isoforms (p90RSK1-3) by ErkMAPKinase has also been postulated to be important for the regulation of translation. In contrast, activation of the JNK/SAPK and p38 MAPKinases induces growth arrest and apoptosis in a human immature B cell line following BCR-ligation (Graves *et al.*, 1996).

To understand how these signalling pathways influence cellular responses, the regulation of the cell cycle and the induction of programmed cell death, or apoptosis, will be discussed.

1.2.4 Cell cycle and apoptosis

Signalling via the BCR has been shown to play a pivotal role in the activation of B cells that can lead to various distinct cellular responses, including proliferation, differentiation, anergy and cell death. Despite numerous investigations, it is still not clear how the biochemical signalling events can be translated into these responses. What is clear is that any cellular response is regulated by a number of enforced checkpoints. Checkpoints serve a critical role in the control of cell cycle progression and the damage response system, as they provide the cell with an opportunity to assess the appropriateness of response. Thus a cellular response is the integration of biochemical signals that promote and inhibit the progression of the cell cycle.

The cell cycle is the period of time from one cell division to the next. This complex process relies on the assembly of nuclear proteins that integrate signals and programme cell cycle progression (**Figure 1.10**) (O'Connor *et al.*, 2000). There are fours main stages of the cycle, during which a cell must duplicate its contents and divide. In G_1 (gap 1) the cell increases in size, separates its centrioles and prepares to copy its DNA. DNA duplication occurs in the S phase (synthesis). After chromosome replication a second gap period, G_2, allows the cell to monitor DNA integrity and cell growth prior to M (mitosis) when the cell finally divides. The daughter cell products immediately enter G_1 and may go through the full cycle again or alternatively stop cycling temporarily and enter the G_0 phase (reviewed in Planas-Silva and Weinberg, 1997). Cell cycle progression is driven by two essential components, cyclins and cyclin dependent kinases (Cdks). The kinases include Cdk4 and Cdk6 which associate with the cyclins D1, D2 and D3 whilst, Cdk2 associates with cyclin E (Pavletich, 1999). At the G_1 checkpoint cells have to decide whether to commit to DNA synthesis. Here, D-type cyclins bind to Cdks 4 or 6 and the resulting complex promotes G_1/S transition by releasing the braking effect of retinoblastoma protein, pRb^{105}. Hypophosphorylated Rb actively blocks cycling by sequestering the transcription factor, E2F, thus blocking expression of necessary S-phase genes (Dyson, 1998). Once phosphorylated by the cyclin-Cdk complexes, E2-F is released and S phase genes are transcribed (**Figure 1.10**).

In response to cellular stress or damage, various external signals and inhibitory proteins can restrain the cycle progression at the G_1/S boundary and promote growth-arrest and/or apoptosis (see below). Extracellular regulators include the transforming growth factor-β (TGF-β) and interferon-α (IFN-α) (Sangfelt *et al.*, 2000). These pleiotropic factors act in part to suppress Rb phosphorylation, through the inhibition of Cdks and the recruitment of Cdk inhibitors (**Figure 1.10**) (reviewed in Sherr and Roberts, 1999). The INK4 (p15, p16, p18 and p19) and the WAF1 (p21, p27 and p57) families of Cdk inhibitors act to block Cdk activity at various stages of the cell cycle. p15, $p16^{INK4A}$ and $p27^{Kip1}$ have all been demonstrated to inhibit the Cyclin D-Cdk 4/6 complex, whilst $p19^{ARF}$ and

$p21^{WAF1}$ are thought to induce cell cycle arrest through interactions with the tumour suppresser gene, p53.

Activation of the tumour suppresser gene, p53, in response to DNA damage from both endogenous and exogenous sources results in cell cycle arrest in the G_1 phase. This is presumably to allow an opportunity for DNA repair to occur before replication or mitosis (Hartwell and Kastan, 1994). However, in some cell types including immature B cells, p53 activation results in apoptosis (Wu *et al.*, 1998). The final outcome of p53 activation thus depends on the action of a variety of downstream effector genes transactivated by p53. The protective role of p53 is highlighted by the fact that around 50% of all cancers have lost, or possess an inactive form of p53. Indeed, the inactivation of various cell cycle proteins including Rb^{105}, $p16^{INK4A}$ and p15 has been implicated in the progression of human cancers. The net effect of their loss being deregulation of the cell cycle and, in turn, survival and excessive proliferation of the cell.

The growth and proliferation of cells must be tightly regulated to prevent the production of excessive cell numbers. Programmed cell death, or apoptosis, provides a mechanism for the disposal of "unwanted" cells in a co-ordinated manner (reviewed by Hengartner, 2000). This mechanism also protects the system by enabling the destruction of damaged or potentially harmful cells. The classical morphological features of apoptosis include the condensation of chromatin, protein and DNA fragmentation and the formation of apoptotic bodies. Thus, biologically activated apoptosis is quite distinct from necrosis, where cellular death arises due to chemical or physical injury. Most of the observed changes associated with apoptosis (i.e. DNA/protein cleavage, nuclear shrinking, loss of cell shape) are implemented by a set of cysteine proteases, caspases, which are specifically activated in apoptotic cells (reviewed by Thornberry and Lazebnik, 1998).

The caspases are a family of proteases that possess an active-site cysteine and cleave substrates after aspartic residues. The four residues amino-terminal to the cleavage site determines the distinct substrate specificity of different

caspases (Thornberry *et al.*, 1997). Proteolytic cleavage by caspases can lead to diverse results depending on the nature of the substrate and the exact cleavage site position. Thus, caspases are able to mediate both activation and inactivation of downstream target proteins. Most caspases are activated by proteolytic cleavage of an inactive pro-caspase form (**Figure 1.11**). Each pro-caspase contains in its prodomain a protein-protein interaction module, which allows it to bind and associate with upstream regulators. Initiator caspases-8 and -10 contain a death-effector domain (DED), permitting interaction with CD95 and the adapter molecule FADD, whilst the effector caspases-2 and -9 contain a caspase activation and recruitment domain (CARD). Activation can occur simply by exposure to another previously activated caspase, resulting in a 'caspase cascade' of activation.

Many of the functions of the cascade appear to converge on mitochondria, where the induction of apoptosis has been shown to result in a rapid loss of mitochondrial membrane potential and organelle swelling. However, a cells fate is often decided through the activation of the Bcl-2 family of apoptosis regulator proteins (**Figure 1.12**) (Jacobson, 1997). Homo- or heterodimers of pro- and anti-apoptotic Bcl-2 members, act at the surface of the mitochondria and compete to regulate cytochrome c release which, in association with the adapter Apaf-1, has been implicated in the activation of caspase-9 in the cytosol. This complex results in the activation of the effector caspase-3 and can be antagonised by the expression of inhibitors-of-apoptosis (IAPs). However, the Smac/DIABLO protein, also released from the mitochondria, negatively regulates IAP family members ensuring that they do not inhibit caspase activation once a cell is committed to apoptosis.

Cleavage of the pro-apoptotic Bcl-2 family member, Bid, by caspase-8 releases an active, truncated form (tBid) which translocates to the mitochondria and promotes the release of cytochrome c (Porter, 1999) (**Figure 1.13**). In addition, interaction of the pro-apoptotic regulator Bad with anti-apoptotic regulators Bcl-2 or Bcl-X_L at the mitochondrial surface promotes apoptosis. In contrast, phosphorylation of Bad by the survival protein Akt promotes the association of

Bad with the phospho-serine binding protein, 14-3-3 and its removal from the mitochondria. The blocking of the interaction of Bad with other Bcl-2 family members thus promotes cell survival (Franke and Cantley, 1997).

Thus, the regulation of the cell cycle and the apoptotic pathways exemplify the high degree of crosstalk and integration between cell signalling pathways that are involved in deciding cell fate.

1.2.5 B cell co-receptors and signalling

A hallmark of the immune response is the maintenance of equilibrium between antigen reactivity and cellular quiescence. Tight regulation and control over a potentially inappropriate response is critical. On the cellular level, many aspects of positive signalling in the generation of a controlled immune response have been investigated. In contrast, detailed knowledge of counteracting inhibitory pathways has been quite limited. Recent studies into inhibitory cell surface receptors have highlighted the requirement for the balance of activatory and inhibitory pathways to maintain this control. Indeed, loss of suitable inhibitory signalling is frequently associated with the development of inflammatory responses and, in some cases, autoimmunity.

This balance of control is exemplified by the fine-tuning of B cell responses to antigen, resulting in differential biological responses. Extracellular mediators which can alter BCR mediated signalling, include; the concentration of the antigen, the avidity with which it is bound, the timing and duration of antigen encounter and co-stimulation by other cells, receptors or antigenic sources. (reviewed by Healy and Goodnow, 1998). Similarly, the differential expression of signalling molecules at different B cell maturation stages or the use of alternative signalling pathways by the recruitment of different adapter molecules leads to the generation of a full repertoire of responses.

The involvement of B cell co-receptors and their modulation of the cellular response have been widely studied (reviewed by Tsubata, 1999). Molecules

such as CD19, CD22, CD72 and the Fcγ receptor, FcγRIIb, appear to alter the signalling threshold of the BCR either by facilitating positive signalling or by downmodulating BCR function.

1.2.5.1 CD19

CD19 is a cell surface glycoprotein that forms a non-covalent complex with CD21 (complement receptor type 2, CR2), CD81 (TAPA-1) and Leu-13 on B cells. Co-ligation of this complex on B cells results in a reduction of the signalling threshold required for B cell activation, such that CD19-deficient mice are hyporesponsive to stimulation via the BCR (Engel *et al.*, 1995). CD19-BCR co-ligation results in an increase in the release of intracellular calcium, DNA synthesis, ErkMAPK activation and antibody production (Tedder *et al.*, 1997). CD19 is uniformly expressed throughout B cell development, with an increase in expression shown by mature B cells. Alteration in the levels of CD19 expression has little effect on B cell development until maturity, where an overexpression of CD19 by immature B cells results in a significant reduction of conventional B cells in the periphery (Engel *et al.*, 1995). This reduction is presumably as a result of a hyper-responsive antigen receptor leading to enhanced negative selection in the bone marrow.

In some cases, CD19-overexpression can lead to hyper-gammaglobulinema and autoimmune disease, which is believed to be due to CD19-mediated lowering of the threshold for BCR-mediated signalling. Thus, overexpression of CD19 may permit autoreactive B cells to overcome their anergic state. CD19-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 (Fujimoto *et al.*, 1999). This suggests that CD19-signalling is important for T-cell dependent B cell responses.

Following BCR-ligation the PTK Lyn phosphorylates the cytoplasmic tail of CD19 and subsequently leads to the creation of SH2 binding sites and the recruitment and activation of various downstream signal-transducing molecules

(Fujimoto *et al.*, 1999). Src-family PTKs, PLC-γ, PI-3-Kinase, the GEF Vav and the adapter molecule, Shc, have all been associated with direct binding to activated CD19 (Tuveson *et al.*, 1993; Weng *et al.*, 1994). Studies into these complex associations have lead to the belief that CD19 may be responsible for many of the initial early signalling events following BCR co-ligation, as CD19-deficiency leads to a reduction in the tyrosine phosphorylation of multiple effector molecules downstream of BCR activation (Fujimoto *et al.*, 1999). The association of adapter molecules with CD19 has also helped to explain the ability of CD19 to promote events normally associated with positive BCR signalling. Thus, CD19 can regulate BCR coupling to PLC-γ and sustained calcium influx via amplification of the Src-family PTK signal. The phosphorylation of CD19 by Lyn initiates additional rounds of PTK activation including the activation of Syk and Btk, leading to the recruitment of the BLNK/PLC-γ/calcium pathway.

However, calcium influx in CD19-deficient B cells is sustained at later time points when it normally decreases in wild-type B cells. This may be due to a reduction in the tyrosine phosphorylation of multiple effector molecules downstream of the BCR following BCR-ligation in CD19-deficient cells. This leads to decreased levels of PLC-γ or the decreased phosphorylation of the SH2 domain containing inositol phosphatase, SHIP. SHIP negatively regulates calcium mobilisation by decreasing the availability of the major PLC-γ substrate, PtdIns(3,4,5)P_3 and is an important mediator in the negative regulation of B cell activation by FcγRIIb (see section **1.3**). Additional studies have also accounted for the ability of CD19 to augment BCR-mediated ErkMAPK activation by the synergistic enhancement of MEK1 activity, in a Ras- and PKC-independent manner (Li and Carter, 1998).

1.2.5.2 CD22

CD19 can also interact with other regulators of BCR signalling such as CD22, which is a B cell specific protein that appears to be both a negative and a positive regulator of B cell activation (Sato *et al.*, 1996). In contrast to CD19, co-

ligation of CD22 with the BCR results in the specific suppression of ErkMAPK and the modulation of JNK activity. Thus, CD22-deficient mice show enhanced B cell responses, including augmented intracellular calcium mobilisation. Following BCR ligation, CD22 is tyrosine phosphorylation via the PTK Lyn and recruits the tyrosine phosphatase SHP-1 to its cytoplasmic ITIM. SHP-1 is a strong candidate for mediating the negative effects of CD22, as there are increased levels of SHP-1 recruited by CD22 following BCR ligation. Indeed, recent data suggests that CD22 may function as a molecular "scaffold" that specifically coordinates the docking of multiple effector molecules, in addition to SHP-1, in a context necessary for BCR-dependent JNK stimulation. (Poe *et al* , 2000).

A role for CD22 in the induction of autoimmunity has also been suggested since the gene for CD22 maps within a region associated with the development of autoimmune disease in certain strains of mice. The CD22 protein expressed in these autoimmune mice is markedly underphosphorylated and binds very little SHP-1 (Cornall *et al.*, 1998). However, the disease phenotype displayed is milder than that of SHP-1 deficient mice, confirming that SHP-1 may also regulate B cell signalling by affecting other signalling molecules. The transmembrane protein tyrosine phosphatase CD45 has also been implicated in the regulation of CD22, since CD45 deficient mice display increased resting CD22 phosphorylation. Consistent with this, cross-linking of CD45 leads to a decrease in tyrosine phosphorylation of CD22 and subsequent SHP-1 recruitment (Greer and Justement, 1999).

1.2.5.3 CD72

CD72, a type II membrane protein, is also thought to negatively regulate B cell activation via the recruitment of SHP-1 (reviewed by Parnes and Pan, 2000). However, the interaction of SHP-1 with CD72 is thought to have a different function than that displayed by other SHP-1 dependent inhibitory receptors. The tyrosine phosphorylation of CD72 in response to BCR ligation correlates strongly with BCR-induced growth arrest and/or apoptosis in B cell lines and primary B cells. Thus, CD72 acts to send a signal resulting in cell death by

decreasing the activation threshold level of the BCR in immature B cells. However, if CD72 is pre-ligated its tyrosine phosphorylation levels are decreased and B cell tolerance levels are increased preventing BCR mediated apoptosis. Thus, SHP-1 acts to keep CD72 dephosphorylated and prevent BCR signalling from causing cell death.

1.3 Fc Receptors

Produced by B cells, antibodies (immunoglobulins, Ig) are constructed from heavy and light polypeptide chains. Two heavy (H) chains and two light (L) chains are joined by disulphide bridges and a flexible hinge region to construct a Y-shaped molecule (**Figure 1.14**). Proteases have been used to dissect the structure of antibody molecules and to determine which parts of the molecule are responsible for its various functions. The $F(ab')_2$ fragment has exactly the same binding characteristics as the original antibody but is unable to interact with any effector molecules or receptors. This is exemplified by the fact that intact antibodies that experimentally ligate the BCR are poor polyclonal B cell activators, in comparison to $F(ab')_2$ fragments, because the Fc portion is able to interact with an inhibitory Fc receptor (FcγRIIb) expressed by the B cells.

By binding the Fc domain (Fc) of antibodies, Fc receptors (FcRs) provide a critical link between the humoral and cellular arms of the immune system through the targeting of antigen-antibody complexes to effector cells and priming of an immune response. Despite being members of the same family, FcRs exhibit rich structural and functional diversity. Indeed, there exists a separate FcR for every class of Ig; IgA (FcαR), IgD (FcδR), IgE (FcεR), IgG (FcγR) and IgM (FcμR) (Ravetch and Kinet, 1991; Raghavan and Bjorkman, 1996). With the exception of the FcR for IgE (CD23) and the polyimmunoglobulin transporters for IgM/IgA (pIgR) and neonatal gut IgG (FcRn) (Raghavan and Bjorkman, 1996) most human and murine FcR are members of the immunoglobulin domain super family (IgSF) (Ravetch and Kinet, 1991; Daeron, 1997a; Daeron, 1997b). This group includes the high affinity receptors for IgE (FcεRI), IgA (FcαRI) and all the IgG receptors (FcγR).

Recent studies have also reported the identification of a new family member, Fcα/μR, that binds to both IgM and IgA (Shibuya *et al.*, 2000). Fcα/μR is the first IgM receptor to be characterised on a molecular level in lymphocytes and macrophages and may play an essential role in both innate and adaptive immunity.

With the exception of some classes of FcγRII (section **1.3.5**), which possess an integral cytoplasmic signalling motif, the majority of these receptors exist as multisubunit receptor complexes (**Figure 1.15**). These complexes comprise a ligand binding chain and an associated signalling chain, such as the γ-chain. The ligand binding chains of these receptors are all type I membrane glycoproteins. The highly conserved extracellular regions contain two or three immunoglobulin (V-class) domains formed by disulphide loops of varying length. The transmembrane and cytoplasmic regions are less conserved. However, FcγRIIIb (section **1.3.6**) lacks a transmembrane region and is tethered to the membrane via a glycosylphosphatidyl linkage (GPI), preventing is association with a signalling chain.

1.3.1 Fcγ receptors

The Fcγ receptors (FcγRs) are specific for the Fc domain of immunoglobulin G (IgG). They comprise a multimembered family of structurally homologous but distinct receptors and are expressed on the vast majority of leukocytes. (reviewed by Daeron, 1997a) (**Figure 1.15**). Three main classes of FcγR exist, FcγRI, FcγRII and FcγRIII (Ravetch and Kinet, 1991; Hulett and Hogarth, 1994). In the mouse, single genes encode each of the classes whereas in humans multiple genes have been described for each class, encoding several forms of these receptors. With the exception of the murine FcγRI gene on chromosome 3, the FcγR genes are located on chromosome 1 in both humans and mice. As products of alternative transcript splicing or receptor proteolysis, FcγRs are able to exist as both membrane receptors and as soluble molecules.

Soluble immunoglobulin binding factors (IBFs) specific for each immunoglobulin isotype have also been described (Fridman *et al.*, 1992). Most FcγR expressing

cells can produce binding factors in response to various cytokines and the interaction with immunoglobulin. Factors that influence the expression of the membrane forms of FcγR correlate with the production of soluble FcγRs. Soluble FcγRs arise by different mechanisms; via stop codons in the extracellular domains (FcγRIb1 and FcγRIc), via alternative splicing events (FcγRIIa2) or via proteolytic cleavage (FcγRIIIa and FcγRIIIb). Proteolytic cleavage of the murine membrane bound FcγRIIb2, between the transmembrane and first cytoplasmic domains, creates a soluble form of FcγRIIb3. (Sautes *et al.*, 1992). Interestingly, the shedding of FcRs by human B cells directly correlates with the upregulation of trypsin like serine-proteases within activated B cells (Sarmay *et al.*, 1991).

Although the biological relevance of soluble FcγRs or soluble IgG-binding factors is unclear, the immunomodulatory potential of these receptors has generated substantial interest. Binding factors have been shown to modulate the *in vitro* synthesis of immunoglobulins and to regulate antibody production in an isotype-specific manner. Human serum contains high levels of soluble FcγRIIIb, released by serine protease activity on neutrophils. Increased levels of sFcγRIIIb have been shown to be associated with significantly lower risk of infection, which may be accounted for by the ability of sFcγRIIIb to induced cytolysis in certain cell types (Hoover *et al.*, 1995). Moreover, sFcγRII has been shown to suppress antibody production in cell culture (Varin *et al.*, 1989).

1.3.2 FcγRs display different binding affinities for IgG

The three distinct classes of FcγR, FcγRI, FcγRII and FcγRIII, are defined by their cellular distribution, structure and affinity for the IgG subclasses (**Figure 1.15** and **Table 1.2**) (Hulett and Hogarth, 1994). There are four known subclasses of IgG, IgG1, IgG2, IgG3 and IgG4, differing only slightly in their amino acid sequence. Each subclass varies in its size, carbohydrate content, half life and binding affinity to the individual FcγRs. Humans possess all four subclasses whilst the murine system lacks IgG4 and produces IgG2 variants (IgG1, IgG2a, IgG2b and IgG3). The high affinity FcγRI (CD64) receptor, is the only class of FcγR capable of recognising and binding monomeric IgG at

physiological concentrations (section **1.3.4**) (Allen and Seed, 1989). In humans, FcγRI binds IgG1 with higher affinity than IgG3 or IgG4 but is unable to bind IgG2. In contrast, FcγRII (CD32) (section **1.3.5**) and FcγRIII (CD16) (section **1.3.6**) are low affinity receptors which can only recognise IgG in the form of an immune complex (Van de Winkel and Capel, 1993; Raghavan and Bjorkman, 1996; Hulett and Hogarth, 1994). Interestingly, whilst human FcγRIIa and FcγRIIb display high homology in their extracellular domains they differ in their IgG subclass binding affinities; FcγRIIa is unable to bind IgG4 whilst FcγRIIb is unable to bind IgG2 (Van de Winkel and Capel, 1993). Thus, IgG subclass plays an important role in the regulation of the immune response as it dictates which FcγRs are ligated and therefore determines the effector mechanism elicited. In particular, a variation in subclass type may affect the rate of immune complex clearance from the system, which has implications for immune complex-mediated diseases (see **Chapter 4**).

IgG subclass can also affect the activation of complement by immunoglobulin. The term complement constitutes a complex group of serum proteins that mediate inflammatory reactions and are essential for innate immunity against bacterial pathogens. In particular, complement is thought to aid host defence by directly opsonising and lysing bacteria and by activating other immune cells. IgG1 and IgG3 mediate many of the effector mechanisms involved in this cascade, whilst IgG2 is less effective and IgG4 displays no binding affinity for the functional proteins within the complement system. Thus, IgG subclass will directly influence whether a host is susceptible to infection by its ability to recruit complement or FcγR bearing effector cells.

1.3.3 FcγR biological responses

The diversity of biological responses elicited by FcγR classes is presumably accounted for by the divergence in cytoplasmic tail domains of these receptors, as the extracellular domains are structurally related. However, when expressed in different types of cells, FcγR tend to trigger a cell type specific response, suggesting that the response triggered by FcγR depends on the cell type more so than on the receptor (Daeron, 1997a). Ligand occupation and subsequent

aggregation of FcγR can result in ligand internalisation, by a process of endocytosis or phagocytosis, and/or cell activation. FcγR-mediated cell activation can lead to cytokine release, antibody directed cellular cytotoxicity (ADCC), degranulation and induction of cytokine gene transcription.

Despite being structurally and functionally different, the initiation of cell activation by the FcγRs is similar to that of other immunoreceptors. The signalling pathways recruited by several FcγRs depend upon the conserved immunoreceptor tyrosine based activation motif (ITAM) (Reth, 1989), previously discussed in the context of the BCR signalling accessory molecules Ig-α/Ig-β. The ITAM motif is present in the cytoplasmic tails of certain FcγRs or in their associated signalling molecules, such as the γ-chain (**Figure 1.15**). An atypical ITAM is found in the tails of FcγRIIa and FcγRIIc, where 12 variable residues, instead of 7, separate the conserved Yxx(I/L) sequences (Van den Herik-Oudijk *et al.*, 1995). Phosphorylation of the tyrosine residues in these ITAM motifs is necessary and sufficient to initiate signals transduction by these FcγRs (Cambier, 1995). The phosphorylated ITAMs then recruit and activate further downstream effector molecules, in a manner similar to that of the BCR.

As studies into ITAM-containing immune receptors and their associated signalling pathways continued, it was discovered that an additional group of FcγRs were capable of producing inhibitory signals. Studies on FcγRIIb, a known inhibitor of B cell activation, led to the discovery of the alternative immunoreceptor tyrosine-based inhibition motif (ITIM) (Amigorena *et al.*, 1992a and 1992b; Muta *et al.*, 1994). Subsequent studies found an identical motif within the cytoplasmic domains of other 'inhibitory' receptors, including the NK cell inhibitory receptors (KIRs), the B cell co-receptor, CD22, and the T cell negative regulator CTLA-4 (Daeron, 1997b; Fong and Cambier, 1999). The consensus sequence of the ITIM is reminiscent of the ITAM, with a leucine residue at position Y+3 following the critical tyrosine residue (**Table 1.1**), although the characteristic double YxxL motif of the ITAM is replaced by a single YxxL motif. Further analysis of the sequences flanking the inhibitory motif resulted in the observation of a conserved valine or an isoleucine residue at

position Y -2. Thus, the consensus motif V/IxYxxL constitutes the minimal structural requirement for the ITIM, such that mutation of the tyrosine is sufficient to abrogate the inhibitory properties.

When activated by immune complexes, ITIM containing FcγRs are involved in capping, endocytosis, and phagocytosis. However, inhibitory FcγRs are more commonly associated with negative regulation of other ITAM containing receptors. For example, FcγRIIb inhibition of the BCR on B cells and FcεRI and FcγRIII on murine mast cells (Ono *et al.*, 1996; Ujike *et al.*, 1999). Thus, FcγRs can positively or negatively regulate cell activation depending upon the cytoplasmic consensus motif that they employ for mediating signal transduction (**Figure 1.16**). A third group of FcγRs neither trigger nor inhibit cell activation. These include the polyimmunoglobulin transporters, involved in the transcytosis of immunoglobulins and FcγRIIIb, which lacks any intrinsic signalling capability but contributes to cell signalling via its association with other FcR (Raghavan and Bjorkman, 1996).

The main focus of this thesis centres on the expression and signalling aspects of FcγRIIb. However, the structural and signalling similarities to other members of the FcγR will also be discussed.

1.3.4 FcγRI

FcγRI (CD64) is a 70 kDa, type I membrane glycoprotein. It is constitutively expressed on the surface of macrophages, monocytes and can be induced on IFN-γ stimulated neutrophils and eosinophils (Van de Winkel and Capel, 1993; Hulett and Hogarth, 1994). FcγRI is a high affinity receptor and the only FcγR to bind monomeric IgG at physiological concentrations (Allen and Seed, 1989). IgG binding by FcγRI can result in ligand internalisation, ADCC or degranulation depending on the cell type. Three human FcγRI genes have been identified and mapped to chromosome 1q21.1 (Van de Winkel and Capel, 1993; Hulett and Hogarth, 1994). The receptor structure is comprised of three extracellular IgSF, V-like domains, a single transmembrane domain and a short cytoplasmic

domain with no known signalling motifs. The third extracellular domain is distinct, whereas the first two are homologous to the extracellular domains of FcγRII and FcγRIII, suggesting that the unique IgG binding characteristics of FcγRI are conferred by third domain (Allen and Seed, 1989).

Signal transduction occurs through the non-covalent association of FcγRI with γ-chain homodimers or FcγRIIa, both of which possess ITAMs necessary for activation of soluble tyrosine kinases. In the monocytic cell line, U937, the recruitment of FcγRII or the γ-chain depends on the differentiation state of the cell. In the monocytic-like cells, FcγRI mediates signal transduction via the γ-chain whilst the macrophage-like cells require FcγRIIa (Cameron *et al.*, 2001). The signal transduction unit employed has been shown to determine the signalling pathway and thus the cellular response elicited.

1.3.5 FcγRII

In contrast to FcγRI, the FcγRII (CD32) receptors display low affinity for IgG (K_A <1 x 10^7 M^{-1}). With the exception of FcγRIIa, which can signal by association with FcγRI, FcγRII isoforms are only activated through aggregation. This occurs *in vivo* when IgG is presented as an immune complex (Raghavan and Bjorkman, 1996; Van de Winkel and Capel, 1993; Hulett and Hogarth, 1994). In humans three distinct genes FcγRIIA, IIB and IIC, mapped to q23-24 on chromosome 1, encode a total of six transcripts. Separate exons encode the transmembrane and cytoplasmic domains; thus alternative splicing of mRNA transcripts can create several isoforms (Brooks *et al.*, 1989). All isoforms, with the exception of FcγRIIa2 (see below), are type 1 integral membrane glycoproteins comprising two extracellular IgSF domains, a transmembrane domain and a cytoplasmic domain of varying length. Due to the homologous nature (>95%) of the extracellular domains, divergence between the receptors is contained within the cytoplasmic domain signalling motifs (sections **1.3.2** and **1.3.3**). Together, FcγRII receptors are an interesting example of how different cellular responses can be initiated by structural similar proteins. Thus, placing significant importance on the signalling pathways that they recruit.

uman FcγRIIA encodes three transcripts, two of which encode identical membrane receptors (FcγRIIa1, known as FcγRIIa) whilst the third encodes a putative soluble product (FcγRIIa2). Primarily expressed by cells of the myeloid lineage, including monocytes, macrophages and neutrophils, the ITAM-containing FcγRIIa isoforms are able to mediate phagocytosis and endocytosis upon receptor ligation.

Human FcγRIIB encodes three transcripts, FcγRIIb1, FcγRIIb2 and FcγRIIb3, which arise by alternative splicing mechanisms. The mature forms of FcγRIIb1 and FcγRIIb3 are identical but they differ in their peptide leader sequences (Brooks *et al.*, 1989). FcγRIIb3 lacks the information required for surface expression, encoded on the S2 exon, and is thus not expressed. FcγRIIb2 is capable of mediating rapid IgG endocytosis by means of clathrin-coated vesicles. However, an insertion of 19 amino acids in the cytoplasmic tail of FcγRIIb1 disrupts the cytoskeletal attachment domain responsible for modulating receptor internalisation (**Figure 1.17**) (Daeron, 1997a). FcγRIIb isoforms are widely expressed by cells of haematopoetic origin, with preferential expression of FcγRIIb1 on B cells and FcγRIIb2 on myeloid cells. All isoforms of FcγRIIb possess an inhibitory ITIM motif in their cytoplasmic domains and *in vitro* and *in vivo* studies have shown that FcγRIIb acts as a negative regulator of immune complex-triggered activation (section **1.4**) (Ono *et al.*, 1996; Ujike *et al.*, 1999).

Human FcγRIIC encodes only a single transcript, which predicts a receptor comprising of the extracellular domains of FcγRIIb bearing an ITAM containing cytoplasmic domain, similar to that of FcγRIIa (Van de Winkel and Capel, 1993). Four known splice variants of FcγRIIc exist (FcγRIIc1-4) although their biological importance is unknown. Expression of FcγRIIc, in association with FcγRIIIa (section **1.3.6**), has recently been reported in NK cells (Metes *et al.*, 1994).

In contrast to humans, a single gene encodes FcγRII in mice, which maps to the Ly-17 locus on chromosome 1. Three integral membrane glycoproteins have

been isolated FcγRIIb1, FcγRIIb2 and FcγRIIb1'. No ITAM bearing FcγRII receptor isoforms exist in mouse. Similar to the human FcγRIIb isoforms, murine FcγRIIb1 and FcγRIIb2 are identical except for a 47 amino acid insertion in the first cytoplasmic exon of FcγRIIb1, which inhibits its endocytic capacity. An additional murine isoform exists as a result of a cryptic splice donor site in the first cytoplasmic exon, creating a murine homologue of human FcγRIIb1 (named FcγRIIb1').

1.3.6 FcγRIII

FcγRIII (CD16) is a low affinity IgG receptor that exists as a multimeric signalling complex (Daeron, 1997b; Van de Winkel and Capel 1993; Hulett and Hogarth, 1994). Two isoforms, with differential cell expression, exist in humans. FcγRIIIa is expressed mainly on NK cells and macrophages, whilst FcγRIIIb is present almost exclusively on neutrophils. FcγRIII are structurally similar to FγRII however they exhibit unique forms of membrane anchoring (Ravetch and Perussia, 1989). Whilst FcγRIIIa is an integral membrane glycoprotein, FcγRIIIb exists as a glycosylphosphatidylinositol (GPI)-linked receptor lacking any transmembrane or cytoplasmic domains. Signalling by FcγRIIIa is mediated by ITAM containing accessory molecules, recruiting γ-chain homodimers in macrophages and γ-chain or ξ-chain homo- or heterodimers in NK cells. No other Fc subunits or ITAM containing accessory molecules are known to associate with FcγRIIIb, but it is thought to signal in co-operation with FcγRIIa and complement receptors. The murine FcγRIII exists as a single, transmembrane bound isoform, with no FcγRIIIb equivalent. Signalling and cell-surface expression is mediated via its co-association with γ-chain subunits only.

1.4 Mechanisms of FcγRIIb-mediated negative regulation

Negative regulation of BCR-mediated activation was first recognised when passively administered antigen-specific IgG antibodies were shown to inhibit *in vivo* primary responses to that antigen (Chan and Sinclair, 1971). A molecular basis for this inhibition was revealed following the cloning of the genes for murine low-affinity IgG receptors, FcγIIb and FcγRIII (Ravetch *et al.*, 1986).

Inhibition by IgG-containing immune complexes required the co-ligation of the BCR with the Fc receptor, FcγRIIb (reviewed by Chan and Sinclair, 1971; Van de Winkel and Capel, 1993). Further studies demonstrated that FcγRIIb inhibited BCR-mediated activation when the two receptors were co-aggregated by antigen-IgG antibody complexes and/or anti-idiotypic antibodies (reviewed by Sinclair and Panoskaltsis, 1987). Similarly, on mast cells, co-aggregation of FcγRIIb with FcεRI (the high affinity receptor for IgE) or FcγRIII was shown to inhibit mast cell degranulation and the release of inflammatory mediators (Ono *et al.*, 1996; Fong *et al.*, 1996). Co-ligation with FcεRI is via IgE:antigen complexes, for which FcγRIIb has demonstrated low affinity (Choi *et al.*, 1996) and inhibition is dependent on the recruitment of SHIP by FcγRIIb (Ono *et al.*, 1996, Ujike *et al.*, 1999). Although the mechanisms underlying the negative regulation of BCR signalling are poorly understood, several of the well characterised cellular responses of BCR stimulation; phosphoinositide hydrolysis, influx of extracellular calcium, cellular proliferation and immunoglobulin secretion are all inhibited following co-engagement of FcγRIIb.

Following the discovery of the ITIM motif within the cytoplasmic domain of FcγRIIb and other inhibitory receptors, distinct pathways of inhibition have been described (Scharenberg and Kinet, 1998; Gupta *et al.*, 1997). The signalling pathways involve the tyrosine phosphorylation of the ITIM by protein tyrosine kinases and the recruitment of Src-homology domain 2 (SH2) containing proteins to the phosphorylated ITIM. The protein tyrosine phosphatases, SHP-1 and SHP-2 (Long *et al.*, 1997; Burshtyn and Long, 1999) and the inositol 5' phosphatase, SHIP (Ono *et al.*, 1996) have been shown to be the major proteins recruited by ITIM containing inhibitory receptors such as FcγRIIb, CD22 and CD72 in B cells, CTLA-4 in T cells and the family of Leukocyte Inhibitory Receptors (LIRs) (Daeron, 1997a/b; Unkeless and Jin, 1997; Vely *et al.*, 1997). These SH2-domain containing phosphatases act to dephosphorylate key signalling components downstream of the inhibiting receptors, thereby inhibiting normal cellular responses.

Early *in vitro* studies on FcγRIIb indicated that recruitment of the tyrosine phosphatases, SHP-1 and SHP-2, mediated the inhibitory effect of this receptor in B cells (D'Ambrosio *et al.*, 1995). SHP-2 has a broad expression profile and has been shown to mediate both positive and negative regulatory effects. Its association with the platelet-derived growth factor β (PDGF-β) receptor, leads to the recruitment of the Grb-2-SoS complex and MAPKinase activation (Bennett *et al.*, 1994). However, in human B cells, SHP-2 is thought to mediate FcγRIIb inhibition of the Ras/MAPKinase and PI-3-Kinase pathways by dephosphorylating its potential substrates SHIP and Shc (Koncz *et al.*, 1999).
In contrast, expression of SHP-1 appears to be limited to cells of the haematopoetic lineage. SHP-1 is widely accepted as a negative regulator of many immunoreceptors, including the B cell receptor, CD22, CD72, and the natural killer cell inhibitory receptor (KIR) (Daeron, 1997a/b; Vely *et al.*, 1997). The ligation of KIR on NK cells, by major histocompatibility (MHC) class I complexes, inhibits NK mediated cell lysis of target cells and cytotoxic T cell function (Burshtyn and Long, 1997; Long *et al.*, 1997; Vely *et al.*, 1997). This inhibitory effect requires the phosphotyrosine-dependent association of SHP-1; indeed the inhibitory function is prevented by expression of a dominant negative SHP-1 mutant. SHP-1 dephosphorylates the SH2 domain-containing leukocyte protein-76 (SLP-76), which is normally required for optimal activation of cytotoxic lymphocytes (Binstadt *et al.*, 1998). A newly identified ITIM bearing receptor, gp49B, is also expressed by NK and mast cells. Studies show that like KIR, gp49B signalling involves SHP-1 and this association is dependent on tyrosine phosphorylation of the ITIM motif (Wang *et al.*, 1999). The significance of this finding is, as yet, undetermined.

The negative regulatory role of SHP-1 was highlighted in SHP-1 deficient motheaten (*me*) and motheaten viable (me^v) mice. These mice, which are SHP-1 null or express a catalytically inactive form of SHP-1, respectively (Tsui and Tsui, 1994) display a phenotype of widespread autoimmunity as a result of multiple haematopoetic cell defects (Shultz *et al.*, 1997). In particular, B cells from *me* and me^v mice are hyper-responsive to stimulation via the BCR. Indeed, studies of these mice with transgenic sIg specific for hen egg lysozyme (HEL),

showed that B cell deletion could be induced using levels of antigen that would normally only result in anergy (Cyster and Goodnow, 1995). This suggests that by removing SHP-1, the threshold of activation of B cells is substantially lowered. Hence, SHP-1 deficient mice possess a reduced number of bone marrow $B220^+$ progenitor B cells and a decreased resting B cell population. A high percentage of the residual peripheral B cells are also spontaneously activated with a skewing towards the B-1 subset. As B-1 B cells are associated with autoantibody production and are often expanded under conditions of autoimmunity (Hayakawa and Hardy, 2000), it would explain the observation that these mice exhibit many autoreactive features such as hyper-gammaglobulinemia, increased autoantibody titres and immune complex deposition. Moreover, a recent study by Dustin *et al.*, 1999 showed that expression of catalytically inactive SHP-1 increased BCR-mediated calcium mobilisation and ErkMAPK activation suggesting that SHP-1 regulates both early and late events in B cell activation.

However, studies in these mice and chimeric receptor experiments in murine (A20) and chicken (DT40) B cell lines demonstrated that FcγRIIb-mediated inhibition was still functional. Thus, *in vivo,* FcγRIIb does not necessarily recruit SHP-1 or SHP-2 but rather recruits the inositol 5' phosphatase, SHIP (Chacko *et al.*, 1996; Ono *et al.*, 1997; Liu *et al.*, 1998). Thus, SHP-1 was shown to be dispensable for FcγRIIb-mediated inhibition of B cell antigen receptor activation (Nadler *et al.*, 1997). Conversely, the KIR cytoplasmic domain was shown to only associate with SHP-1. Interestingly, the binding of SHP-1 to the phosphorylated ITIM of FcγRIIb seems to be correlated with the amount of receptor aggregation (Sato and Ochi, 1998; Lesourne *et al.*, 2001). Thus, in conditions of low receptor aggregation SHIP appears to be preferentially utilised. However, when there are high levels of immune complexes and superclustering of receptors both SHIP and SHP-1 are recruited to FcγRIIb. Thus, FcγRIIb-associated SHP-1 activity may be involved in downregulating BCR signalling for example, by deactivating the Src-family PTK, Lyn. It is possible, therefore, that the conditions of BCR-FcγRIIb co-ligation determine the

selectivity of these phosphatases and that SHP-1 may act to enhance the downstream inhibitory effects of SHIP.

Studies with dominant negative SHIP mutants and knockout models have confirmed the inhibitory role of SHIP in mediating the FcγRIIb negative signal (Gupta *et al.*, 1997, Liu *et al.*, 1998, Huber *et al.*, 1998). SHIP is a 145 kDa, SH2-domain containing, 5'-inositol-polyphosphate phosphatase. It was initially cloned by its association with the adapter protein Shc in response to multiple cytokines (Ware *et al.*, 1996, Damen *et al.*, 1996). The catalytic domain bears homology to several 5'-inositol phosphatases, whilst the c-terminal region contains several PTB (phospho-tyrosine binding) domains and a proline rich region. *In vitro*, SHIP preferentially targets substrates that are phosphorylated on the D3 position of the inositol ring thus converting phosphatidylinositol 3,4,5 phosphate (PtdIns(3,4,5)P_3) to phosphatidylinositol 3,4 phosphate (PtdIns(3,4)P_2) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) to inositol 1,3,4-trisphosphate (Ins(1,3,4)P_3). Post-translational C-terminal truncated forms of SHIP, 135-, 125- and 110-kDa in size, have recently been identified (Damen *et al.*, 1998). The 110 kDa form possesses the same inositol 5' phosphatase activity as the 145 kDa form. It is therefore possible that different forms of SHIP are generated *in vivo* and perform distinct functions within haematopoetic cells. Similarly to SHIP-1, SHIP-2 has been shown to associate with FcγRIIb following ligation in A20 B cells and displays Shc binding activity (Muraille *et al.*, 2000). Moreover, its discovery in activated B cells may provide an alternative inhibitory mechanism for FcγRIIb on memory and plasma cells compared to resting B cells (Bruhns *et al.*, 2000).

Recruitment of SHIP to the tyrosine-phosphorylated ITIM of FcγRIIb, following co-ligation with the BCR, leads to a drastic reduction in levels of phosphoinositide hydrolysis, influx of extracellular calcium, cellular proliferation and immunoglobulin secretion. FcγRIIb abrogation of BCR activation by the hydrolysis of PtdIns(3,4,5)P_3 (Ono *et al.*, 1996) was thought to occur by the direct conversion of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 by SHIP or by inactivation of PI-3-Kinase (Damen, *et al.*, 1996). It was subsequently demonstrated that

SHIP-mediated hydrolysis of PtdIns(3,4,5)P_3 disrupts PH domain phosphoinositol lipid interactions and prevents the association of Btk and PLC-γ with the plasma membrane (Bolland *et al.*, 1998). The deletion of SHIP increases PtdIns(3,4,5)P_3 levels, resulting in increased Btk membrane association and hyper-responsive BCR signalling. The negative effects of SHIP on Btk recruitment can also be overcome by the expression of Btk as a membrane-associated chimera. Thus, SHIP-1 acts to inhibit the pathways leading to calcium mobilisation by interrupting PLC-γ recruitment to the membrane.

The ability of FcγRIIb to modulate calcium levels was supported by the observation that SHIP-deficient B cells display enhanced calcium mobilisation in response to BCR ligation (Okada *et al.*, 1998). FcγRIIb and BCR co-aggregation does not affect the initial mobilisation of calcium from B cell intracellular stores, probably because of direct G-protein mediated BCR-PLC-γ coupling to calcium release. However, FcγRIIb ligation affects the subsequent influx of calcium. SHIP has also been shown to convert Ins(1,3,4,5)P_4 to Ins(1,3,4)P_3 (Bolland *et al.*, 1998, Scharenberg, *et al.*, 1998). Ins(1,3,4,5)P_4 is thought to regulate capacitive calcium entry though a membrane channel and hence dephosphorylation of Ins(1,3,4,5)P_4 by SHIP would prevent extracellular calcium flux (Luckhoff and Clapham, 1992). Furthermore, BCR-mediated production of Ins(1,3,5)P_3 induces the release of calcium from intracellular stores (Berridge, 1993). However, SHIP-mediated hydrolysis of Ins(1,3,4,5)P_4 produces an alternative form of InsP_3 (Ins(1,3,4)P_3) that is unable to bind InsP_3R in the endoplasmic reticulum (Bolland, *et al.*, 1998, Scharenberg, *et al.*, 1998) (**Figure 1.18**). FcγRIIb may also attenuate these activation signals by interfering with the G-protein regulation of BCR-PLC-γ coupling, resulting in a reduction of the calcium influx (Bijsterbosch and Klaus, 1985; Bijsterbosch *et al.*, 1986).

Through its catalytic phosphatase domain, SHIP-1 also results in the partial inhibition of the survival factor Akt/PKB by hydrolysis of PtdIns(3,4,5)P_3 (Jacob *et al.*, 1999; Aman *et al.*, 1998) (**Figure 1.19**). However, the hydrolysis product, PtdIns(3,4)P_2 has also been shown to be involved in the positive regulation of

Akt activity (Downward, 1998). Thus, the inhibition of Akt may be as a result of upstream deregulation by the alternative inositol phosphatase, PTEN (reviewed by Cantley and Neel, 1999), which is able to dephosphorylate the 3' position of its inositol substrates (Maehama and Dixon, 1998). Indeed, a study by Stambolic *et al.,* 1998 confirmed that PTEN can negatively regulate Akt/PKB by dephosphorylating PtdIns(3,4,5)P_3. Furthermore, PTEN has also been shown to dephosphorylate PtdIns(3,4)P_2 suggesting that PTEN may directly counteract PI-3-kinase activation of Akt/PKB (Haas-Kogan *et al.*, 1998).

FcγRIIb ligation also inhibits BCR-mediated activation of the RasMAPK pathway. However, there is a lack of consensus as to the mechanism underlying this downstream effect. There are currently three proposed models; the first concerns the GTPase exchange factors (GEFs), SOS and Vav. SOS and Vav interact with PtdIns(3,4,5)P_3 at the plasma membrane via PH domain interactions. As discussed above, by hydrolysing PtdIns(3,4,5)P_3, SHIP-1 may prevent Ras activation by preventing the translocation of exchange factors to the plasma membrane.

The second and most popular of the models involves the ability of SHIP-1 to also act as an adapter protein and engage in PTB binding domain interactions. Phosphorylated SHIP-1 can recruit the adapter molecules Shc (p52/46) and Grb-2 and the association of SHIP-1 with Shc is enhanced upon BCR-FcγRIIb coligation (Chacko *et al.*, 1996). Thus, SHIP may indirectly inhibit RasMAPK activation by competing with SOS/Grb2 complexes for Shc binding (Tridandapani *et a l.*, 1997 and 1998). However, this interaction is not considered sufficient to mediate the inhibitory action of SHIP-1 on the RasMAPK pathway as multiple stimuli, including BCR aggregation and cytokine activation, can induce the association between SHIP and Shc, possibly via PTB binding domain interactions (Lamkin *et al.*, 1997).

Thirdly, a Ras GTPase activating protein (RasGAP) has been shown to be recruited by the adapter protein, p62Dok, in a SHIP-dependent manner following FcγRIIb co-ligation with the BCR (Tamir *et al.*, 2000a). By activating

RasGAP via p62Dok, FcγRIIb may enhance the conversion of active Ras-GTP to inactive Ras-GDP, thus preventing activation of the ErkMAPK pathway.

Overall, the recruitment and activation of SHIP by FcγRIIb results in the inhibition of multiple B cell signalling pathways and the modulation of both BCR-induced B cell activation and antigen internalisation (**Figure 1.20**). In addition, ligation of FcγRIIb alone on B cells has been found to be capable of generating an apoptotic signal (Ashman *et al.,* 1996; Ono *et al.,* 1997). Studies in the B cell line, DT40, demonstrated that this response was independent of SHIP recruitment, suggesting that FcγRIIb may directly couple to an apoptotic pathway in the absence of BCR-ligation. Additional work by Pearse *et al.,* 1999 demonstrated that failure to recruit SHIP, either by deletion of SHIP or mutation of FcγRIIb, resulted in enhanced FcγRIIb-triggered apoptosis. Further studies demonstrated that this SHIP-independent pathway lead to the Btk-dependent activation of JNK (**Figure 1.20**). Overall, these studies suggest that aggregation of FcγRIIb in B cells results in a stress response that leads to apoptosis and that SHIP recruitment following co-ligation with the BCR 'rescues' the cells from apoptosis. Interestingly, SHIP-1 may mediate this effect by recruiting the p85 subunit of PI-3-Kinase upon BCR-ligation and could act to regulate downstream events such as B cell activation-induced apoptosis. (Gupta *et al.*, 1999).

Pearse *et al.,* 1999 postulate that this mechanism, which is unique to B cells, may be of importance in the germinal centre, where FcγRIIb may be an active determinant in the negative selection of B cells. B cells that have undergone somatic hypermutation of their antigen-receptors are selected on the ability of their mutated receptors to bind antigen presented within the germinal centre. This process discriminates between B cells that display high-affinity cognate antigen binding and those with low-affinity, potentially cross-reactive specificities. Antigen that interacts with FcγRIIb alone will result in B cell apoptosis whilst antigen co-engagement of FcγRIIb with the BCR will promote survival. Thus, selection of B cells may represent the sum of opposing signals generated by the interaction of immunocomplexes with the BCR and FcγRIIb through pathways modulated by SHIP.

In addition, co-ligation of FcγRIIb1 with the BCR blocks the processing and presentation of BCR-bound antigen and thus can indirectly inhibit antigen dependent T-cell stimulation (Minskoff, *et al.*, 1998). In contrast, co-ligation of the endocytosis competent isoform, FcγRIIb2, does not inhibit these BCR-mediated functions. A potential model was recently proposed by Aman, *et al.*, 2001 to explain this negative inhibition of BCR endocytosis. It was believed that FcγRIIb1 might be excluded from the lipid rafts in resting B cells and that co-ligation resulted in the destabilisation of raft-dependent BCR signalling. However, a fraction of FcγRIIb1 was shown to be constitutively localised in the rafts which increased upon BCR-FcγRIIb1 co-ligation. Furthermore, SHIP was shown to be actively recruited to the lipid rafts following receptor co-ligation.

1.5 Aims and objectives of this study

To maintain homeostasis and tolerance to self-antigens, B cells require a balance of signals via activatory and inhibitory co-receptors. Indeed, animal models have demonstrated that a loss of suitable inhibitory signalling is frequently associated with the development of inflammatory responses and, in some cases, autoimmunity. By binding the Fc domain (Fc) of antibodies, Fc receptors (FcRs) provide a critical link between the humoral and cellular arms of the immune system through the targeting of antigen-antibody complexes to effector cells and priming of an immune response. Interestingly, B cells express only one form of Fc receptor for IgG, the low affinity IgG receptor, FcγRIIb. In contrast to the priming situation described above, ligation of FcγRIIb, by means of IgG-containing antigen-antibody complexes, negatively regulates the BCR response to antigen.

Moreover, *in vitro* and *in vivo* studies have shown that FcγRIIb displays differential inhibitory activities in B cells dependent on the nature of receptor-aggregation (**Figure 1.20**). Homo-aggregation of FcγRIIb, via non-cognate antigen-antibody complexes, generates a pro-apoptotic signal. In contrast, co-ligation of FcγRIIb with the BCR, by cognate antigen-antibody complexes, promotes B cell survival. However, co-ligation also results in the inhibition of BCR-mediated phosphoinositide hydrolysis, influx of extracellular calcium, cellular proliferation and immunoglobulin secretion. Furthermore, co-ligation acts as a negative feedback mechanism to switch off ongoing B cell responses once the pathogen has been cleared.

Experimentally the BCR can be mitogenically stimulated with $F(ab')_2$ fragments of anti-Ig antibodies alone. In contrast, intact anti-Ig antibodies result in the co-ligation of the BCR with FcγRIIb, resulting in the induction of anergy and commitment to apoptosis. Stimulation of B cells with a combination of both $F(ab')_2$ fragments and intact anti-Ig antibodies produces an alternative co-ligation event which leads to the abortive activation of BCR-mediated proliferation and mimics the negative feedback regulation of ongoing B cell

responses. The primary aim of this study was to investigate the differential signalling mechanisms following the two methods of FcγRIIb co-ligation in primary B cells and to define how FcγRIIb co-ligation modulates:

- BCR-mediated proliferation and survival signalling pathways, in particular the mitogen-activated protein kinase (MAPK) and PI-3-Kinase/Akt pathways.

- BCR-mediated regulation of cell cycle machinery and the recruitment of nuclear transcription components required for proliferation and the induction of immunomodulatory genes.

Having examined FcγRIIb signalling mechanisms in B cells from normal mice, the secondary aim of this study was to investigate FcγRIIb expression and function in B cells from established murine models of autoimmune disease. In particular, to investigate whether aberrant FcγRIIb-mediated negative regulation of B cell activation becomes defective during onset and progression of disease in the collagen-induced arthritis (CIA) and murine systemic lupus erythematosus (MRL-*lpr/lpr)* models.

B cells encode two isoforms of FcγRIIb, FcγRIIb1 and FcγRIIb2. While both isoforms of FcγRIIb are able to mediate negative regulation of B cell activation, FcγRIIb2 can also internalise IgG containing immune complexes. A sequence insertion in the cytoplasmic tail of FcγRIIb1 disrupts its ability to modulate receptor endocytosis. Therefore, it is possible that differences in the relative expression levels of the two isoforms of FcγRIIb may influence the regulation of B cell responses by immune-complexes and the overall susceptibility of an individual to developing autoimmune disease.

Thus, the final objective of this study was to investigate the expression levels of the specific FcγRIIb isoforms in peripheral blood cells from autoimmune patients. To determine whether differential FcγRIIb isoform expression is related to, or indicative of, disease susceptibility and progression.

Figure 1.1 Summary of the developmental stages of conventional B cells.

The stages of B cell development are marked by a series of changes in location and in the expression of immunoglobulin heavy and light genes, intracellular proteins, and surface markers. B cell development starts in the bone marrow (or foetal liver) with the commitment of haematopoetic stem cells (HSCs) to the B cell lineage, in an antigen-independent manner. Rearrangement of the heavy chain locus genes begins in the early pro-B stage. Cells are allowed to progress to the next stage if a productive rearrangement has been achieved. Although no functional immunoglobulin is expressed in late pro-B cells expression of accessory Ig-α/Ig-βheterodimers on the surface in association with calnexin has been demonstrated (Gong and Nussenzweig, 1996; Nagata *et al.*, 1997). The antigen-independent stage continues within the bone marrow, where pre-B cells express a pre-BCR consisting of cytoplasmic μ chain in combination with a surrogate light chain, V_{preB} and λ5. Successful light-chain gene rearrangements result in the surface expression of a complete IgM molecule. The immature B cells then enter the antigen-dependent stage of B cell development where recognition of self-antigen can lead to clonal deletion, receptor editing or clonal inactivation (anergy). Once in the periphery, the mature B cells migrate to the lymphoid follicles and following further selection stages, enter the mature B cell pool until they encounter antigen. Upon interacting with their specific antigen in conjunction with co-stimulatory signals from T_H cells, the B cell is activated. Depending on the nature of the signals, the mature B cell gives rise to antibody generating plasma cells or long-lived memory cells which contribute to lasting protective immunity (Adapted from Alt *et al.*, 1997).

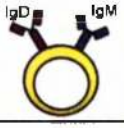
	B cells		Heavy-chain genes	Light-chain genes	Intra-cellular proteins	Surface Marker proteins	
ANTIGEN INDEPENDENT	Stem cell		Germline	Germline		CD34 CD45	BONE MARROW
	Early pro-B cell		D-J rearranged	Germline	RAG-1 RAG-2 TdT λ5, VpreB	CD34, CD45 MHCII CD10, CD19 CD38	
	Late pro-B cell		V-DJ rearranged	Germline	TdT λ5, VpreB	CD45R, CD40 MHCII, CD10, CD19 CD38, CD20	
	Large pre-B cell		VDJ rearranged	Germline	RAG-1 RAG-2 μ λ5, VpreB	CD45R, CD40 MHCII, preBCR CD10, CD19 CD38, CD20	
	Small pre-B cell		VDJ rearranged	V-J rearranged		CD45R MHCII, preBCR CD19, CD38, CD20, CD40	
	Immature B cell		VDJ rearranged μ heavy chain	V-J rearranged		CD45R MHCII, IgM CD19, CD20, CD21, CD40	
ANTIGEN DEPENDENT	Mature naive B cell		VDJ rearranged μ heavy chain	V-J rearranged		CD45R, MHCII IgM, IgD CD19, CD20, CD21, CD40	PERIPHERY
	Lympho-blast		VDJ rearranged secreted μ chains	V-J rearranged	IgM	CD45R MHCII CD19, CD20, CD21, CD40	
	Memory B cell		Isotype Switch to Cγ, Cα or Cε hypermut^n	V-J rearranged Somatic hypermut^n		CD45R, MHCII IgG, IgA CD19, CD20, CD21, CD40	
TERMINAL DIFFERENTIATION	Plasma cell		Secreted γ, α or ε chains	V-J rearranged	Ig	Plasma cell antigen -1 CD38	

Figure 1.2 The developmental stages of the B cell receptor (BCR) for antigen.

The progression of B cells from the pro-B cell stage to the mature B cell, is accompanied by changes in surface expression of the BCR accessory molecules, Ig-α/Ig-β, and associated molecules such as the chaperone molecule, calnexin, and the immunoglobulin heavy chain, μm. Progression through the developmental stages is driven by incremental signalling through these Ig-α/Ig-β-containing receptors, thus these molecules must be expressed as receptor complexes for normal B cell development. Successful B cell development is dictated by (1) expression of Ig-α/Ig-β in association with calnexin at the late pro-B cell stage. (2) Intact μ heavy chains expressed in an immunoglobulin-like "pre-BCR" complex with surrogate light chains, λ5 and VpreB at the pre-B cell stage. (3) A mature BCR consisting of μ heavy chain, conventional light chains and accessory Ig-α/Ig-β molecules from the immature B cell stage onwards.

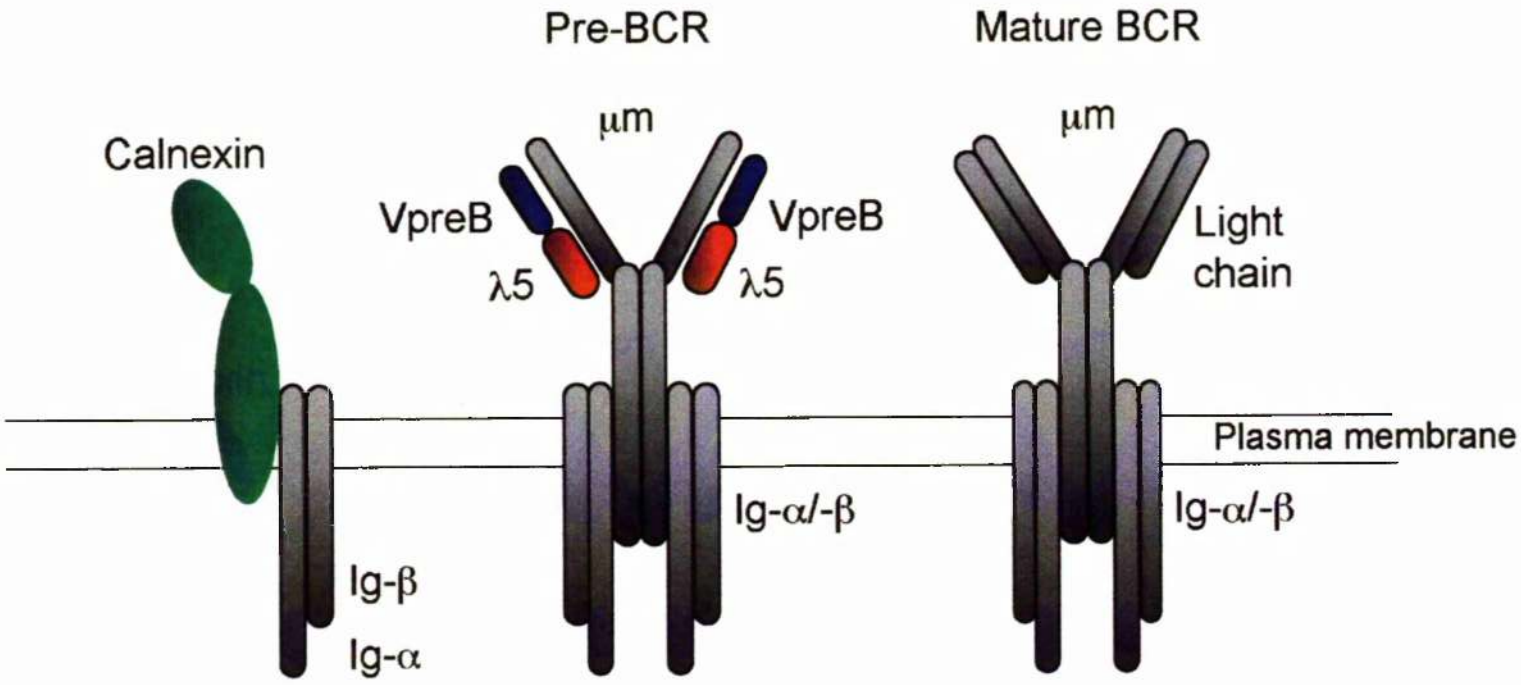

Pre-BCR
Mature BCR
μm
μm
Calnexin
VpreB
VpreB
λ5
λ5
Light
chain
Plasma membrane
Ig-α/-β
Ig-α/-β
Ig-β
Ig-α

Figure 1.3 Schematic representation of B cell activation and selection within a germinal centre.

Following T cell dependent activation (1) B cells migrate into the primary lymphoid follicles and form germinal centres. Here the proliferating B cells (2) differentiate into centroblasts (3) where they form the dark zone of the germinal centre. The rapidly dividing centroblasts undergo somatic hypermutation of their immunoglobulin variable-domain genes before differentiating into centrocytes (4). Within the light zone of the germinal centre, the small, non-dividing centrocytes are programmed to die unless they interact with follicular dendritic cells (FDC) that display complexed antigen on their cell surface. Positive selection of centrocytes is dependent on the affinity of their mutated antigen receptors. Centrocytes with low affinity or autoreactive antigen receptors undergo spontaneous apoptosis. The positively selected centrocytes move to the outer edge of the light zone and interact with CD40 ligand expressing T cells (5). Here the centrocytes may undergo CD40-mediated isotype switching, become protected from Fas-induced apoptosis and finally differentiate into either memory B cells (6) or plasma cells (7).

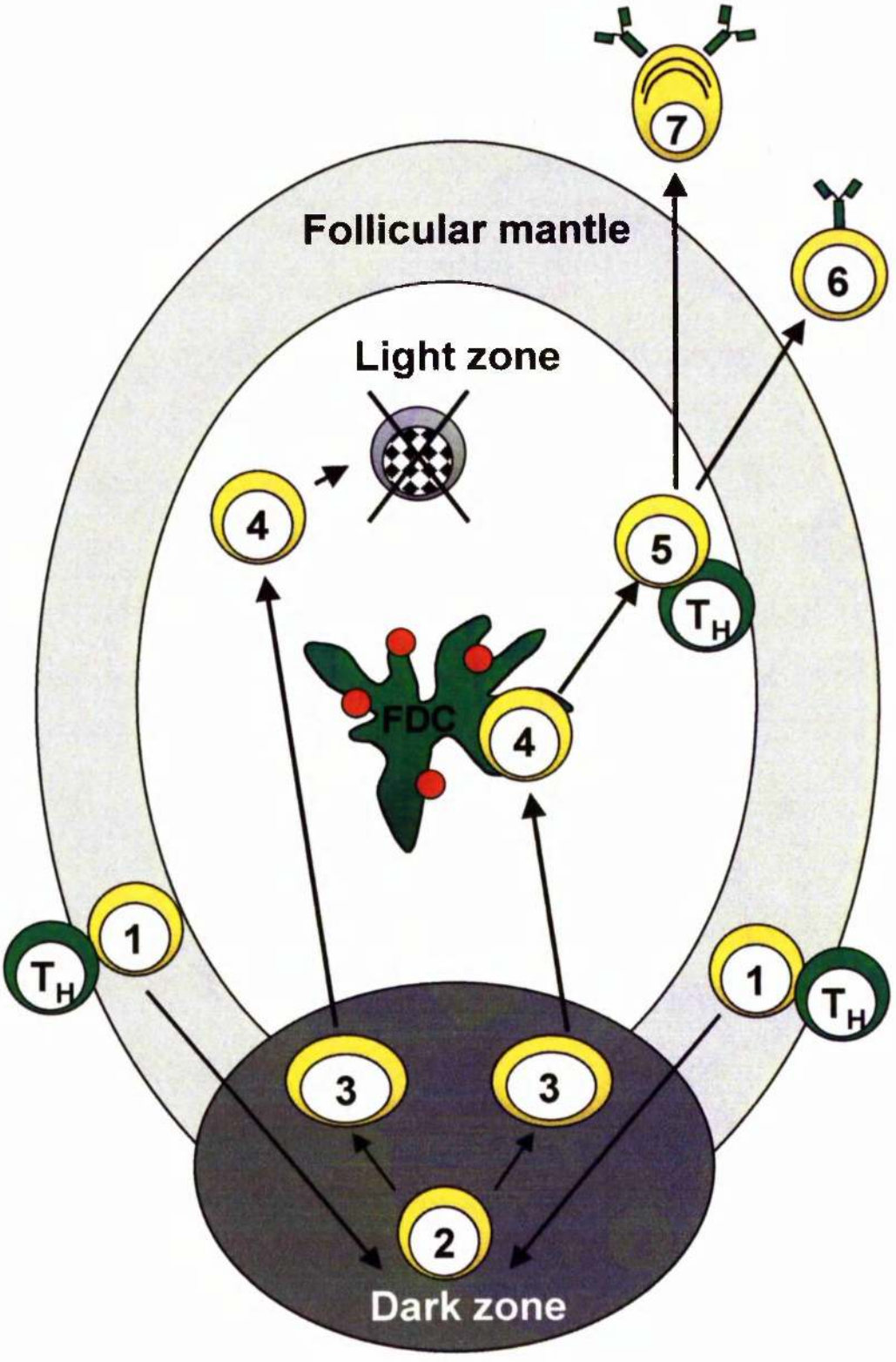

7
Follicular mantle
6
Light zone
4
5
T_H
FDC
4
1
T_H
1
T_H
3
3
2
Dark zone

Figure 1.4 The mature B cell receptor (BCR) for antigen.

The mature B cell receptor for antigen (BCR) is functionally divided into the ligand binding, immunoglobulin molecule (sIg) and the signal transducing heterodimers, Ig-α (CD79a) and Ig-β (CD79b). Conserved immunoreceptor tyrosine-based activation motifs (ITAMs), present in the cytoplasmic domains of the accessory molecules, are essential for the signal transducing capacity of the receptor. The ITAMs aid the recruitment and activation of three distinct types of non-receptor protein tyrosine kinases (PTKs).

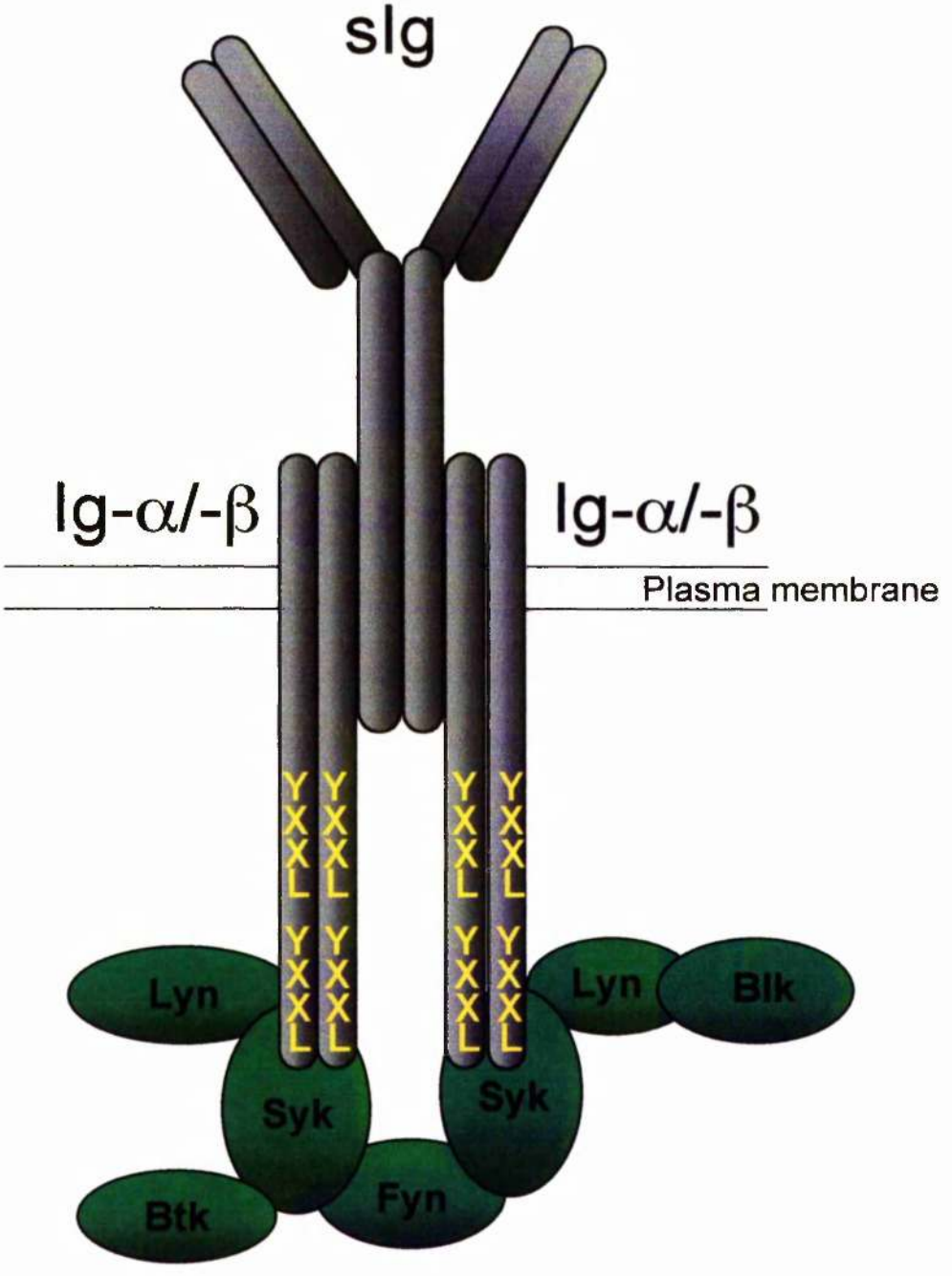

sIg
Ig-α/-β
Ig-α/-β
Plasma membrane
YXXL
YXXL
YXXL
YXXL
YXXL
YXXL
YXXL
YXXL
Lyn
Lyn
Blk
Syk
Syk
Fyn
Btk

Table 1.1 Immunoreceptor ITAM and ITIM sequence motifs.

Consensus and key receptor sequences for the immunoreceptor tyrosine-based activation motif (ITAM) and the immunoreceptor tyrosine-based inhibition motif (ITIM). Conserved residues are displayed in red.

Receptor or subunit chain	Motif	Motif sequence	Residues between consensus motifs
Consensus	ITAM	D/EXXYXXLX(7-11)YXXL/I	7-11
BCR Ig-α chain	ITAM	ENLYEGLNLDDCSMYEDI	7
BCR Ig-β chain	ITAM	DHTYEGLDIDQTATYEDI	7
Consensus	ITIM	XXXI/VXYXXLXXXXX	n/a
Human FcγRIIb	ITIM	ENTITYSLLMHPDA	n/a
Murine FcγRIIb	ITIM	ENTITYSLLKHPEA	n/a

Figure 1.5 The domain-structure and activation of the BCR-associated non-receptor protein tyrosine kinases.

(A) Three distinct types of non-receptor protein tyrosine kinases (PTKs) are known to be recruited and activated by the BCR ITAMs following BCR-ligation. These include the Src-PTKs (including Lyn, Blk and Fyn), Syk and the Tec-family kinase, Bruton's tyrosine kinase (Btk). Src-homology 2 (SH2) domains are modules that bind to phospho-tyrosine containing proteins, whilst Src-homology 3 (SH3) domains preferentially bind proline-rich proteins. Pleckstrin-homology (PH) domains permit association of the protein with phosphoinositides.

(B) In resting B cells, phosphorylation of the inhibitory site within Src-family PTKs, by C-terminal Src kinase (Csk), keeps the kinase domain in a catalytically inactive conformation. Following BCR ligation, the inhibitory site is thought to be dephosphorylated by the transmembrane protein tyrosine phosphatase CD45. Release of inhibition and phosphorylation at the auto-phosphorylation site results in the activation and unfolding of the Src-family PTKs and phosphorylation of key tyrosine residues within the kinases. Activated PTKs are then able to phosphorylate the tyrosines within the BCR accessory molecules. A cascade of PTK activation then occurs, leading to the phosphorylation and activation of key downstream targets in the BCR-associated signal transduction pathways.

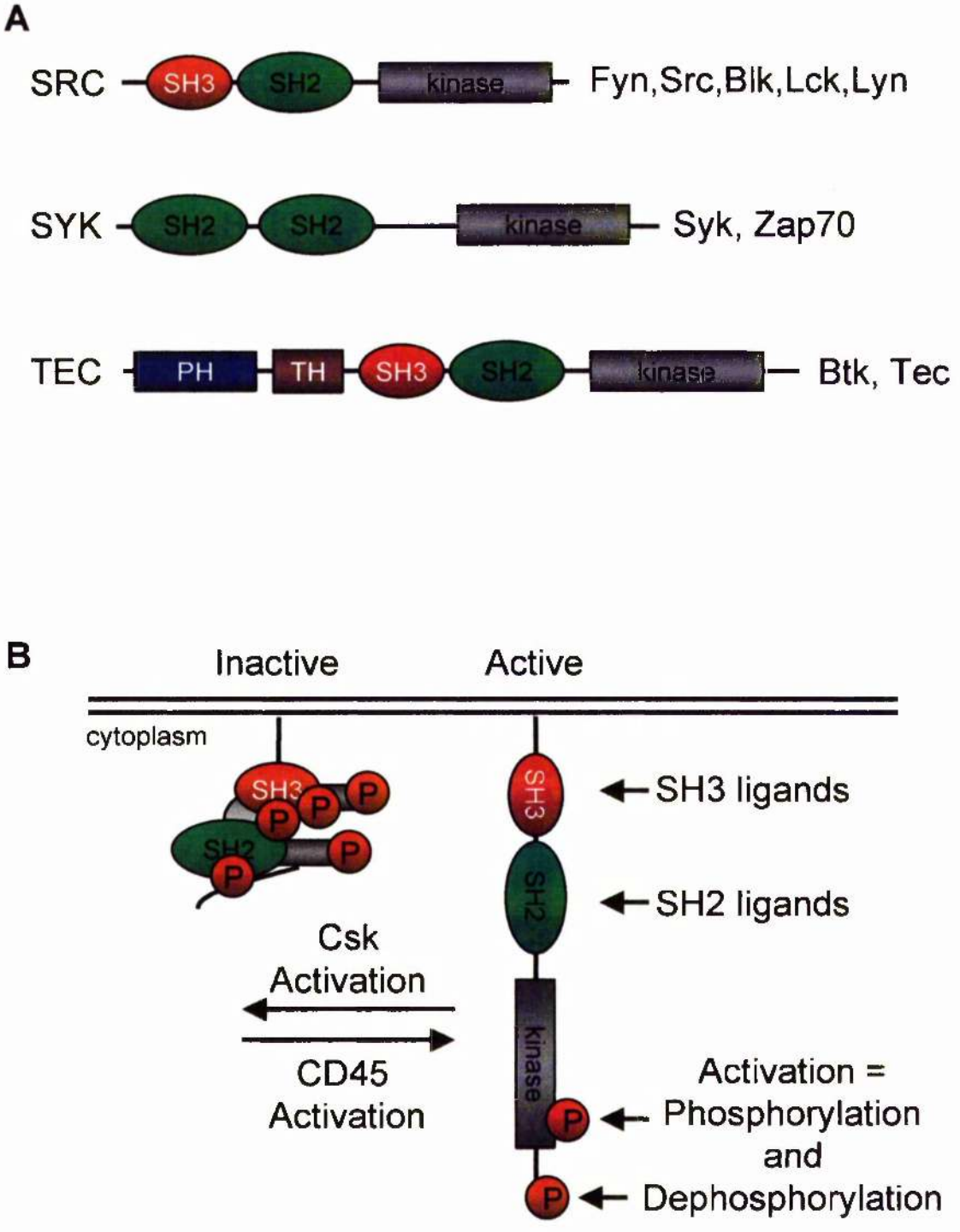

A
SRC
SH3
SH2
kinase
Fyn,Src,Blk,Lck,Lyn
SYK
SH2
SH2
kinase
Syk, Zap70
TEC
PH
TH
SH3
SH2
kinase
Btk, Tec
B
Inactive
Active
cytoplasm
SH3
SH2
P
Csk
Activation
CD45
Activation
SH3 ligands
SH2 ligands
kinase
Activation =
Phosphorylation
and
Dephosphorylation

Figure 1.6 B cell receptor (BCR) signal transduction pathways.

A schematic representation of the parallel cascades initiated following ligation of the B cell receptor (BCR) on mature B cells. The tyrosine phosphorylation of conserved ITAMs, present in the cytoplasmic domains of the sIg accessory molecules Ig-α and Ig-β, results in the recruitment of sIg associated PTKs. These include the Src-PTK family (Blk, Fyn, Lck, and Lyn), Syk, and Btk. Following activation of these kinases three parallel, but potentially cross-regulatory, pathways are recruited to the activated BCR complex. The phospholipase C-γ (PLC-γ) pathway results in the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2), to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($InsP_3$). The phosphatidylinositol 3-kinase (PI-3-Kinase) pathway generates phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P_3) whilst the classical Ras/MAPKinase cascade leads to the activation of ErkMAPK. These pathways converge on the nucleus and initiate activation-state dependent effector responses.

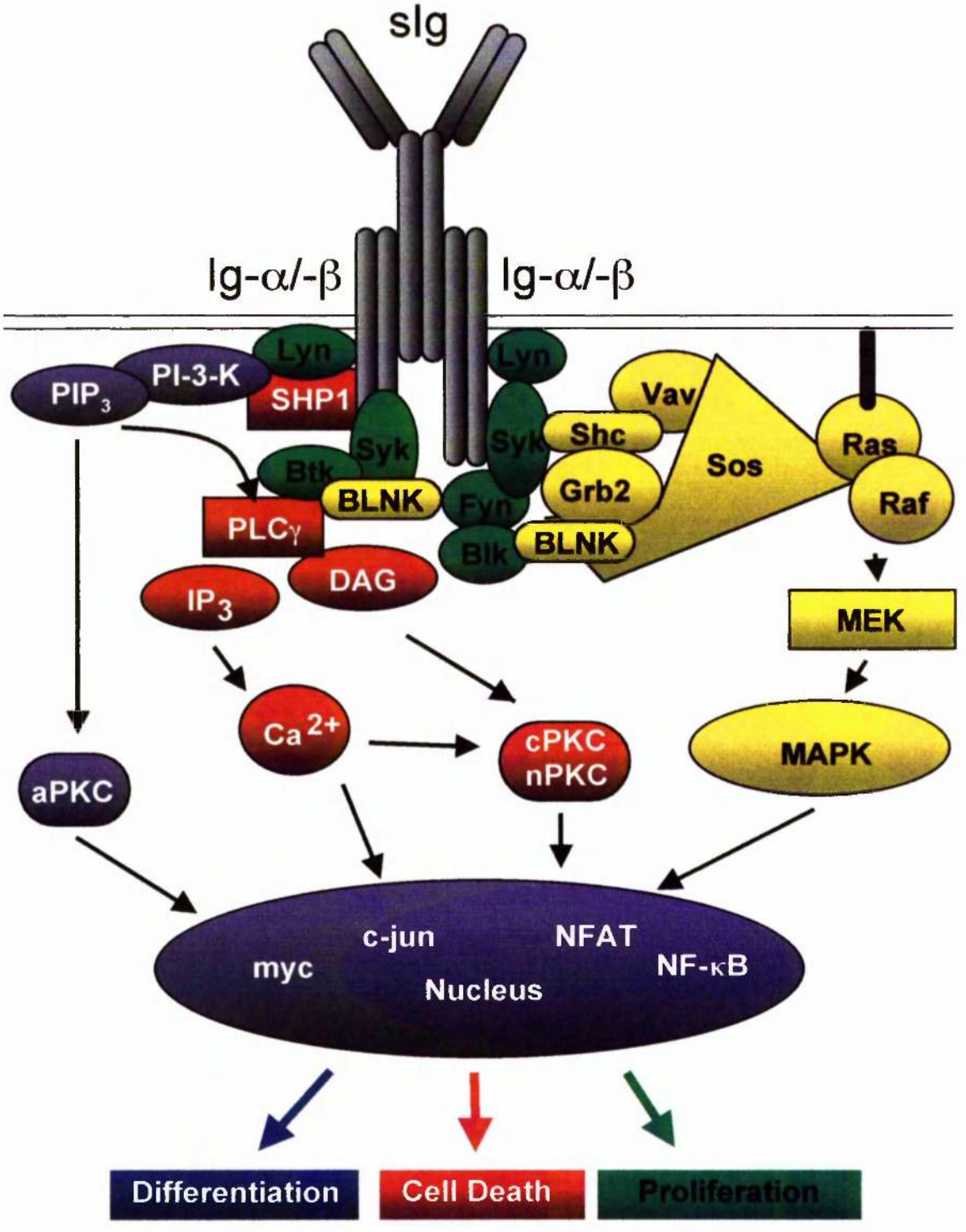

sIg
Ig-α/-β
Ig-α/-β
Lyn
PI-3-K
PIP3
SHP1
Lyn
Vav
Shc
Ras
Syk
Syk
Sos
Btk
BLNK
Grb2
Raf
Fyn
PLCγ
Blk
BLNK
DAG
IP3
MEK
Ca2+
cPKC
nPKC
MAPK
aPKC
c-jun
NFAT
myc
NF-κB
Nucleus
Differentiation
Cell Death
Proliferation

Figure 1.7 Schematic diagram of phosphoinositide signalling.

Phosphatidylinositol 3-kinase (PI-3-Kinase) catalyses the phosphorylation of the inositol phospholipids PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 on the 3' position of the inositol ring to produce PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are important regulators of membrane localisation signals. Several PH domain containing proteins selectively bind and are regulated by these phosphoinositides, including PLC-γ isoforms, small G-protein exchange factors (e.g. Vav), Tec family tyrosine kinases (e.g. Btk) and the serine/ threonine kinase, PDK1/2 and Akt/PKB. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are also substrates for the inositol 3' phosphatase, PTEN, which selectively dephosphorylate the 3' positions of the inositol ring. The inositol 5' phosphatase, SHIP can also dephosphorylate the 5' position of PtdIns(3,4,5)P_3. Thus, these two phosphatases act to antagonise the actions of PI-3-Kinase.

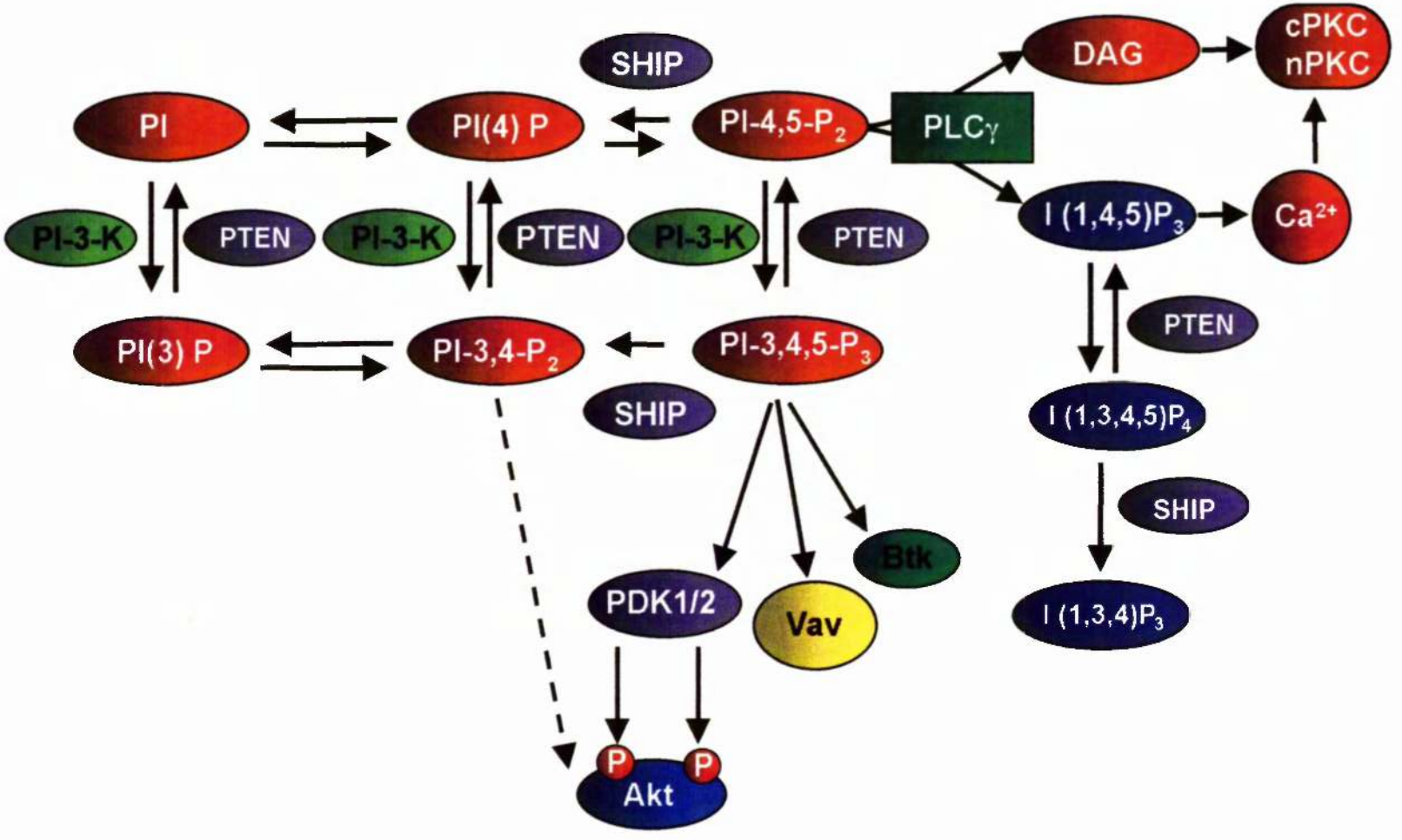

SHIP
PI
PI(4) P
PI-4,5-P2
PLCγ
DAG
cPKC
nPKC
PI-3-K
PTEN
PI-3-K
PTEN
PI-3-K
PTEN
I (1,4,5)P3
Ca2+
PI(3) P
PI-3,4-P2
PI-3,4,5-P3
PTEN
SHIP
I (1,3,4,5)P4
SHIP
Btk
PDK1/2
Vav
I (1,3,4)P3
P
P
Akt

Figure 1.8 The mitogen-activated protein kinase (MAPKinase) signalling pathways.

The mitogen-activated protein kinases (MAPKinase) are a family of serine-threonine protein kinases. They are activated by a wide range of extracellular stimuli and are able to mediate a wide range of cellular functions from proliferation and activation to growth arrest and cell death. The MAPKinase family is further classified into three sub-groups; the classical extracellular signal-regulated kinases (ErkMAPKinase), the c-Jun N-terminal kinases, also known as the stress activated protein kinases (JNK/SAPK) and the p38 MAPKinases. Activation of each sub-group is determined by distinct upstream MAPKinase kinases (MEKs), which are themselves activated by specific MAPKinase kinase kinases (MEKK). MAPKs are activated by dual phosphorylation on tyrosine and threonine residues, located in a T-x-Y motif. Each sub-group of MAPKinase is independently activated and regulated by a specific MEKK and MEK following cell stimulation but extensive cross-talk mechanisms exist. Following MAPKinase activation there occurs independent activation of downstream transcription factors thus, ErkMAPKinase has been shown to activate Elk-1 and c-myc, JNK activates c-Jun and ATF-2 and p38 MAPKinase activates ATF-2 and MAX.

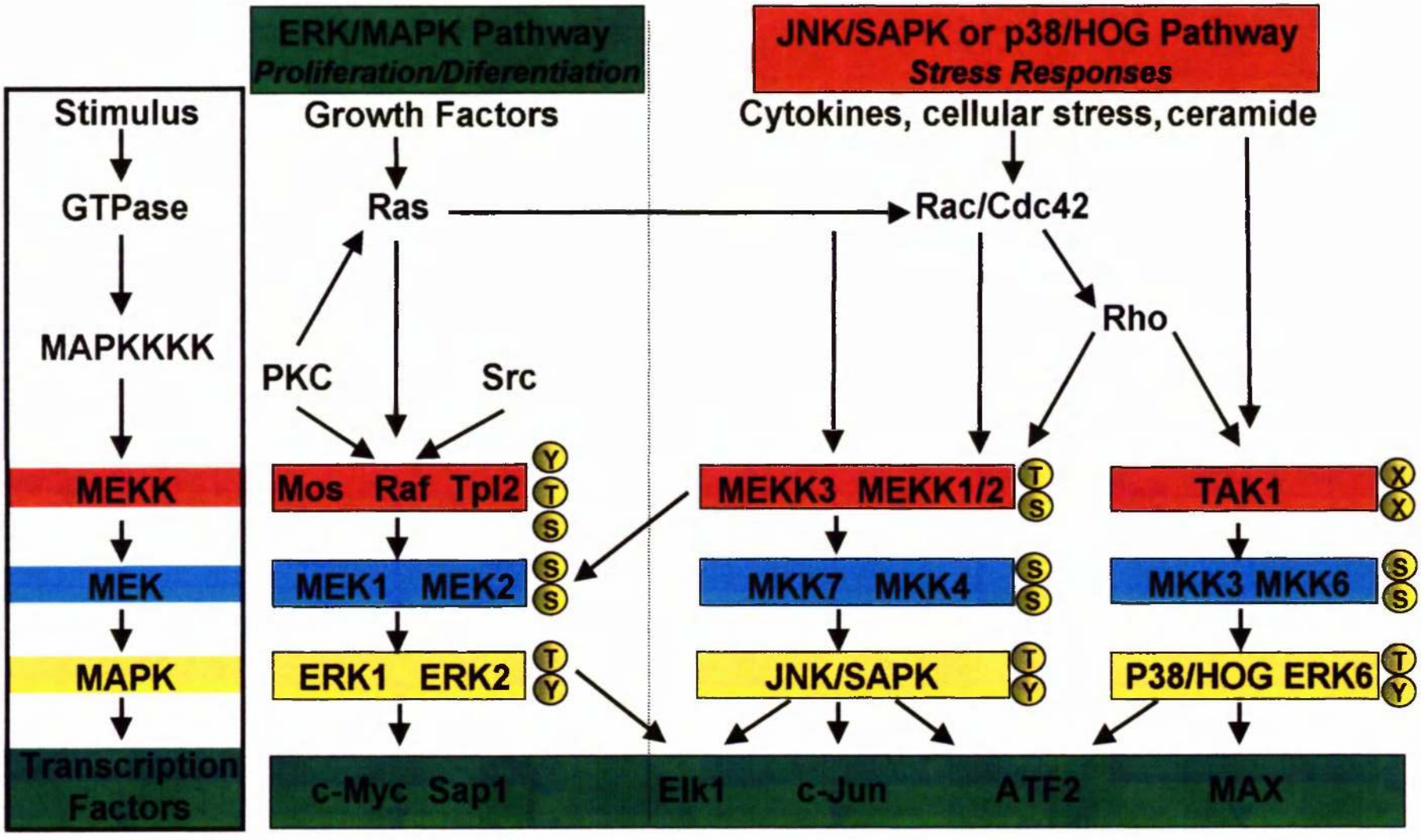

ERK/MAPK Pathway
Proliferation/Diferentiation
JNK/SAPK or p38/HOG Pathway
Stress Responses
Stimulus
GTPase
MAPKKKK
MEKK
MEK
MAPK
Transcription Factors
Growth Factors
Cytokines, cellular stress, ceramide
Ras
Rac/Cdc42
Rho
PKC
Src
Mos Raf Tpl2
MEK1 MEK2
ERK1 ERK2
MEKK3 MEKK1/2
MKK7 MKK4
JNK/SAPK
TAK1
MKK3 MKK6
P38/HOG ERK6
c-Myc Sap1
Elk1
c-Jun
ATF2
MAX

Figure 1.9 Regulation of GTPase activity by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs).

Small GTPases, such as Ras, Rac-1 and Rap-1, cycle between inactive GDP-bound and active GTP-bound states and thus function as signal relays linking membrane receptors to signal transduction pathways. GEFs (guanine nucleotide exchange factors) SoS, Vav and C3G, promote the exchange of GDP for GTP resulting in the production of an active GTPase to turn on downstream signalling effectors. GAPs (GTPase activating proteins) RasGAP, RacGAP and RapGAP, accelerate the normally slow intrinsic GTPase activity, thereby inactivating their target GTPases.

Following BCR ligation Shc-Grb-2-SOS complexes acts to displace GDP from the GTPase Ras to generate an active GTP-bound form. Ras is then able to regulate a wide range of downstream cellular processes, from cytoskeletal reorganisation through to modulation of transcription. In particular, active Ras can bind and derepress Raf-1, a serine/threonine kinase, resulting in the activation of MAPKinase kinases (MEK). Co-recruitment of the GEF Vav and PLC-γ by BLNK and Syk can also result in the activation of the Rho-family GTPases (Rac, RhoA and Cdc42) providing a critical link for the activation of the stress-activated MAPKs, JNK and p38. Activation of the alternative GTPase Rap-1 by the GEF C3G may lead to the sequestering of the downstream effector Raf-1, due to the structural similarities between the effector binding domains of Ras and Rap-1. This may lead to the down-regulation of MAPKinase activation, as shown for the induction of T cell anergy.

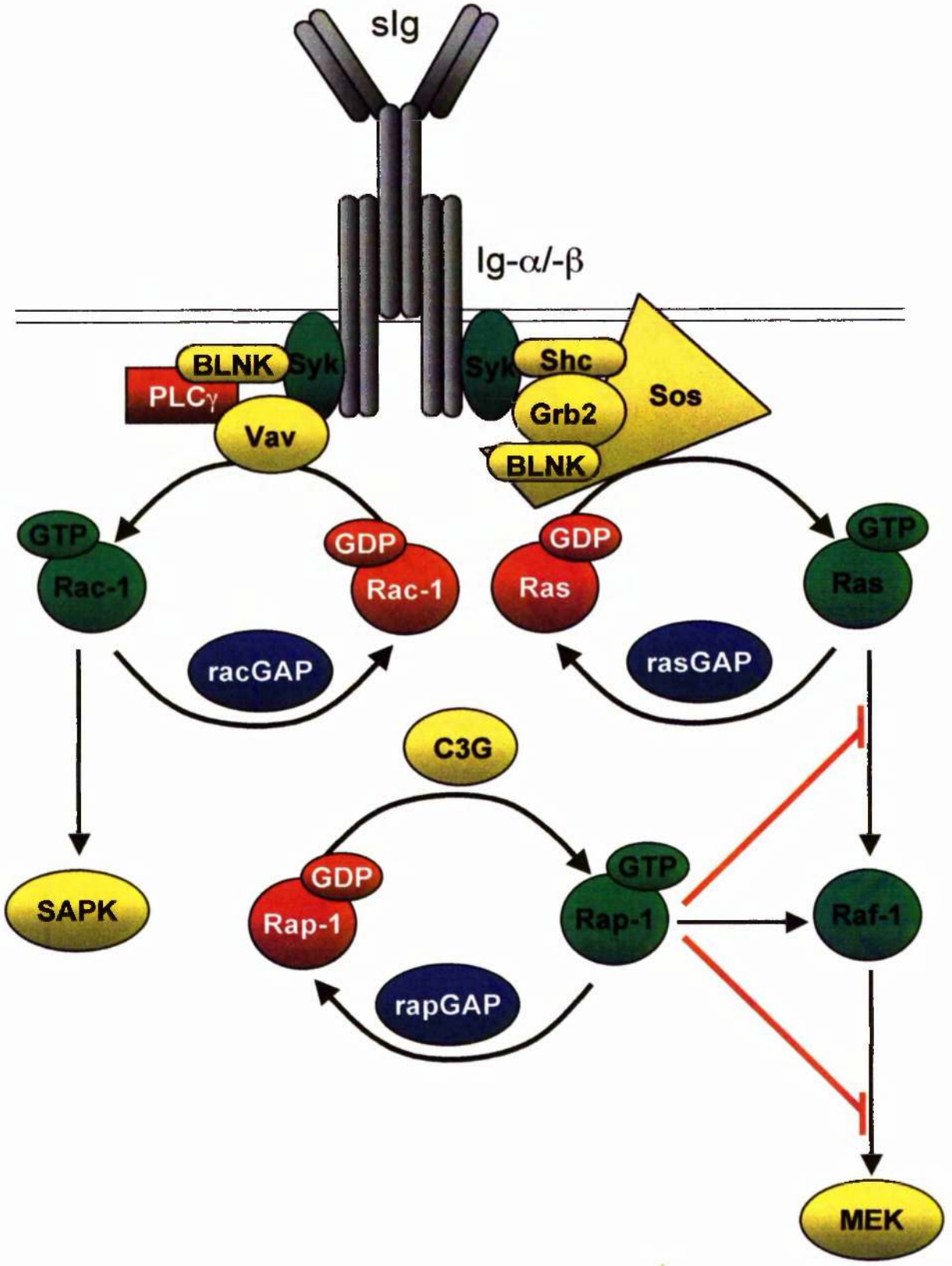

sIg
Ig-α/-β
BLNK
Syk
PLCγ
Vav
Syk
Shc
Grb2
Sos
BLNK
GTP
Rac-1
GDP
Rac-1
racGAP
GDP
Ras
GTP
Ras
rasGAP
C3G
SAPK
GDP
Rap-1
GTP
Rap-1
Raf-1
rapGAP
MEK

Figure 1.10 Important cell cycle stages.

The cell cycle comprises four main stages, during which a cell must duplicate its contents and check the integrity of duplication prior to division. In G_1 (gap 1) the cell increases in size, separates its centrioles and prepares to copy its DNA. DNA duplication occurs in the S phase (synthesis). After chromosome replication a second gap period, G_2, allows the cell to monitor DNA integrity and cell growth prior to M (mitosis) when the cell finally divides. The daughter cell products immediately enter G_1 and may go through the full cycle again or alternatively stop cycling temporarily and enter the G_0 phase. Cyclins and their activation of cyclin-dependent kinases (Cdks) are essential for the regulation of the cell cycle. At the G_1 checkpoint cells have to decide whether to commit to DNA synthesis. Here, D-type cyclins bind to Cdks 4 or 6 and the resulting complex promotes G_1/S transition by releasing the braking effect of retinoblastoma protein, pRb[105]. Hypophosphorylated Rb actively blocks cycling by sequestering the transcription factor, E2F, thus blocking expression of necessary S-phase genes. Once phosphorylated by the cyclin-Cdk complexes, E2-F is released and S phase genes are transcribed. Cdk inhibitory proteins of the INK4 (p15, p16, p18 and p19) and the WAF1 (p21, p27 and p57) families act to block the activity of Cdks at various stages of the cell cycle. Their inhibitory actions can restrain the cycle progression at the G_1/S boundary by promoting growth-arrest and/ or commitment to apoptosis.

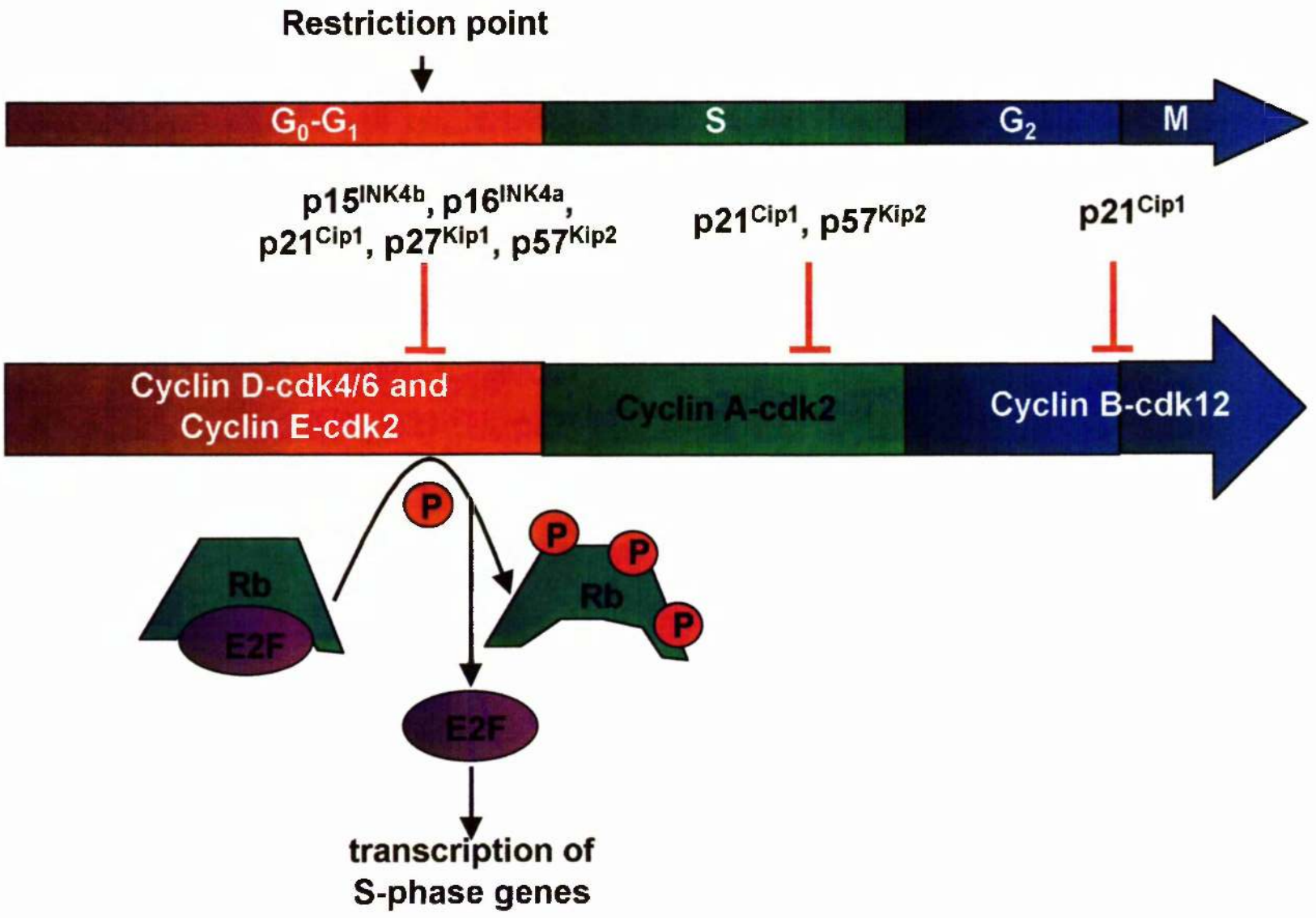

Restriction point
G0-G1
S
G2
M
p15INK4b, p16INK4a,
p21Cip1, p27Kip1, p57Kip2
p21Cip1, p57Kip2
p21Cip1
Cyclin D-cdk4/6 and
Cyclin E-cdk2
Cyclin A-cdk2
Cyclin B-cdk12
P
Rb
E2F
P
P
Rb
P
E2F
transcription of
S-phase genes

Figure 1.11 The cascade pathway of caspase activation.

The caspases are a family of cysteine proteases that are activated specifically in apoptotic cells. Most caspases are activated by proteolytic cleavage of an enzymatically inactive pro-caspase form. Each pro-caspase contains in its prodomain a protein-protein interaction module, which allows it to bind and associate with upstream regulators. Initiator caspases-8 and -10 contain a death-effector domain (DED), permitting interaction with death receptors such as CD95 (Apo-1/Fas) and the adapter molecule FADD (Fas-associated death domain protein). The effector caspases-2 and -9 contain a similar domain, a caspase activation and recruitment domain (CARD). Activation can occur simply by pre-exposure to another previously activated caspase, resulting in a 'caspase cascade' of activation. Upon ligand binding, CD95 aggregates and forms membrane bound signalling complexes. These complexes recruit, via the adapter molecule FADD the pro-caspase-8 molecule, resulting in caspase-8 activation through induced proximity. Following activation of the initiator caspases (8 and 10) a cascade of activation occurs. Caspase-8 activation also induces cytochrome c release from the mitochondria (**Figure 1.13**) and thus initiates a second apoptotic pathway. These two pathways converge in the activation of effector caspases-3, -6 and –7, which finally cleave various death substrates. Caspase-8 can also be activated by caspase-6 following cleavage by caspase-9, thereby amplifying the apoptotic signal.

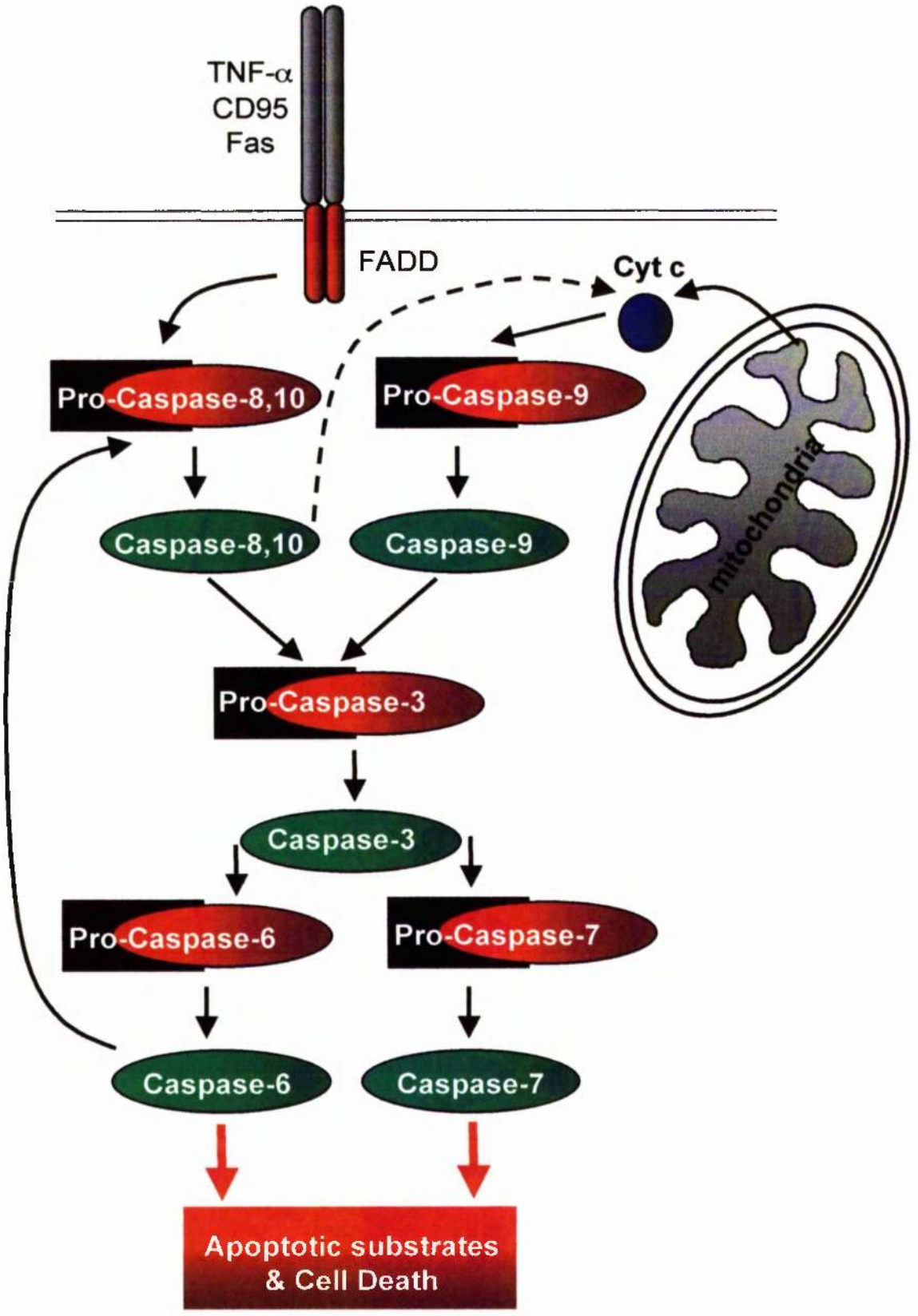
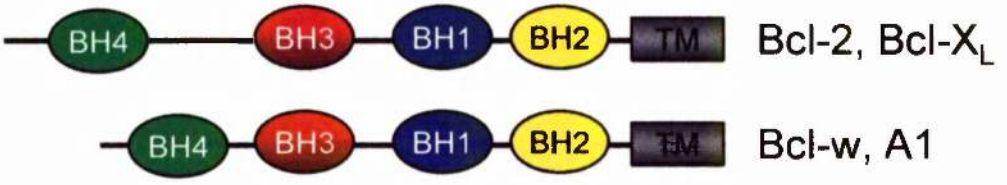

TNF-α
CD95
Fas
FADD
Cyt c
Pro-Caspase-8,10
Pro-Caspase-9
mitochondria
Caspase-8,10
Caspase-9
Pro-Caspase-3
Caspase-3
Pro-Caspase-6
Pro-Caspase-7
Caspase-6
Caspase-7
Apoptotic substrates
& Cell Death

Figure 1.12 The Bcl-2 family of apoptotic regulators.

The Bcl-2 family of apoptotic regulators is comprised of over a dozen proteins, which have been classified into three functional groups. All family members are structurally distinct due to conserved Bcl-2 homology (BH) domains. Group I members all possess anti-apoptotic activity, and protect cells from death whereas members of group II and III promote cell death. Many family members can homodimerise, but more importantly, pro- and anti-apoptotic members can form heterodimers. For example, pro-apoptotic Bax can heterodimerise with the anti-apoptotic protein, Bcl-2. This interaction blocks the anti-apoptotic capabilities of the Bcl-2 protein and induces a pro-apoptotic response.

Anti-apoptotic Bcl-2 family members (Group I)



Pro-apoptotic Bcl-2 family members (Groups II/III)

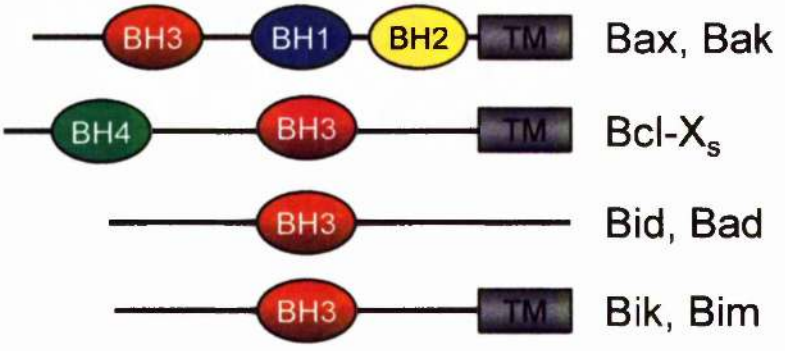


Figure 1.13 Role of mitochondria in the apoptotic response pathway.

Many of the signals leading to cell death appear to converge on the mitochondria, which plays a pivotal role in the decision of a cell's fate. For many, but not all of these apoptotic signals, activation of apoptosis regulator proteins, the Bcl-2 family is central to the mechanism. The Bcl-2 family proteins form homo- or heterodimers and integrate competing pro- and anti-apoptotic signals at the surface of the mitochondria and other sites within the cell. The pro-apoptotic members of the Bcl-2 family have been proposed to act by inactivating dimers of anti-apoptotic Bcl-2 family members, which act to preserve mitochondrial integrity. For example, following apoptotic signals, the pro-apoptotic regulator Bax, is believed to heterodimerise with the anti-apoptotic regulator Bcl-2. This interaction blocks the anti-apoptotic capabilities of the Bcl-2-like proteins. Similarly, the pro-apoptotic regulator Bad is believed to interact with Bcl-2 or Bcl-X_L, thus promoting apoptosis. Phosphorylation of Bad by the survival factor Akt promotes the association of Bad with the phospho-serine binding protein, 14-3-3 and its removal from the mitochondria. The blocking of the interaction of Bad with its other family members thus promotes cell survival. Pro-apoptotic signals also result in the cleavage of Bid by caspase-8 which releases an active, truncated form (tBid). tBid then translocates to the mitochondria, where it is involved in promoting the release of cytochrome c. The released cytochrome c associates with the adapter Apaf-1 and together activate caspase-9 in the cytosol. The death and mitochondrial pathways converge at the level of caspase-3 activation. Caspase 3 activation and activity is antagonised by the expression of inhibitors-of-apoptosis (IAPs) which in turn are inhibited by Smac/DIABLO proteins, also released from the mitochondria. The induction of apoptosis exemplifies the high degree of cross talk and integration between cell signalling pathways that are involved in the decision of a cells fate.

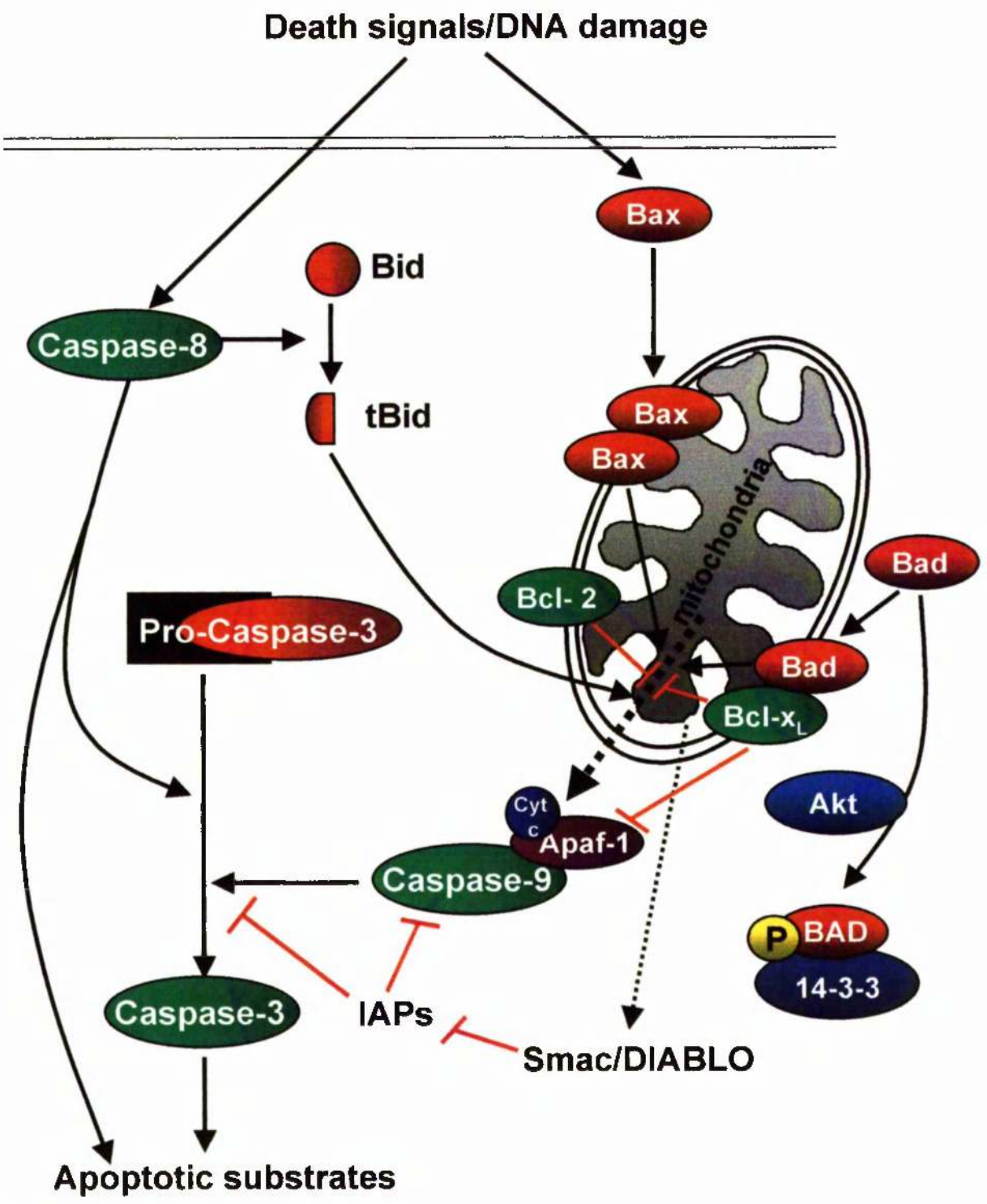

Death signals/DNA damage
Bax
Bid
Caspase-8
tBid
Bax
Bax
mitochondria
Bad
Bcl- 2
Pro-Caspase-3
Bad
Bcl-x$_L$
Cyt c
Apaf-1
Akt
Caspase-9
P
BAD
14-3-3
Caspase-3
IAPs
Smac/DIABLO
Apoptotic substrates

Figure 1.14 Schematic diagram of the domain structure of IgG.

Immunoglobulin G (IgG) is comprised of two heavy chains (H) linked to two light chains (L) by interchain disulphide bonds (red lines). The variable (V) and constant (C) domains are as indicated. Green circles represent N-linked carbohydrate groups, which vary between the isoforms of IgG. Each rectangular unit represents a complete immunoglobulin super-family (IgSF) domain. The Cγ domains are unique to IgG and, though very similar to each other, small structural differences confer specialised functions.

Proteases, such as pepsin and papain, have been used to dissect the structure of antibody molecules and to determine which parts of the molecule are responsible for its various functions. The variable Fab fragments (Fragment antigen binding) contain the antigen-binding activity of the antibody. In contrast, the Fc fragment (Fragment crystallisable) contains no antigen-binding ability but is able to interact with effector molecules of the complement cascade and Fc receptor (FcR) expressing cells. The $F(ab')_2$ fragment has exactly the same binding characteristics as the original antibody but is unable to interact with any effector molecules or receptors.

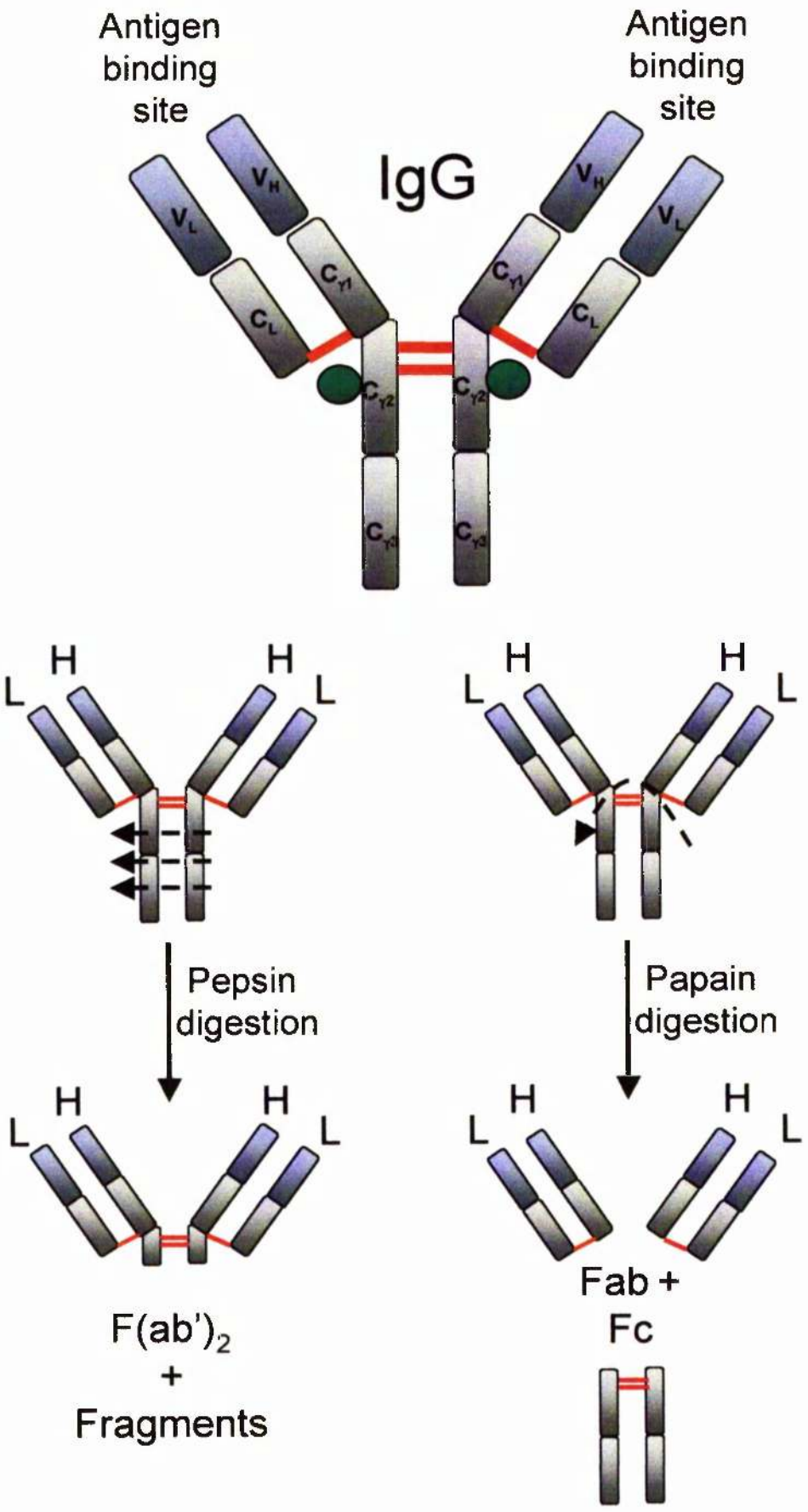

Antigen binding site
Antigen binding site
IgG
V_H
V_L
C_L
$C_{\gamma 1}$
$C_{\gamma 2}$
$C_{\gamma 3}$
H
L
Pepsin digestion
Papain digestion
$F(ab')_2$ + Fragments
Fab + Fc

Figure 1.15 Structure of Fc receptors and their associated signalling chains.

Most human and murine Fc receptors are members of the immunoglobulin domain super family (IgSF). This group includes the high affinity receptors for IgE (FcεRI), IgA (FcαRI) and all the IgG receptors (FcγR). The Fcγ receptors (FcγRs) are specific for the Fc domain of immunoglobulin G (IgG). They comprise a multi-membered family of structurally homologous but distinct receptors and are expressed on the vast majority of leukocytes. Three classes of FcγR exist, FcγRI, FcγRII and FcγRIII. As products of alternative transcript splicing or receptor proteolysis, FcγRs are able to exist as both membrane receptors and as soluble molecules.

Activation (ITAM) and or inhibition (ITIM) signalling motifs are located within the cytoplasmic domains of the Fc receptors or their associated signalling molecules, as indicated. With the exception of some classes of FcγRII, which possess an integral cytoplasmic signalling motif, the majority of these receptors exist as multi-subunit receptor complexes. These complexes comprise a ligand binding chain and an associated signalling chain, such as the γ-chain, as shown. FcεRI expresses an additional β-chain, whilst FcγRIIIa is also capable of associating with the ζ-chain. The ligand binding chains of these receptors are all type I membrane glycoproteins. The highly conserved extracellular regions contain two or three immunoglobulin (V-class) domains formed by disulphide loops of varying length. The transmembrane and cytoplasmic regions are less conserved. FcγRIIIb lacks a transmembrane region and is tethered to the membrane via a glycosylphosphatidyl linkage (GPI), preventing is association with a signalling chain.

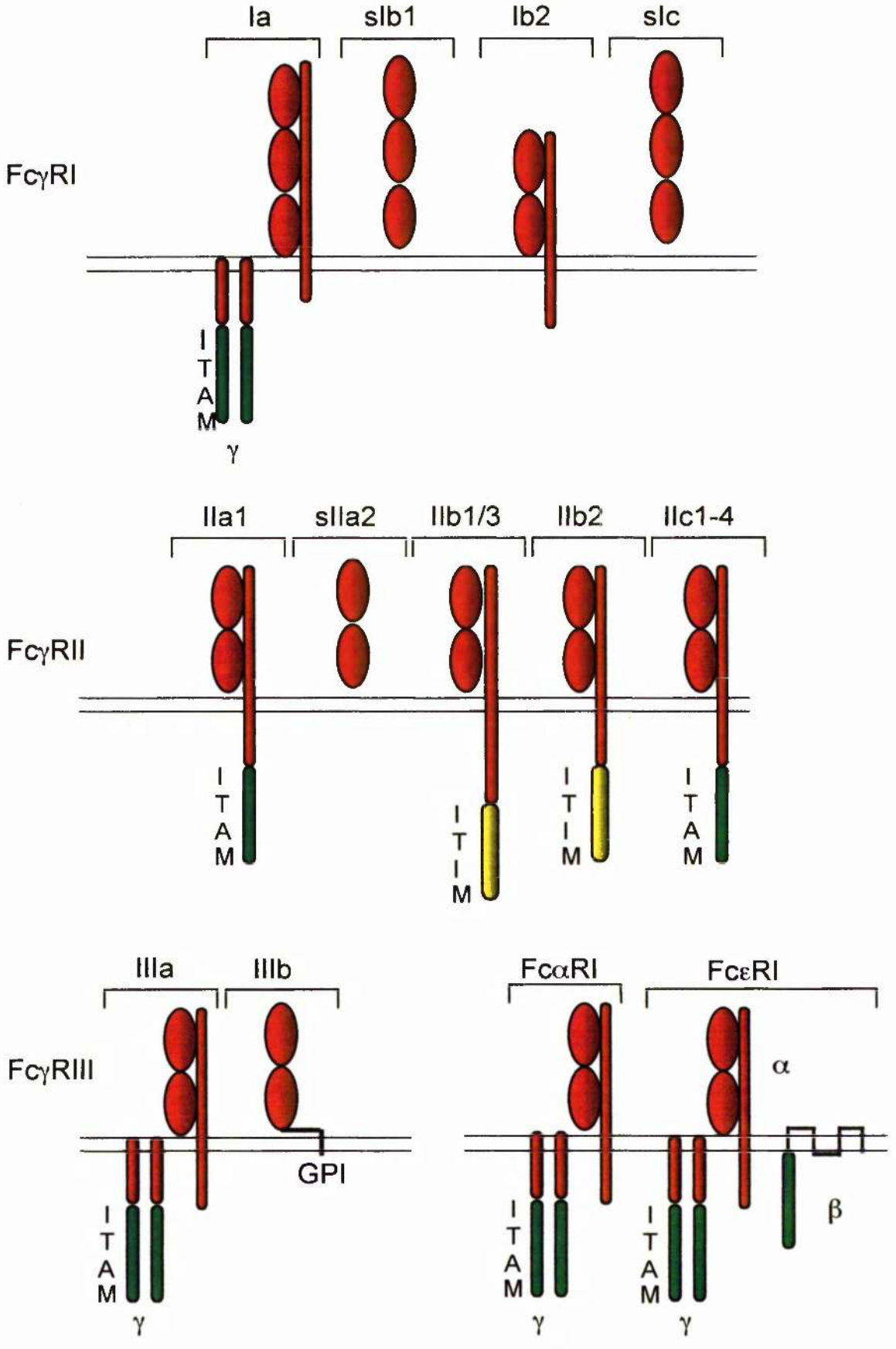

Ia
sIb1
Ib2
sIc
FcγRI
ITAM
γ
IIa1
sIIa2
IIb1/3
IIb2
IIc1-4
FcγRII
ITAM
ITIM
ITIM
ITAM
IIIa
IIIb
FcγRIII
GPI
ITAM
γ
FcαRI
FcεRI
α
β
ITAM
γ
ITAM
γ

Table 1.2 Fcγ receptor binding affinities and cellular distribution.

Fc receptors are defined by their cellular distribution, structure and affinity for their specific immunoglobulin isotypes. Three classes of FcγR exist, that have varying affinities for the four known subclasses of IgG (IgG1, IgG2, IgG3 and IgG4). The high affinity FcγRI (CD64) receptor, is the only class of FcγR capable of recognising and binding monomeric IgG at physiological concentrations. In humans, FcγRI binds IgG1 with higher affinity than IgG3 or IgG4 but is unable to bind IgG2. In contrast, FcγRII (CD32) and FcγRIII (CD16) are low affinity receptors which can only recognise IgG in the form of an immune complex. Interestingly, whilst human FcγRIIa and FcγRIIb display high homology in their extracellular domains they differ in their IgG subclass binding affinities; FcγRIIa is unable to bind IgG4 whilst FcγRIIb is unable to bind IgG2. Thus, IgG subclass plays an important role in the regulation of the immune response as it dictates which FcγRs are ligated and therefore determines the effector mechanism elicited. For the low affinity receptors, FcγRII and FcγRIII, the binding affinities refer to immune complexes and not monomeric immunoglobulin.

Species	Fc receptor	IgG binding affinity (Ka)	Cell distribution
Human/ Murine	FcγRI (CD64)	High $2\text{-}5 \times 10^{8}\ M^{-1}$ Human 3>1>4>>>2 Murine 2a =3>>>1,2b	monocytes, macrophages, IFN-γ induced neutrophils, eosinophils
Human	FcγRIIa (CD32)	Low $2 \times 10^{6}\ M^{-1}$ Human 3>1>>>2,4	monocytes, macrophages, neutrophils, platelets
Human/ Murine	FcγRIIb1/2 (CD32)	Low $2 \times 10^{6}\ M^{-1}$ Human 3>1>4>2 Murine 2a =2b>1	B cells, myeloid cells
Human	FcγRIIc (CD32)	Low $2 \times 10^{6}\ M^{-1}$ Human 1>3 =4>>>2	NK cells
Human/ Murine	FcγRIIIa (CD16)	Low $5 \times 10^{5}\ M^{-1}$ Human 1=3>>>2,4 Murine 3>2a>2b>>1	NK cells, macrophages, mast cells
Human/ Murine	FcγRIIIb (CD16)	Low $5 \times 10^{5}\ M^{-1}$ Human 1=3>>>2,4 Murine 3>2a>2b>>1	neutrophils

Figure 1.16 Activatory versus inhibitory Fcγ Receptors.

The diversity of biological responses between FcγRs is accounted for by the divergence in cytoplasmic tail domains of these receptors, as the ligand-binding domains are structurally related. Two general classes of FcγR exist that can either positively or negatively regulate cell activation. The activation FcγR possess a cytoplasmic ITAM consensus motif whilst the inhibitory FcγR employ an ITIM motif for mediating signal transduction (Adapted from Ravetch and Bolland, 2001)

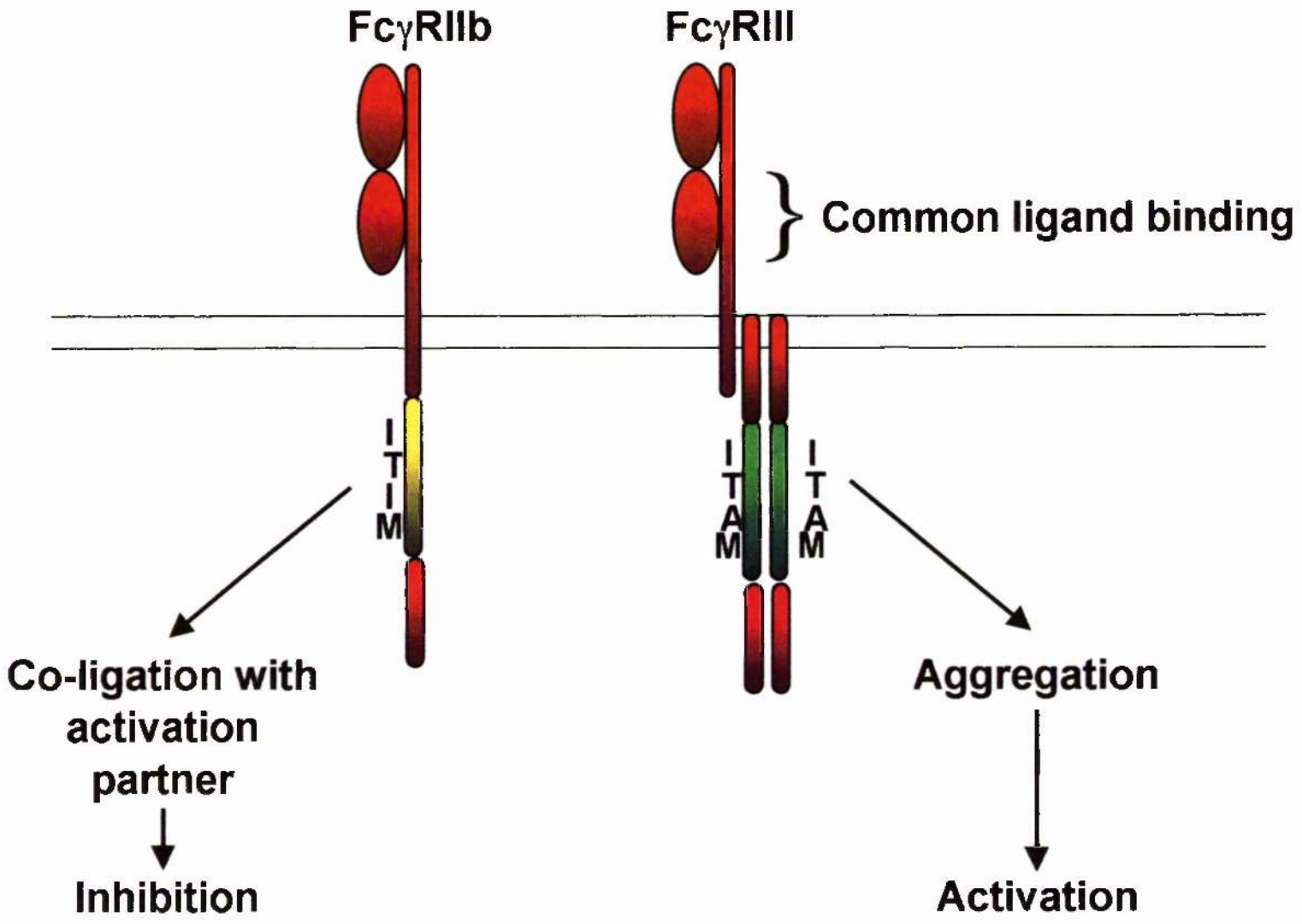

FcγRIIb
FcγRIII
Common ligand binding
ITIM
ITAM
ITAM
Co-ligation with activation partner
Inhibition
Aggregation
Activation

Figure 1.17 FcγRIIb genes and isoforms.

Separate exons encode the transmembrane and cytoplasmic domains of FcγRIIb; thus several isoforms can be created by alternative splicing of mRNA transcripts. Widely expressed by cells of haematopoetic origin, all isoforms of FcγRIIb possess an ITIM in their cytoplasmic domains and are therefore able to mediate inhibitory signalling mechanisms. *In vitro* and *in vivo* studies have shown that FcγRIIb acts as a negative regulator of immune complex-triggered activation, with preferential expression of FcγRIIb1 on B cells and FcγRIIb2 on myeloid cells.

Human FcγRIIb encodes three transcripts, FcγRIIb1, FcγRIIb2 and FcγRIIb3, which arise by alternative splicing mechanisms. The mature forms of hFcγRIIb1 and hFcγRIIb3 are identical but differences in their peptide leader sequences prevent the expression of hFcγRIIb3. Upon IgG binding, hFcγRIIb2 is capable of mediating rapid endocytosis by means of clathrin-coated vesicles. However, an insertion of 19 amino acids in the cytoplasmic tail of hFcγRIIb1 disrupts the cytoskeletal attachment domain responsible for modulating receptor internalisation.

In contrast to humans, a single gene encodes FcγRII in mice and three integral membrane glycoproteins have been isolated, FcγRIIb1, FcγRIIb2 and FcγRIIb1'. Similar to the human FcγRIIb isoforms, murine FcγRIIb1 and FcγRIIb2 are identical except for a 47 amino acid insertion in the first cytoplasmic exon of FcγRIIb1, which inhibits its endocytic capacity. An additional murine isoform exists as a result of a cryptic splice donor site in the first cytoplasmic exon, creating a murine homologue of human FcγRIIb1 (named FcγRIIb1').

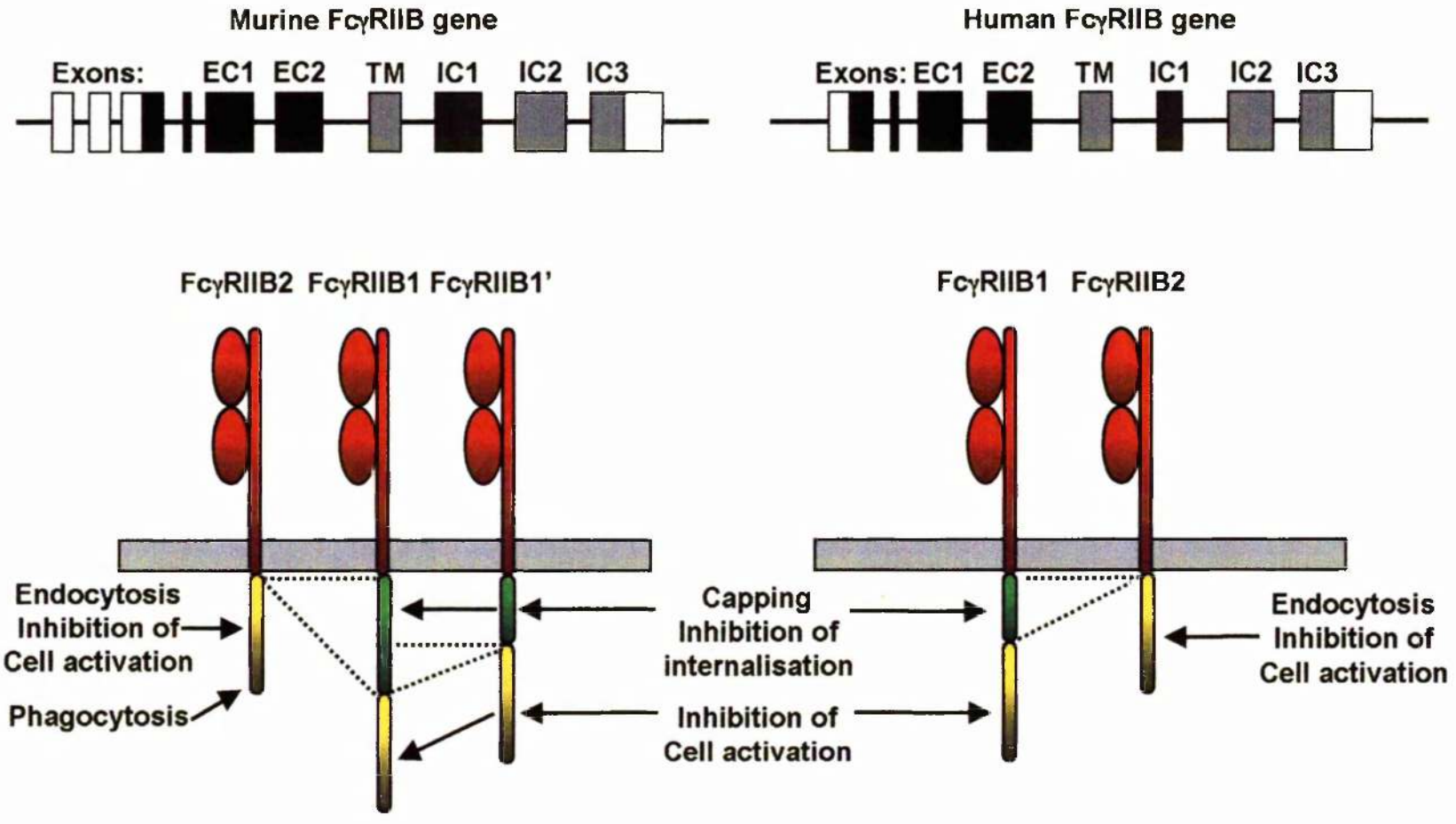

Murine FcγRIIB gene
Exons:
EC1
EC2
TM
IC1
IC2
IC3
Human FcγRIIB gene
Exons:
EC1
EC2
TM
IC1
IC2
IC3
FcγRIIB2
FcγRIIB1
FcγRIIB1'
Endocytosis
Inhibition of
Cell activation
Phagocytosis
Capping
Inhibition of
internalisation
Inhibition of
Cell activation
FcγRIIB1
FcγRIIB2
Endocytosis
Inhibition of
Cell activation

Figure 1.18 SHIP-mediated inhibition of B cell activation.

Co-ligation of the BCR and FcγRIIb by immune complexes leads to tyrosine phosphorylation of the ITIM, the recruitment of SHIP and the inhibition of BCR-mediated calcium mobilisation and cellular proliferation. SHIP is able to inhibit calcium mobilisation by hydrolysing the membrane inositol phosphate, PtdIns(3,4,5)P_3. In the absence of PtdIns(3,4,5)P_3, PH domain binding proteins, Btk and PLC-γ, are released from the membrane and a sustained calcium signal is blocked as the influx of extracellular calcium is prevented. FcγRIIb recruited SHIP is also able to abrogate BCR-mediated proliferation by the downregulation of the MAPK pathway and inactivation of the survival factor Akt/PKB (Adapted from Ravetch and Lanier, 2000).

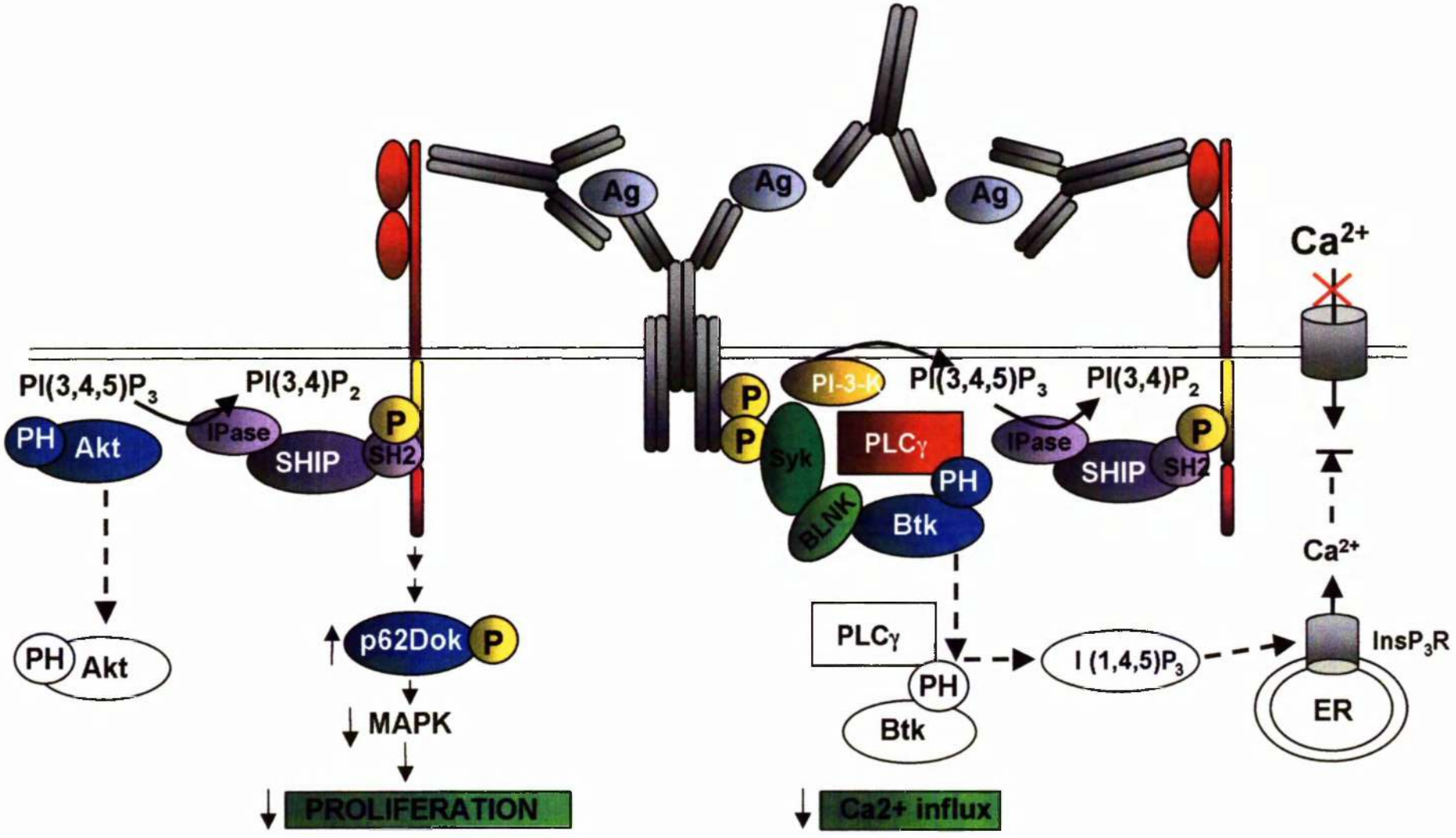

Ag
Ag
Ag
Ca2+
PI(3,4,5)P3
PI(3,4)P2
PH
Akt
IPase
SHIP
P
SH2
PI-3-K
PI(3,4,5)P3
PI(3,4)P2
P
P
Syk
PLCγ
PH
BLNK
Btk
IPase
SHIP
P
SH2
Ca2+
PH
Akt
p62Dok
P
MAPK
PROLIFERATION
PLCγ
PH
Btk
I (1,4,5)P3
InsP3R
ER
Ca2+ influx

Figure 1.19 Regulation of the survival-promoting factor Akt/PKB.

The generation of PtdIns(3,4,5)P_3 by PI-3-kinase can activate the survival-promoting factor Akt/PKB via the recruitment of the protein-serine/threonine kinases, PDK1 and PDK2, to the plasma membrane. PDK1 and PDK2 activate Akt via phosphorylation of Thr^{308} and Ser^{473}, respectively, within its activation loop. Akt then promotes cell survival by phosphorylating and compromising multiple targets of the cell-death pathway, including the pro-apoptotic Bcl-2 family member BAD and the cell-death pathway enzyme caspase-9. In addition to promoting cell survival, Akt may regulate cell proliferation by phosphorylation of the pro-apoptotic serine/threonine kinase, glycogen synthase kinase-3 (GSK-3). GSK-3 promotes cyclin D proteolysis, thus by phosphorylating and inhibiting GSK-3, Akt contributes to cyclin D accumulation and cell cycle entry. The inositol 5'-phosphatase, SHIP-1, partially inhibits Akt/PKB activation by converting PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 whilst the inositol 3'-phosphatase, PTEN, converts PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2. Since PtdIns(3,4)P_2 is also capable of activating Akt/PKB, the sequential activation of SHIP and PTEN potentially counteracts PI-3-kinase activity and terminate downstream signalling.

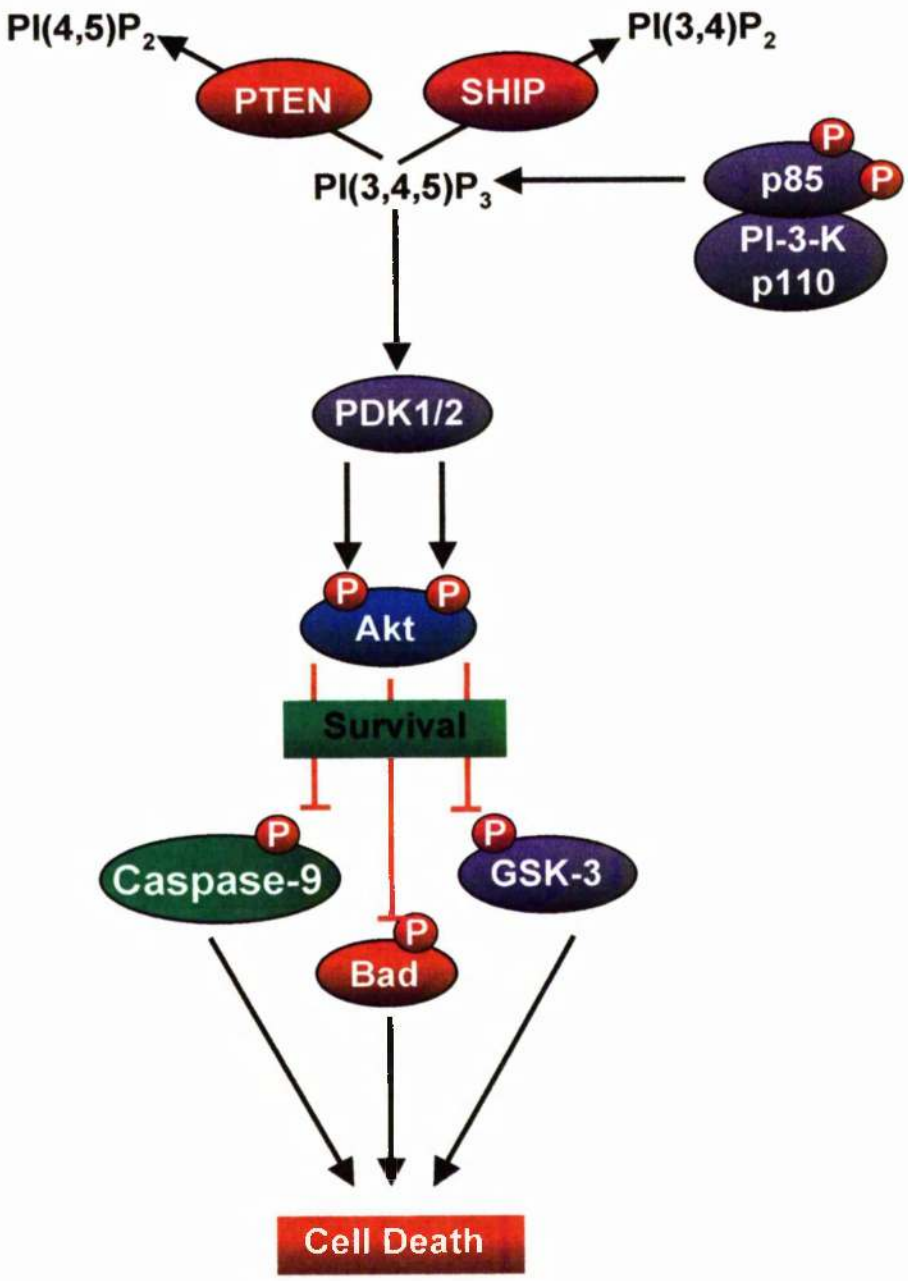
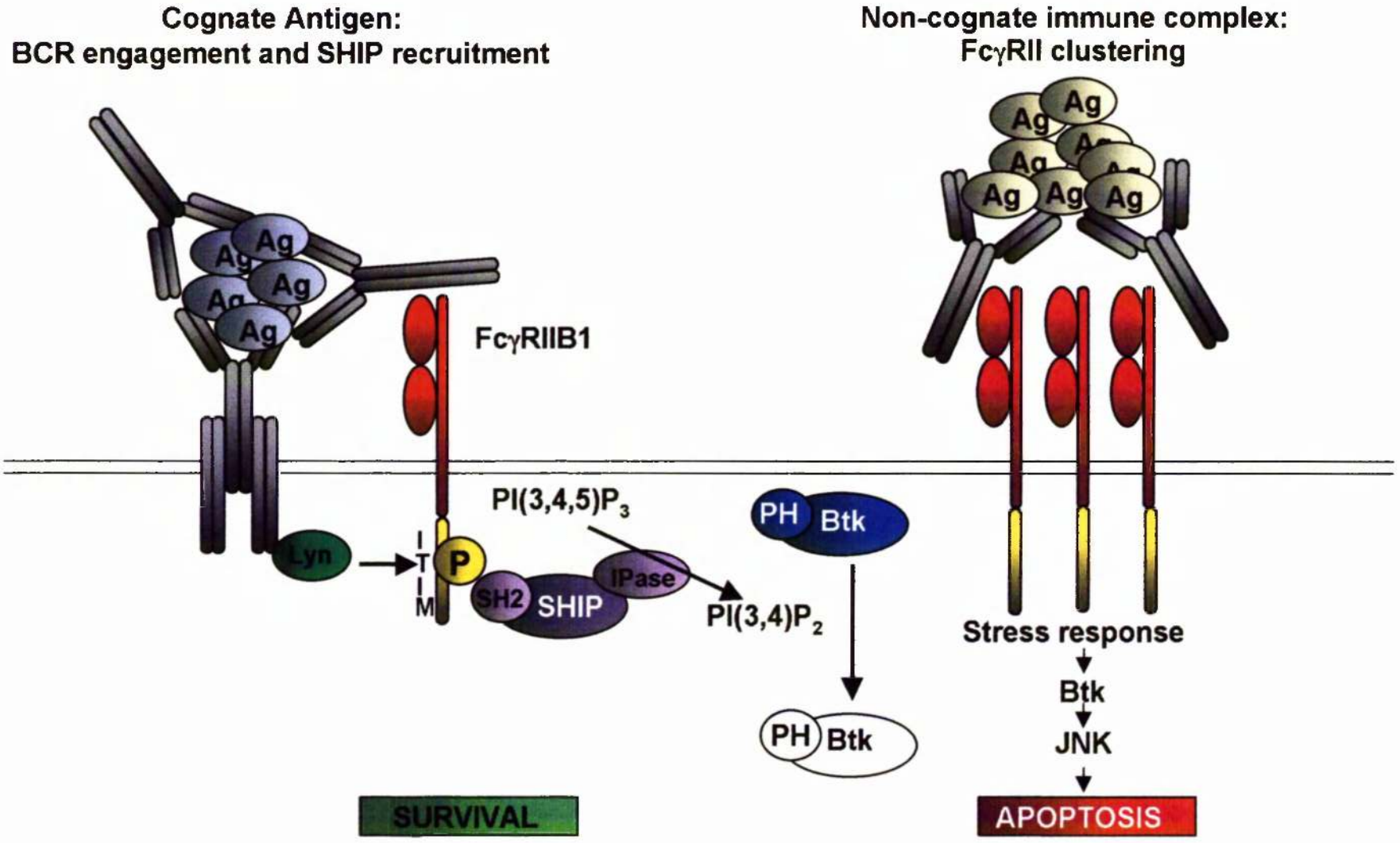

PI(4,5)P2
PTEN
SHIP
PI(3,4)P2
PI(3,4,5)P3
P
p85
P
PI-3-K
p110
PDK1/2
P
P
Akt
Survival
P
Caspase-9
P
GSK-3
P
Bad
Cell Death

Figure 1.20 Antigen-antibody recognition by FcγRIIb can determine B cell fate.

FcγRIIb displays three separate inhibitory activities dependent on the nature of antigen-ligation. Co-ligation of the BCR and FcγRIIb by cognate antigen-antibody complexes leads to tyrosine phosphorylation of the ITIM by the Src-family kinase Lyn, recruitment of SHIP and the inhibition of BCR-triggered calcium mobilisation and arrest of cellular proliferation. Aggregation of FcγRIIb alone, via non-cognate antigen-antibody complexes, generates a pro-apoptotic signal that is mediated via Btk. Rather surprisingly, this pro-apoptotic signal is blocked by the recruitment of SHIP to FcγRIIb following BCR co-ligation. This novel FcγRIIb-mediated mechanism has been proposed to act as a means of maintaining B cell peripheral tolerance in the germinal centre, by promoting apoptosis of B cells with low-affinity, potentially cross-reactive antigen-receptors. Antigen that interacts with FcγRIIb alone will result in B cell apoptosis whilst antigen co-engagement of FcγRIIb with the BCR will promote survival. Thus, selection of B cells may represent the sum of opposing signals generated by the interaction of immunocomplexes with the BCR and FcγRIIb through pathways modulated by SHIP (Adapted from Ravetch and Lanier, 2000).


Cognate Antigen:
BCR engagement and SHIP recruitment
Non-cognate immune complex:
FcγRII clustering
Ag
FcγRIIB1
Lyn
ITIM
P
SH2
SHIP
IPase
PI(3,4,5)P3
PI(3,4)P2
PH
Btk
Stress response
Btk
JNK
SURVIVAL
APOPTOSIS

Chapter 2 - Materials and Methods

2.1 Cell culture reagents and antibodies

All cell culture reagents were purchased from Gibco-BRL. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated, and were of the highest grade available. See **Tables 2.6** and **2.7**.

2.2 Animals

2.2.1 Balb/c

Male Balb/c mice, at least 8 weeks of age, were used unless otherwise stated. These mice were maintained at the animal facility of the Department of Immunology, University of Strathclyde, Glasgow or the Central Research Facility (CRF), University of Glasgow. Mice, arriving from Harlan UK Ltd, were quarantined for 7 days prior to commencing any experiment.

2.2.2 MRL/Mp-*lpr/lpr*

Male MRL/Mp-*lpr/Ola/Hsd*, (H-2^k) mice (Harlan UK Ltd), aged 14-26 weeks, were maintained at the Central Research Facility (CRF), University of Glasgow. The strain was developed by E.D. Murphy (Jackson Laboratory, Bay Harbor, USA) by backcrossing the congenic mutant gene *lpr* (*lymphoproliferation)* by 5-cycles of cross-intercross matings from substrain MRL/1 to substrain MRL/n (now designated MRL/$Mp^{+/+}$). A gradual onset of disease was observed from 18 weeks and animals were monitored for a further 8 weeks for the development of disease characteristics, such as enlarged lymph nodes. Timepoints for *ex vivo* analysis were selected on the basis of disease progression. Spleens were removed at the times indicated and purified B cells were cultured as described (see **Chapter 4**). Mice were quarantined for 7 days prior to commencing the study.

2.2.3 Collagen-induced arthritis (CIA) model

Male DBA/1 mice (Harlan UK Ltd), aged 6-8 weeks, were maintained at the Joint Animal Facility (JAF), University of Glasgow, in accordance with the UK Home Office animal care guidelines. Dr. Bernard Leung (Department of Immunology, University of Glasgow) performed all procedures. Mice were quarantined for 7 days prior to commencing the study.

CIA was induced as described previously (Ruchatz, *et al.,* 1998). Bovine type II collagen (Sigma-Aldrich) was solubilised in 0.01 M acetic acid (4 mg/ml) overnight at 4°C. The resultant suspension was emulsified in an equal volume of freshly prepared Freund's complete adjuvant (Difco) supplemented with 5 mg/ml of heat killed *Mycobacterium tuberculosis*, H37RA (Difco).

Mice were immunised intradermally at the tail base with a total of 100 µl of the prepared emulsion (CII, 200 µg) or CFA alone. An intraperitoneal CII challenge (CII, 200 µg in PBS) was carried out on day 21. A gradual onset of arthritis was observed 7-10 days after the intraperitoneal challenge and animals were observed for up to 5 weeks for the development of arthritis (**Figure 2.1**).

2.2.4 ES-62/CIA prophylactic model

CIA was induced in male DBA/1 mice as previously described (see section **2.2.3**). Dr. Bernard Leung (Department of Immunology, University of Glasgow) undertook all procedures, clinical measurements and histological assessments. ES-62, a glycoprotein that is a major ES product of the rodent filarial parasite *Acanthocheilonema viteae*, was prepared as previously described (Houston, *et al.*, 1997) and kindly supplied by Dr. William Harnett (Department of Immunology, University of Strathclyde, UK). Mice were immunised sub-cutaneously (2 µg/animal) with ES-62 at d-2, d0 and d21 or PBS as control. Extra treatments of ES-62 were administered to a third group (ES-62 multi) on d22-24, plus an additional treatment once every three days until the end of the study. Animals were observed for up to 4 weeks for the development of arthritis. Spleens were removed at the time indicated and purified B cells cultured as described (see **Chapter 4**).

2.2.5 *In vivo* ES-62 release model

To mimic the *in vivo* release of the filarial nematode excretory-secretory product, ES-62, ALZET osmotic mini-pump model 2002 (Charles River UK Ltd.) was used as directed by the manufacturer (Theeuwes and Yum, 1976). All procedures were performed by Dr. Maureen Deehan (Department of Immunology, University of Glasgow, UK) or Emma Wilson (Department of Immunology, University of Strathclyde, UK). Pumps were loaded with 200 µl of 100 or 400 µg ES-62 or PBS, pH 7.4 and left overnight at room temperature in sterile 0.9 % saline solution. Male Balb/c mice, aged 6-8 weeks and weighing at least 20 g, were anaesthetised with Halothane-RM (Aventis Pasteur MSD). The pumps were inserted into a mid-scapular incision made in the back of the neck, the wounds sutured and the animal observed until consciousness was regained. Animals were sacrificed on day 14. Spleens and lymph nodes were removed and cultured as described.

2.3 Purification of murine B cells

Primary B cells were prepared from murine spleens or lymph nodes using the CD43-magnetic bead negative-selection method of Miltenyi Biotec. All procedures were performed at 4°C. A single cell suspension was prepared by mashing the spleens or nodes through a wire mesh (Sigma-Aldrich), in RPMI-1640 media. The resultant suspension was centrifuged (400 x g, 7 min, 4°C) and the pellet resuspended in 9 mls of red blood cell removal buffer (0.168 M NH_4Cl, pH 7.2). The suspension was carefully layered over 1 ml heat-inactivated foetal calf serum (FCS) and incubated on ice for 7 min to permit red blood cell lysis and lipid precipitation. The supernatant was removed, carefully layered over 1 ml FCS in a fresh tube and centrifuged again (400 x g, 7 min, 4°C).

The cell pellet was resuspended in 9 ml dead cell removal buffer (HEPES-buffered, mouse tonicity, balanced salt solution (BSS) supplemented with 0.12 M Sorbitol, 20 mM Glucose) (Shortman *et al.*, 1972; Von Boehmer and Shortman, 1973). The suspension was immediately filtered through two prepared dead cell removal columns (absorbent cotton wool plugged, short-

form, glass pipettes, wetted with 0.5 ml BSS/2% FCS). Cells were recovered from the column into a 15 ml tube on ice containing 1 ml BSS/2% FCS. The cells were centrifuged (400 x g, 7 min, 4°C) and resuspended in 50 ml ice-cold MACS buffer (phosphate buffered saline (PBS), 0.5% BSA, 2 mM EDTA) counted by Trypan blue exclusion, and pelleted by centrifugation (400 x g, 7 min, 4°C).

The cells were resuspended in ice-cold MACS buffer (2 x 10^8 cells/ml) and passed through gauze, to produce a single cell suspension for labelling. Cells were incubated for 25 min at 4°C with anti-CD43 (Ly-48) beads (100 µl CD43+ beads/2 x 10^8 cells). Labelled cells were passed through gauze again and applied to a CS-type negative selection magnetic column (Miltenyi Biotec) in a strong magnetic field. Purified mature B cells (CD43-) were eluted from the column by washing with 50 mls ice-cold MACS buffer. The cells were centrifuged (400 x g, 7 min, 4°C), resuspended in RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine, counted and assessed for purity by FACS analysis. Typically, B cells were obtained with a purity >98 % B220 positivity (as assessed by flow cytometry).

High density "resting" and low-density "activated" B cells were separated by Percoll density gradient centrifugation. Percoll (100%) was prepared by the addition of 2 ml 10 x PBS to 18 ml Percoll and dilutions (85%, 65% and 50%) were prepared by the addition of 1 x PBS. Percoll dilutions were layered (2.5 ml aliquots) into 13 ml centrifuge tubes to form a discontinuous gradient onto which the prepared B cells were loaded (approximately 6 x 10^7 cells/tube in 2 ml RPMI-1640) and the tubes centrifuged (1230 x g, 20 min, room temperature). High density "resting" B cells were harvested from the 85%-65% interface and low-density "activated" B cells from the 65%-50% interface. The cells were washed twice by centrifugation (400 x g, 7 min, 4°C), resuspended in RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine, counted and assessed for purity and activation status (size and granularity) by FACS analysis.

2.4 Isolation of human peripheral blood mononuclear cells

Peripheral blood samples were obtained from rheumatoid arthritis (RA) patients attending the Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow, who satisfied the 1987 American College of Rheumatology diagnostic criteria, (Arnett *et al.*, 1988). Total RNA from peripheral blood mononuclear cells of systemic lupus erythematosus (SLE) patients and controls was kindly provided by Dr. H. Siew (Dept. of Rheumatology, Allergy and Immunology, Tan Tock Seng Hospital, Singapore).

Blood samples (7-14 ml) were collected in heparinised vials and mononuclear cell fractions were obtained by density gradient centrifugation through Lymphoprep (Nycomed). Briefly, blood samples were diluted 1:1 with warm RPMI-1640 and layered over Lymphoprep cushions (6 ml diluted blood per 6ml cushion). Following centrifugation (400 x g, 30 min, 20°C) cells at the interface ('fluffy' mononuclear layer) were removed to a clean 15 ml tube and washed with warm RPMI-1640 (400 x g, 10 min, 20°C). Mononuclear cells were counted by Trypan blue exclusion and total RNA isolated.

2.5 Ethical considerations

Peripheral blood samples were surplus to clinical requirements following routine therapeutic or diagnostic procedures and would otherwise have been discarded. These samples were collected by Dr. Iain McInnes (Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow) only when clinically indicated and informed consent was obtained from patients prior to research use of samples obtained.

All animal and disease model experiments was performed under project procedure licence 60/2217, provided following UK Home Office review and were used only to address questions not amenable to *in vitro* study of human tissue.

2.6 [^{3}H]-Thymidine DNA synthesis assay

Proliferation of primary cells was assessed by a DNA synthesis assay. Briefly, purified cells (2 x 10^5 cells/well) were cultured in triplicate in round bottomed

microtitre plates in RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate and 50 μM 2-Mercaptoethanol (2-Me). Cells were stimulated in the presence of the appropriate agonists in a final well volume of 200 μl. Cells were cultured for 48 hours at 37°C in a 5% (v/v) CO_2 atmosphere at 95% humidity.

Proliferation was assessed by pulsing with 0.5 μCi/well [6-^{3}H]-thymidine (Amersham Pharmacia Biotech) for the last four hours of culture, to allow incorporation into cellular DNA. Cells were harvested onto glass fibre filter mats (Wallac) using a Betaplate 96-well harvester (Amersham Pharmacia Biotech). Incorporated label was assessed by liquid scintillation counting and results are expressed as counts per minute (c.p.m) incorporated ± SD.

2.7 Cell stimulation and preparation of cell lysates

Splenic B cells (10^7) were stimulated for the times indicated at 37°C. Control stimulations were carried out in the presence of medium alone (RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine). Following incubation, cells were lysed by the addition of ice-cold, modified RIPA 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, 10 mM sodium orthovanadate plus 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml CLAP [chymostatin, leupeptin, antipain and pepstatin]). Whole cell lysates were incubated on ice for 30 min to permit protein extraction. Cellular debris was removed by centrifugation (20000 x g, 30 min, 4°C) and the supernatants transferred to fresh tubes, either for immediate use or stored at -20°C until required.

2.8 Preparation of nuclear protein extracts

Nuclear extracts were prepared using a modified protocol, as described previously (Dignam, *et al.,* 1983; Lee *et al.,* 1988). Stimulated B cells (10-30 x 10^6) were washed twice with ice-cold TBS and then resuspended in 400 μl of ice-cold buffer A (10 mM HEPES buffer, pH 7.9, containing 10 mM KCl, 0.1 mM

EDTA, 0.1 mM EGTA, 1 mM DTT, 0.15 mM spermine, 0.75 mM spermidine, 0.5 mM PMSF and 100 μM sodium orthovanadate). Cells were allowed to swell on ice for 15 min, after which 25 μl of a 10% (v/v) IGEPAL solution was added and the cells vortexed for 15 seconds. Following centrifugation (13,000 x g, 30 s, 4°C) the nuclear pellet was resuspended in 100 μl ice-cold buffer C (20 mM HEPES buffer, pH 7.9, containing 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 100 μM sodium orthovanadate) and samples were incubated on ice for 15 min with vigorous agitation. The nuclear extract was harvested by centrifugation (13,000 x g, 5 min, 4°C) and the supernatant collected and frozen in aliquots at -70°C until required.

2.9 Immunoprecipitation

A BCA Protein Assay kit (Bio-Rad Laboratories Ltd) was used to assess the protein concentration of the whole cell lysates. Samples were normalised to 1 μg/μl with 2 x modified RIPA buffer and then pre-cleared with 10 μl of a 50% protein-G-sepharose:RIPA slurry for 30 min at 4°C with constant rotation. Following centrifugation (13,000 x g, 15 min, 4°C), the pre-cleared supernatant was transferred to a fresh tube and incubated with the appropriate immunoprecipitating antibody (1-2 μg/sample) overnight at 4°C with constant rotation.

The protein-antibody complex was pulled down with 10 μl of protein G-sepharose for 2 hours at 4°C with constant rotation. Protein-agarose complexes were harvested by centrifugation (13,000 x g, 30 min, 4°C) and washed three times in ice-cold modified RIPA buffer. The samples were then used immediately and assayed for phosphatase activity or dissociated from the sepharose beads by boiling in 50 μl of 2 x sample loading buffer (100 mM Tris pH 6.8, 4% (v/v) bromophenol blue, 0.2% SDS and 20% v/v glycerol) containing 5% 2-Me for 10 min at 100°C. Samples were pulse centrifuged, and resolved by SDS-PAGE, or stored at -20°C until required.

2.10 SDS-Polyacrylamide Gel Electrophoresis

Standard gel electrophoresis apparatus and glass plates were cleaned with a deproteinating detergent and 70% ethanol prior to assembling according to the manufacturers instructions. Cell lysates or immunoprecipitates were resolved by Tris-glycine, SDS-PAGE (Laemlli, 1970).

A resolving gel of an appropriate percentage for protein resolution was poured into the prepared gel apparatus (**Table 2.1**) and overlaid with 70% ethanol solution to promote setting. The gel was allowed to set for at least 30 min. The ethanol layer was discarded and the gel interface washed with distilled water. Prior to pouring of the stacking gel, a well comb was inserted. The stacking gel was allowed to set for 30 min. The comb was carefully removed and the resultant wells were washed with electrophoresis buffer (25 mM Tris, 250 mM glycine and 0.1% SDS (w/v)).

Equal protein loadings of cell lysates or immunoprecipitates were resuspended in an equivalent volume of 2 x sample buffer containing 5% 2-Me and denatured by heating for 10 min at 100°C. Samples were loaded into the individual wells. Prestained wide-range molecular weight rainbow markers (Sigma-Aldrich) were used to elucidate the molecular weights of the unknowns. The samples were then electrophoresed into the stacking gel at 100V and then run at 200V through the resolving gel.

2.11 Western Blotting

Protein equivalent samples were resolved on a 7.5-12% polyacrylamide gel under reducing conditions and transferred to PVDF nitro-cellulose membrane (Millipore) using a transblot system (Bio-Rad Laboratories Ltd), cooled by the presence of an ice-block. Ponceau Red staining determined even protein loading and sample recovery of gels. The PVDF membranes were then incubated in modified TBS buffer I (TBS, pH 7.4, containing 10% non-fat milk and 0.1% Tween-20) for at least 1 hour at 4°C to block non-specific protein binding. Primary antibodies were diluted, at concentrations as recommended by the manufacturer, in TBS buffer II (TBS, pH 7.4, containing 5% non-fat milk

and 0.1% Tween-20) and incubated with the membranes for 1 hour at room temperature, or overnight at 4°C. Membranes were washed four times in TBS buffer (TBS, pH 7.4, and 0.1% Tween-20) before incubating with the appropriate HRP-conjugated secondary antibody in modified TBS buffer II for 1 hour at room temperature. The membranes were then washed thoroughly in TBS buffer and the immuno-reactive protein bands visualised using the Enhanced ChemiLuminescence (ECL) Western Blotting Detection System (Amersham Pharmacia Biotech) and exposed to X-OMAT film (Kodak).

2.12 Purification of anti-FcγRII/III antibody

Equal volumes of saturated ammonium sulphate solution and tissue culture supernatant (2.4G2) were mixed and the immunoglobulin fraction allowed to precipitate overnight at 4°C. The immunoglobulin precipitate was harvested by centrifugation and then dialysed exhaustively in PBS. A column, consisting of a glass wool-stoppered syringe containing 1 ml of Protein G-sepharose (capacity: 35 mg/ml)) was prepared, and this was connected to a reservoir made from a 50 ml syringe. The column was washed with 20 ml of PBS, prior to the antibody supernatant, 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 0.2 M acetic acid, into vials containing 100 µl of 1 M Tris buffer, pH 8.8. The elution profile was followed and the amount of protein estimated by measuring A_{280} (OD of 1.4 = 1 mg/ml of protein). The appropriate fractions were then pooled and dialysed in PBS overnight, filter sterilised and stored in 0.5 ml aliquots at 4°C or -20°C.

2.13 Flow cytometric analysis

2.13.1 Cell surface markers

Cells (0.5-1.0 x 10^6) were washed twice in a 96-well round bottom plate with 200 µl cold FACS buffer (PBS, 2% FCS, 2 mM EDTA) at 400 x g for 5 min, 4°C. Cells were resuspended in 15 µl of an anti-Fc receptor blocking solution (2.4G2 supernatant) or 15 µl of FACS buffer for 10 min on ice. Cells were incubated

with 30 µl of an appropriate concentration of primary antibody (or 15 µl of 2 x concentration, if Fc receptor block was used) and incubated for 20 min on ice. Cells were washed with 2 x 100 µl FACS buffer (400 x g, 5 min, 4°C) and then resuspended in 300 µl FACS buffer. For non-fluorescence conjugated primary antibodies, the staining procedure was repeated with the addition of the secondary antibody. Cellular fluorescence data was acquired using a Becton Dickinson FACSCalibar™ flow cytometer and analysed using Cell Quest software.

2.13.2 Cell cycle analysis

To determine what percentage of cells were at a particular stage of the cell cycle, propidium iodide (PI) staining of cells was undertaken. Cells (0.5 $x10^6$) were washed twice in ice-cold FACS buffer and stained for appropriate cell surface markers as previously described, see section **2.13.1.** Following washing in FACS buffer, cells were fixed with 100 µl ice-cold 70% ethanol for 15 min at 4°C. The cells were washed in FACS buffer and then incubated with propidium iodide (250 µg/ml) for 35 min at room temperature. The PI fluorescence of the stained cells was measured at an excitation wavelength of 488 nm. Cellular fluorescence data was acquired using a Becton Dickinson FACSCalibar™ flow cytometer and analysed using Cell Quest software.

To determine what percentage of cells were apoptotic, in G_0/G_1 arrest, in S phase or in G_2/M of the cell cycle, linear FL-3 voltage settings were used (**Figure 2.2**) The G_1 peak (2N DNA) was set at 400 fluorescence units on the x-axis and a marker was set at either side of the peak. The upper and lower 2N DNA peak values were then doubled to produce the upper and lower 4N DNA peak values and markers set. Sub-diploid DNA or apoptotic cells were determined by setting markers below the 2N peak, whilst cells in the S phase were taken as those between the 2N and 4N peaks.

2.13.3 Cell division by CFSE dye analysis

Cells (1.25 x 10^7/ml) were washed in sterile PBS/0.1% BSA and incubated with 2.5 mM CFSE (3.5 x 10^7 cells/ml) in PBS/0.1% BSA for 10 min at 37°C in the dark. Cells were then washed twice in PBS/0.1% BSA, resuspended in RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and stimulated as required. A cell sample of 10^4 cells was taken to determine the initial dye intensity loading at timepoint zero. After stimulation, cells were washed twice in FACS buffer and resuspended at 3.5 x 10^7 cells/ml. Cellular fluorescence data was acquired using a Becton Dickinson FACSCalibar™ flow cytometer and analysed using Cell Quest software.

For each stimulation cell division number was determined as a percentage of total dye uptake relative to timepoint zero. Briefly, total dye intensity at timepoint zero was plotted on a histogram and division markers were set so that each marker range was half of the previous marker setting (e.g. 8000, 4000, 2000, 1000 etc.). The markers represented each cell division resulting in daughter cells with half the dye intensity. The percentage of cells within each marker range was then determined.

2.13.4 Mitochondrial membrane potential

Incorporation of the cationic lipophilic dye $DiOC_6(3)$ into the mitochondria is proportional to the mitochondrial transmembrane potential, $\Delta\psi m$ (Zamzami *et al.*, 1995). Cells were stained for surface markers, as previously described, and then washed with FACS buffer. Cells were then incubated with 2.5 µM $DiOC_6(3)$ for 30 min at room temperature, washed with FACS buffer and resuspended in PBS/2 mM EDTA. Fluorescence data was acquired using a Becton Dickinson FACSCalibar™ flow cytometer and results were analysed using Cell Quest software (**Figure 2.3**).

2.14 Inositol phosphate hydrolysis

2.14.1 Preparation and characterisation of Dowex-formate

For use in the assessment of inositol phosphate hydrolysis, Dowex-chloride was formate exchanged and the inositol phosphate elution profile characterised. Briefly, 100 g of Dowex-chloride (1x8-400, Sigma-Aldrich) was washed twice with 500 ml of distilled water in a glass beaker to remove any broken beads. The slurry was subsequently washed with 2 l of 2 M sodium hydroxide, 1 l of distilled water, and 500 ml of 1 M formic acid. The exchanged Dowex was washed repeatedly with distilled water (approximately 5 l) until the pH remained constant (pH 5.5). Exchanged Dowex was kept covered with distilled water at 4°C to prevent drying prior to use.

For characterisation, glass wool-plugged Pasteur pipettes were filled with 0.5 ml of a 1:1 Dowex-formate:water slurry. Standards of [^{3}H]-Ins(1,3,4,5) P_4 and [^{3}H]-Ins(1,3,4)P_3 were made up in 5 mM sodium tetraborate/0.5 mM EDTA, pH 6.7 (3 µl of [^{3}H]/ml). 1 ml of the standard was passed through the column and the eluted load collected in a scintillation vial. The columns were subsequently washed with 3 x 1 ml aliquots of distilled water followed by 5 mM sodium tetraborate/0.5 mM EDTA, pH 6.7. For elution of the bound InsP forms the columns were washed with increasing concentrations of ammonium formate/0.1 M Formic acid (10 x 1 ml, 0.4-2M ammonium formate). High salt scintillation fluid (Uniscint BD, National Diagnostics) was added to each eluted fraction and to an equivalent amount of each standard used and assessed for [^{3}H] incorporation by liquid scintillation counting. The individual InsP forms were typically eluted in the following concentrations of ammonium formate/0.1 M Formic acid; 0.4 M for IP_1/IP_2, 0.65 M for IP_3 and 1 M for IP_4 (**Figure 2.4**).

2.14.2 Dialysis of Calf-Serum for Inositol Labelling

To permit better ^{3}H-inositol labelling of cells, heat inactivated foetal calf-serum was dialysed to remove endogenous inositol. Briefly, 50 ml of FCS was placed in prepared dialysis tubing (pre-boiled in 10 mM EDTA and stored in 20% Ethanol) and dialysed exhaustively against Earle's salts (0.12 M NaCl, 1 mM

KCl, 1 mM $MgSO_4.7H_20$, 1 mM NaH_2PO_4, 8 mM Glucose) at 4°C for 2 days. Dialysed FCS was filter sterilised, aliquoted and stored at -20°C prior to use.

2.14.3 Assessment of inositol tetra-phosphate hydrolysis

Hydrolysis of ^{3}H-labelled inositol 1,3,4,5-tetrakisphosphate ([^{3}H]-Ins (1,3,4,5) P_4), by whole cell lysates, anti-SHIP or anti-PTEN immunoprecipitates derived from splenic B cells was assessed by measurement of released ([^{3}H]-Ins (1,3,4) P_3). Cell lysates were prepared from stimulated splenic B cells (10^7), as previously described, and aliquoted for triplicate immunoprecipitates. To each sample 2 μg of anti-SHIP or anti-PTEN antibody was added and samples incubated overnight at 4°C with constant rotation. Protein-agarose complexes were pelleted by centrifugation (13,000 x g, 30 min, 4°C) and washed three times with ice-cold lysis buffer and once with assay buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$). Agarose-complexes were resuspended in 10μl of assay buffer.

Reaction mixtures (25 μl) containing whole cells, immunoprecipitate or assay buffer plus 16 μM Ins (1,3,4,5) P_4 and 0.5 μl [^{3}H]-Ins (1,3,4,5) P_4 (5000 cpm/μl) were incubated for 10 min at 37°C with constant agitation. The reactions were terminated by the successive addition of 0.22 N HCl and 300 μl chloroform: methanol (1:2, v/v). To separate the phases, 100 μl chloroform and 100 μl water was added and the samples were allowed to extract for 10 min at 4°C. Aliquots of the upper phase were diluted with water and applied to a 0.5 ml 1 x 8-400 Dowex-formate column equilibrated with water and 50 mM ammonium formate/0.1 M formic acid. To elute the [^{3}H]-Ins(1,3,4)P_3 product, columns were washed with 0.6 M ammonium formate/0.1 M formic acid. The non-hydrolysed substrate ($InsP_4$) was eluted with 1.0 M ammonium formate/0.1 M formic acid. For assessment of total inositol phosphates, products were eluted with 1.5 M ammonium formate/0.1 M formic acid only. Eluting fractions of 2 ml were collected and assayed for radioactivity by liquid scintillation counting.

2.15 *In vitro* Phosphatase assay (peptide assay)

Phosphatase activity was measured using a malachite green detection system, as previously described (Harder *et al.*, 1994). Measurement of phosphatase activity was performed using a commercial tyrosine phosphatase assay kit (Upstate Biotechnology) specific for SHP-1 activity. Cell lysates were prepared from stimulated splenic B cells (15×10^6) as previously described (section **2.7**) and immunoprecipitated with the appropriate antibody. Protein-agarose complexes were pelleted by centrifugation and washed four times with 10 mM Tris-HCl buffer, pH 7.4, to remove all traces of contaminating phosphates. Samples were resuspended in 75 µl of the same buffer and aliquoted (3 x 25 µl) for triplicate measurements.

SHP-1 specific phosphopeptide (R-R-L-I-E-D-A-E-pY-A-A-R-G; 350 µM) and 100 µl of a malachite green solution was added to each well and the plate incubated at room temperature for 30 min. The A_{630} was measured for each sample and the phosphate concentration determined from a standard reference curve. Pac-1, PP2A and anti-ErkMAPK associated phosphatase activity was also assessed using the Upstate Biotechnology kit by the hydrolysis of pNPP (para-nitrophenylphosphate) by the appropriate immunoprecipitate. All results are obtained from triplicate assay readings and presented as the mean ± S.D.

2.16 Measurement of Ras activity

A Ras activation assay kit (Upstate Biotechnology) was used to determine the Ras activity in stimulated cell samples. The protocol was followed according to the manufacturer's instructions. Briefly, splenic B cells (10^7) were stimulated, harvested by centrifugation and lysed with 200 µl of 5 x Mg^{2+} lysis buffer. The protein content of the whole cell lysates was determined and the samples normalised to 1 mg/ml with lysis buffer. Samples were precleared with 10 µl of 50% glutathione-agarose:lysis buffer slurry, for 30 min at 4°C with constant rotation.

Samples were then centrifuged (13,000 x g, 10 min, 4°C) and the supernatants were transferred to fresh tubes. Samples were incubated with 5 µl of Raf-1-Ras

binding domain (RBD)-agarose conjugate for 30 min at 4°C with constant rotation. Following centrifugation (13,000 x g, 10 min, 4°C) the protein-agarose complexes were washed 3 times in lysis buffer and denatured by resuspending in 50 µl 3 x sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue) and boiled for 10 min at 100°C. The agarose beads were collected by pulse centrifugation and the supernatants resolved by SDS-PAGE. Proteins were transferred to PVDF membranes and were subjected to Western blotting with 1 µg/ml anti-Ras antibody. Immuno-reactive proteins were visualised using the ECL system and exposure to X-OMAT film.

2.17 Measurement of Rap-1 activity

Rap activity in stimulated B cells was determined using a similar protocol undertaken for the measurement of Ras activity, see section 2.16. Briefly, splenic B cells (10^7) were stimulated, harvested by centrifugation and lysed with 200 µl of 5 x Mg^{2+} lysis buffer (Upstate Biotechnology). Lysates were pre-cleared with glutathione-agarose and incubated for 30 min at 4°C with 5 µg of Ral-Ras binding domain (RBD)-agarose conjugate. The conjugate was prepared in our lab from a Ral-RBD construct, kindly provided by Dr. J. L. Bos (Utrecht University, The Netherlands). Protein-agarose complexes were harvested by centrifugation (13,000 x g, 10 min, 4°C) and washed three times in lysis buffer. The complexes were harvested by centrifugation, resuspended in 3 x sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue), resolved by SDS-PAGE and subjected to Western blotting with 1 µg/ml anti-Rap-1 antibody. Immuno-reactive proteins were visualised using the ECL system and exposure to X-OMAT film.

2.18 Measurement of SAPK activity

Stimulated splenic B cells (10^7) were pelleted by pulse centrifugation and resuspended in 100 µl of modified RIPA lysis buffer (20 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Sodium Orthovanadate, 1mM PMSF, 10 mg/ml

chymostatin, leupeptin, antipain and pepstatin A). Whole cell lysates were incubated on ice for 20 min. Following centrifugation (13,000 x g, 30 min, 4°C), the supernatants were split into 2 fresh Eppendorfs and 2 μg of c-Jun fusion protein was added to one and 2 μg of ATF-2 fusion protein to the other. Samples were incubated overnight at 4°C with continuous rotation.

10 μl protein G sepharose:RIPA buffer slurry was added to each sample and incubated for 2 hours at 4°C, with continuous rotation. Samples were pelleted by centrifugation (13,000 x g, 5 min, 4°C) and were washed twice in RIPA lysis buffer and twice in kinase buffer (25 mM Tris ph7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$). The pellet was resuspended in 50 μl kinase buffer containing 100 mM ATP.

Samples were incubated for 30 min at 30°C and the reactions terminated by the addition of 25 μl 3x sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue). Samples were boiled for 5 min and resolved by SDS-PAGE, transferred to PVDF membrane and Western blotted for phospho-p38 or phospho-JNK.

2.19 Measurement of MEK activity

A modified experimental protocol supplied with a MEK Activation Assay Kit (Upstate Biotechnology) was used to determine the MEK activity in splenic B cells. Stimulated B cells (10^7) were pelleted by pulse centrifugation and resuspended in 100 μl of modified RIPA lysis buffer (20 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mg/ml chymostatin, leupeptin, antipain and pepstatin A) and incubated on ice for 20 min. Following centrifugation (13,000 x g, 30 min, 4°C), supernatants were adjusted to 1 mg/ml and incubated with 2 μg of MEK1/2 antibody overnight at 4°C with continuous rotation.

10 μl of protein G sepharose:RIPA buffer slurry was added to each sample and incubated for 3 hours at 4°C, with continuous rotation. Samples were pelleted

by centrifugation (13,000 x g, 5 min, 4°C) and were washed twice in RIPA lysis buffer and twice in assay dilution buffer (ADB – 20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT). The pellet was resuspended in 20 µl of ADB supplemented with 10 µl of a magnesium/ATP cocktail (ADB, 75 mM $MgCl_2$, 500 µM ATP). The positive control contained 0.5 Units of activated human MEK1. Samples were then incubated for 30 min at 30°C with 1 µg of inactive murine GST-p42 MAPKinase. Reactions were terminated by the addition of 25 µl 3 x sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue) and boiled for 5 min. Following centrifugation (13,000 x g, 2 min, 4°C), supernatants were resolved by SDS-PAGE, transferred to PVDF membrane and Western blotted for phospho-ErkMAPK.

2.20 Measurement of AKT activity

An Akt Kinase Assay Kit (Cell Signalling Technology, Lake Placid, NY) was used to determine the activity of Akt in splenic B cells. Stimulated cells (3×10^7) were washed with PBS, pH 7.4 and pelleted by pulse centrifugation. Cell pellets were resuspended in 200 µl of modified RIPA lysis buffer (20 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 µg/ml leupeptin) and incubated on ice for 10 min. Following centrifugation (13,000 x g, 30 min, 4°C), the supernatants were transferred to fresh tubes and incubated for 3 hours at 4°C with 20 µl of agarose-conjugated Akt antibody (Akt 1G1).

Antibody-agarose complexes were harvested by centrifugation (13,000 x g, 5 min, 4°C) and washed twice in lysis buffer and twice in kinase buffer (25 mM Tris pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$). Sample pellets were resuspended in 40 µl kinase buffer and incubated for 30 min at 30°C in the presence of 200 µM ATP and 1 µg of a GSK-3 fusion protein. Reactions were terminated by the addition of 20 µl of 3 x sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue). Samples were vortexed,

boiled for 5 min and then harvested by centrifugation. Supernatants were resolved by SDS-PAGE, transferred to PVDF membrane and Western blotted for phospho-GSK-3-α/β.

2.21 Total cellular RNA preparation

Total RNA was extracted using RNAzol^{TM} B (Biogenesis) as described (Chomczynski and Sacchi, 1987). Briefly, cells (5 x 10^6) were washed twice with ice-cold PBS (2000 x g, 5 min at 4°C) and the cellular pellet was resuspended in RNAzol^{TM} B (0.2 ml/10^6 cells). Samples were extracted for 5 min on ice and the suspension was transferred to a fresh tube. Chloroform was added (0.1 ml /1 ml of RNAzol^{TM}) and the solution mixed by vigorous shaking for 15 seconds. The samples were incubated on ice for a further 5 min.

Following centrifugation (13,000 x g, 15 min, 4°C) the colourless upper phase was removed to a fresh tube (approximately 50% of the initial RNAzol^{M} volume) and an equal volume of isopropanol was added. RNA was precipitated by incubation on ice for 30 min and separated by centrifugation (13,000 x g, 15 min, 4°C). The RNA pellet was washed with ice-cold 75% ethanol (1 ml/ 1 ml RNAzol^{TM} volume), briefly dried for 10 min at room temperature and finally resuspended in 20-50µl of sterile, distilled water. RNA samples were incubated at 68°C for 5 min prior to concentration assessment by UV spectroscopy. Absorbance readings of diluted samples (1:50) were taken at $A_{260/280}$ and the concentration of RNA calculated (40 µg/ml RNA = A_{260} of 1).

2.22 RT-PCR

Total cellular RNA (5 µg), prepared as previously described, was pre-incubated with 5 µM Oligo(dT)16 (Roche) at 70°C for 10 min and then reverse transcribed in the presence of 50 mM Tris-HCl pH, 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 0.5 mM dNTPs (Gibco-BRL) and 100 U Superscript II RT (Gibco-BRL) at 25°C for 10 min, followed by 42°C for 50 min and 70°C for 10 min. This protocol allowed annealing of the Oligo(dT) and conversion of RNA, via reverse transcription, into cDNA.

PCR amplifications were performed in a total volume of 50 µl, containing 1 µl cDNA sample (250 ng), 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dUTP, 0.5 µM each primer (**Table 2.2**) and 2.5 U Taq DNA polymerase (5 U/µl; Promega). Each amplification was performed under optimised conditions, on a Techne Cyclogene PCR Cycler typically: 5 min at 94°C, followed by a total of 30 three-temperature cycles (30 s at 94°C, 1 min at 55°C and 1 min at 72°C) and a single cycle of 5 min at 72°C. Samples were then stored at -20°C until use.

2.22.1 Agarose gel electrophoresis of nucleic acid

PCR products were routinely analysed by agarose gel electrophoresis. Briefly, an agarose gel, of an appropriate percentage for efficient DNA separation (1-2% w/v) containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich), was poured into the prepared gel apparatus, in the presence of a well comb. The gel was allowed to set for at least 30 min and the comb was carefully removed.

Approximately 10 µl of PCR product with 2 µl of DNA loading buffer was loaded into the individual wells. A prestained, 1 Kb DNA ladder (Promega) was used to elucidate the length of the unknown linear DNA. The samples were electrophoresed into the gel at 60 V, in TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA), until the bands were sufficiently resolved. Gels were analysed under UV light and a photographic image recorded.

2.23 TaqMan®

TaqMan® real-time PCR (Gibson *et al.*, 1996) was performed according to the manufacturers instructions (PE Biosystems) as previously described (Overbergh *et al.*, 1999). See **Figure 2.5**.

Primers and fluorogenic probes were designed using the PrimerExpress™ v1.0 programme purchased from PE Biosystems (**Figure 2.7**). The fluorogenic probes contained a reporter dye (FAM or VIC™) covalently attached at the 5' end and a quencher dye (TAMRA) covalently attached at the 3' end, and were

HPLC purified. Extension from the 3' end was blocked by attachment of a 3' phosphate group See **Tables 2.3** and **2.4**.

cDNA samples were prepared by reverse transcription of total cellular RNA, as previously described. PCR reactions were performed in the ABI-prism 7700 Sequence Detector, which contains a Gene-Amp PCR System 9600 (PE Biosystems). PCR amplifications were performed in a total volume of 25 µl, containing 0.5 µl cDNA sample (125 ng), 50 mM KCl, 10 mM Tris-HCl pH 8.3, 10 mM EDTA, 200 µM dATP, dCTP, dGTP and 400 µM dUTP, 5 mM $MgCl_2$, 300 nM each primer, 0.625 U AmpliTaqGold™ (PE Biosystems) and 0.25 U AmpErase Uracil N-Glycosylase (PE Biosystems). Each reaction also contained 200 nM detection probe. Each amplification was performed in triplicate wells, using the following conditions: 2 min at 50°C and 10 min at 94°C, followed by a total of 45 two-temperature cycles (15 s at 94°C and 1 min at 60°C).

Data was analysed using the Sequence Detection software (PE Biosystems) which calculates the threshold cycle; Ct. This value represents the PCR cycle number in which an increase in fluorescence, over and above threshold, can first be detected. This threshold cycle is determined during the exponential phase of amplification to ensure 100% efficiency. Samples were normalised with respect to a reference reporter gene (HPRT) through the subtraction of respective Ct values to produce a ΔCt value (**Figure 2.6**).

To obtain an absolute value for fold increase, relative to HPRT levels, the formula $2^{-\Delta Ct}$ was used. Multiplication of the absolute value by 100 gives the expression of the gene of interest as a percentage of HPRT. The positive error is the standard deviation of the difference, $s = \sqrt{(s1^2 + s2^2)}$, where s1 and s2 are the standard deviation of the Cts of HPRT and the gene of interest.

2.24 Synthetic oligonucleotide DNA labelling

Synthetic oligonucleotide probes were supplied freeze-dried from Sigma-Genosys. Dried probes were reconstituted by the addition of 500 µl of distilled water and the concentration determined A_{260}, against a water blank. For single

stranded DNA, an absorbance of 1 = approximately 33 μg/ml. Molarity was calculated on the assumption that the average weight of 1 DNA base is equal to 324 Da using the formula:

(1/Total oligo Mr in Da) x oligo concentration in μg/μl x 10^{-6} = moles oligo/μl

40 nmoles (90 μl) of each single strand oligo were incubated together with 20 μl of a high salt buffer (H buffer: Gibco BRL) and boiled at 100°C for 10 min. The probes were allowed to cool slowly for up to 4 hours to permit the annealing reaction.

The resultant double stranded oligonucleotide probes were labelled with [α-^{32}P]-dATP (Amersham-Pharmacia) using a commercially available random priming kit (Gibco-BRL). 2 μl of DNA was incubated together with 2 μl each of 0.5 mM (dCTP, dGTP, dTTP), 5 μl of 10 x H buffer, 2 μl (2 μCi) of [α-^{32}P]-dATP and 1 μl Klenow DNA polymerase (Exonuclease-free, 2 units/μl, Boehringer-Ingelheim) made up to a total volume of 50 μl with distilled water. The reaction was incubated at 37°C for 2 hours and the probes were purified on an 8% non-denaturing polyacrylamide gel, using 0.5 x TBE (90 mM Tris, 90 mM Boric acid, 2 mM EDTA) as the running buffer. Gels were exposed to X-RAY film (Kodak) to detect labelled probes and excised gel pieces were incubated in TE buffer (10 mM Tris, pH 8, 1 mM EDTA) overnight at 37°C.

2.25 Electrophoretic Mobility Shift Assay (EMSA)

5-10 μg of nuclear extract protein was pre-incubated with 200 ng double-stranded poly (dI-dC).(dI-dC) (Amersham-Pharmacia Biotech) in reaction buffer (20 mM HEPES buffer, pH 7.9, containing 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 10% glycerol and 0.1% NP-40) in a total volume of 30 μl for 30 min on ice (Wu *et al.*, 1994). [α-^{32}P]-dATP labelled oligonucleotide probe (0.5 ng) was then added (**Table 2.5**), and the mixture incubated on ice for a further 20 minutes.

DNA-protein complexes were resolved on a non-denaturing 6% PAGE in 0.5 x TBE buffer and the gels were fixed (20% methanol, 10% acetic acid, 10% glycerol) for 20 min at room temperature. Following fixing, gels were vacuum-dried and exposed to film for 5 days at -70°C.

2.26 Suppliers Addresses

Alexis Corporation (UK) Ltd
3 Moorbridge Court
Moorbridge Road East
Bingham
Nottingham NG13 8QG

Amersham Pharmacia Biotech
Amersham Place
Little Chalfont
Buckinghamshire HP7 9NA

Anachem Ltd
20 Charles Street
Luton
Bedfordshire LU2 0EB

Aventis Pasteur MSD
Bridge Avenue
Maidenhead
Berks SL6 1QP

Beckton Dickinson (BD) UK Ltd
21 Between Towns Road
Cowley
Oxford OX4 3LY

Bio/Gene Limited
6 The Business Centre
Harvard Way
Kimbolton
Cambridgeshire PE18 0NJ

Biogenesis
Technology Road
Poole BH17 7DA

Binding Site Ltd
P.O. Box 4073
Birmingham B29 6AT

Bio-Rad Laboratories Ltd
Bio-Rad House
Maylands Avenue
Hemel Hempstead
Hertfordshire HP2 7TD

BioSource UK Ltd
Rue de l'industrie, 8,
B-1400 Nivelles
Belgium

Boehringer-Ingelheim Ltd
Ellesford Avenue
Bracknell
Berkshire RG12 8YS

Caltag
c/o TCS Biologicals Ltd
Botolph Claydon
Buckingham MK18 2LR

Charles River UK Ltd
Manston Road
Margate
Kent CT9 4LT

DAKO
Angel Drove
Ely
Cambridgeshire CB7 4ET

Difco
c/o Beckton Dickinson (BD) UK Ltd

Harlan UK Ltd
Shaw's Farm
Blackthorne
Bicester
Oxon OX25 1TP

Invitrogen (Gibco-BRL/ Life Technologies)
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF

Jackson ImmunoResearch Laboratories
c/o Stratech Scientific
61-63 Dudley Street
Luton
Bedfordshire LU2 0NP

Kodak Ltd
Kodak House
Station Road
Hemel Hempstead
Hertfordshire HP1 1JU

Millipore
The Boulevard
Blackmoor Lane
Watford
Hertfordshire WD1 8YN

Miltenyi Biotech
Almac House
Church Lane
Bisley
Surrey GU24 9DR

Molecular Probes
c/o Cambridge Bioscience
24-25 Signet Court
Newmarket Road
Cambridge CB5 8LA

National Diagnostics
Unit 4
Fleet Business Park
Itlings Lane
Hessle
Hull HU13 9LX

NEN Life Science Products (UK) Ltd
BRU/BRU/40349
P.O Box 66
Hounslow TW5 9RT

New England Biolabs
73 Knowl Piece
Wilbury Way
Hitchin
Hertfordshire SG4 0TY

Nycomed
Oslo, Norway

Oncogene (Calbiochem-Novabiochem (UK) Ltd)
c/o CN Biosciences
Boulevard Industrial Park
Padge Road
Beeston
Nottingham NG9 2JR

PE Applied Biosystems (Applera UK)
Kelvin Close
Birchwood Science Park North
Warrington
Cheshire WA3 7PB

Pharmingen/Transduction Laboratories
c/o Insight Biotechnology Ltd
P.O Box 520
Wembley
Middlesex HA9 7YN

Pierce and Warnier (UK) Ltd
44 Upper Northgate Street
Chester CH1 4EF

Promega/Roche Diagnostics Ltd
Bell Lane
Lewes
East Sussex BN7 1LG

R&D Systems Europe Ltd
4-10 The Quadrant
Barton Lane
Abingdon OX14 3YS

Santa Cruz
c/o Insight Biotechnology Ltd
P.O Box 520
Wembley
Middlesex HA9 7YN

SAPU (Diagnostics Scotland)
Law Hospital
Carluke ML8 5ES

Sigma-Aldrich Company Ltd
Fancy Road
Poole
Dorset BH12 4QH

Sigma-Genosys
London Road
Pampisford
Cambridge CB2 4EF

Serotec Ltd
22 Bankside
Station Approach
Kidlington
Oxford

Upstate Biotechnology
c/o TCS Biologicals Ltd
Botolph Claydon
Buckingham MK18 2LR

Wallac
c/o PE Life Sciences
(Applera UK)

Zymed Laboratories Inc.
c/o Cambridge Bioscience
24-25 Signet Court
Newmarket Road
Cambridge CB5 8LT

Table 2.1 SDS-Polyacrylamide Gel Solutions (Laemmli, 1970)

Solution Components	Component volumes (ml) per gel mould volume of 40 mls			
	Resolving Gel			**Stacking Gel**
	7.5%	**10%**	**12%**	**5%**
distilled water	21.5	15.9	13.2	27.2
30% bis-acrylamide mix	10.2	13.3	16	6.8
1.5 M Tris (pH 8.8)	7.5	10	10	-
1.0 M Tris (pH 6.8)	-	-	-	5
10% SDS	0.4	0.4	0.4	0.4
10% Ammonium persulphate	0.4	0.4	0.4	0.4
TEMED	0.028	0.016	0.016	0.04

Table 2.2 RT-PCR Primer Sequences

Primer	Species	Primer Sequence d5'(-)3'
FcγRIIB1-For	Murine	GGGAAACCATCACGCTAAGG
FcγRIIB1-Rev	Murine	CAGTTTTGGCAGCTTCTTCC
FcγRIIB1-Splic-For	Murine	ACAGGGAAATGGGAGAAACC
FcγRIIB1-Splic-Rev	Murine	GCTTGTTGGCTCCAGTCC
FcγRIIB1-For	Human	CACTGCACAGGAAACATAGGC
FcγRIIB1-Rev	Human	CAAGACAATGGAGACTAAATAC GG
FcγRIIB1-Splic-For	Human	CCCAGGATACCCTGAGTGC
FcγRIIB1-Splic-Rev	Human	GCTGGTTTCTCAGGGAGGG
β-Actin-For	Murine	GGGCTATGCTCTCCCTCACGC CATCCTGCG
β-Actin-Rev	Murine	TTGGCATAGAGGTCTTTACGGA TGTCAACG
β-Actin-For	Human	GGGGTATGCCCTCCCCCATGC CATCCTGCG
β-Actin-Rev	Human	TTGGCGTACAGGTCTTTGCGGA TGTCCACG

Table 2.3 TaqMan® Primer Sequences

Primer	Species	Primer Sequence d5'(-)3'
FcγRIIB1-For	Human	GGC CTT GAT CTA CTG CAG GAA
FcγRIIB1-Rev	Human	GGG AGG GTC TCT CCC ATT TC
FcγRIIB2-For	Human	TGC TGC TGT AGT GGC CTT GA
FcγRIIB2-Rev	Human	CCC CAA CTT TGT CAG CCT CAT
FcγRIIB1-For	Murine	CAT TGT TAT TAT CCT AGT ATC CTT GGT CTA TC
FcγRIIB1-Rev	Murine	GTT TCT CCC ATT TCC CTG TGA TC
FcγRIIB2-For	Murine	AAA AAG CAG GTT CCA GAC AAT CC
FcγRIIB2-Rev	Murine	GAG GAG TGA GTA GGT GAT CGT GTT C
PAC-For	Human	TGG AGA TCT TGC CCT ACC TGT T
PAC-Rev	Human	ACG TTG AGG ACG GCT GTG AT
HPRT-For	Human	Supplied by PE-Biosystems.
HPRT-Rev	Human	GenBank Accession Number: M31642
HPRT-For	Murine	GCA GTA CAG CCC CAA AAT GG
HPRT-Rev	Murine	AAC AAA GTC TGG CCT GTA TCC AA

Table 2.4 TaqMan® Detection Probe Sequences

Probe	Species	Probe Sequence d5'(-)3'
FcγRIIB1	Human	FAM - CGG ATT TCA GCT CTC CCA GGA TAC CCT - TAMRA
FcγRIIB2	Human	FAM -AGG AAA AaG CGG ATT TCA GCA ATC CCA CTA - TAMRA
FcγRIIB1	Murine	FAM -CAA GAA AAA GCA GGT TCC AGC TCT CCC AG - TAMRA
FcγRIIB2	Murine	FAM - TGGAAGAAGCTCCCAAAACTGAGGCTG-TAMRA
PAC	Human	FAM -AGC TGC AGT CAC TCG TCA GAC CTG CAG - TAMRA
HPRT	Murine	FAM -TAA GGT TGC AAG CTT GCT GGT GAA AAG GA - TAMRA
HPRT	Human	VIC-Supplied by PE-Biosystems. GenBank Accession Number: M31642- TAMRA

5' FAM (6-carboxy-fluoresein) labelled

5' VIC™ labelled

3' TAMRA (6-carboxy-tetramethylrhoadmine; quencher) labelled

Table 2.5 EMSA Probe Sequences

Probe	Probe Sequence d5'(-)3'
NF-κB from iNOS promoter	CCC AAC TGG GGA CTC TCC CTT TGG
E2-F from Adenovirus E2A promoter	GCC GTT TTC GCG CTT AAA TTT GAG AAA GGG CGC G

Table 2.6 Antibodies

Protein	Conjugate	Host[1]	Clone	Use[2]	Source
Akt		R		W,IP	NEB
pAkt		R		W,IP	NEB
anti-mouse IgG + IgM $F(ab')_2$		G,R,D		S	Stratech
anti-mouse IgG + IgM Intact		G,R,D		S	Stratech
anti-mouse IgM (μ chain) $F(ab')_2$		G		S	Stratech
anti-IgG Goat, Mouse, Rabbit	HRP	V		W	SAPU
anti-IgG Goat, Mouse, Rabbit, Rat	HRP	V		W	Sigma-Aldrich
anti-Rat IgG	FITC	R		F	Pharmingen
ATF-2 fusion protein			9224S	PD	NEB
B220	APC		RA3-6B2	F	Pharmingen
B220	FITC		RA3-6B2	F	In house
BLNK		M	2B11	W	Santa Cruz
CD11b	PE		M1/70	F	Pharmingen
CD19	FITC			F	Serotec
CD19			1D3	F	Pharmingen
CD3	FITC		KT3	F	In house
CD4	FITC		GK1.5	F	In house
CD43			1B11	F	Pharmingen
cdc42		M	B-8	W	Santa Cruz
Cdk4		M	C-22	W	Santa Cruz
C3G		R	C-19	W	Santa Cruz
Dok-1		R	M-276	W	Santa Cruz
p44/42 ErkMAPK		R	9102	W,IP	NEB
phospho-p44/42 ErkMAPK		M	9106	W,IP	NEB
phospho-p44/42 ErkMAPK		R	9101S	W,IP	NEB
FcγRII/III	FITC	Ra	2.4G2	W,F	In house
Fyn		M	15	W,IP	Santa Cruz

Protein	Conjugate	Host[1]	Clone	Use[2]	Source
GAP		R	L1	W,IP	Transduction
Grb2		M	C-7	W,IP	Santa Cruz
c-Jun		R	9162	W,IP	NEB
phospho-c-Jun		R	9162	W,IP	NEB
c-Jun fusion protein		R	6093S	W,IP	NEB
JNK		R	9252	W,IP	NEB
pJNK(T183/Y185)		R	9251L	W,IP	NEB
Lyn		R	44	W,IP	Santa Cruz
Lyn		M	L05620	W,IP	Transduction
mdm2		M	SMP14	W,IP	Santa Cruz
phospho-p38		M	9219	W,IP	NEB
p38		R	9212	W,IP	NEB
p53		R	FL-393	W	Santa Cruz
p53		M	Pab 240	W	Santa Cruz
p53		M	OPO3	W	Oncogene
p53		S		W	SAPU
phospho-p53 (Ser15)		R		W	NEB
PAC-1		G	C-20, N-19	W,IP	Santa Cruz
PD-1	Biotin	Ra	J43	W,F	Gift
pERK		R	9101S	W	NEB
phospho -Tyrosine		Ra	4G10	W	UBI
phospho -Tyrosine		M	9411	W,IP	NEB
phospho -Threonine		M	13-9200	W,IP	Zymed
phospho - Serine		R	61-8100	W,IP	Zymed
PI-3K		R	Z-8	W,IP	Santa Cruz
PP2A		G	C-20	W,IP	Santa Cruz
PTEN		R	06-894	W,IP	UBI
Rac		R	C-14	W,IP	Santa Cruz
Raf-1		R	C-12	W,IP	Santa Cruz
Raf-1(RBD)				IP	W. Bos
Ral		M		W	
Rap-1 (Krev-1)		R	121	W,IP	Santa Cruz
Rap-1		M	R22020	W,IP	Transduction

Protein	Conjugate	Host[1]	Clone	Use[2]	Source
Rap-1/GAP				W	
Ras		R		W	Santa Cruz
Rb		M	G3-245	W,IP	Pharmingen
Rb		G	C-15-G	W,IP	Santa Cruz
RKIP		R		W	W. Kolch
SHC		M	S68020	W,IP	Transduction
SHC		R	S14630	W,IP	Transduction
SHIP		R	N-1	W,IP	Santa Cruz
SHIP		G	M-14	W,IP	Santa Cruz
SHIP		G	V-19	W,IP	Santa Cruz
SHIP	Agarose	G	V-19	W,IP	Santa Cruz
SH-PTP-1		G	C-19-G	W,IP	Santa Cruz
SH-PTP-1		R	C-19	W,IP	Santa Cruz
SH-PTP-2		R	C-18	W,IP	Santa Cruz
SLP76		G	C-20	W,IP	Santa Cruz
SLP76		M	S60720	W,IP	Transduction
SOS		M	S15520	W,IP	Transduction
Syk		M	4D10	W	Santa Cruz
VAV		R	C-14	W	Santa Cruz
CD43	Magnetic beads	Ra		Pure	Miltenyi Biotech
Fas (CD95)		M	LOB3	F	Serotec

Antibodies were originally raised against the murine form of the protein, unless otherwise indicated.

[1]Host: R = Rabbit, G = Goat, D = Donkey, Ra = Rat, M = Mouse, V = Various.

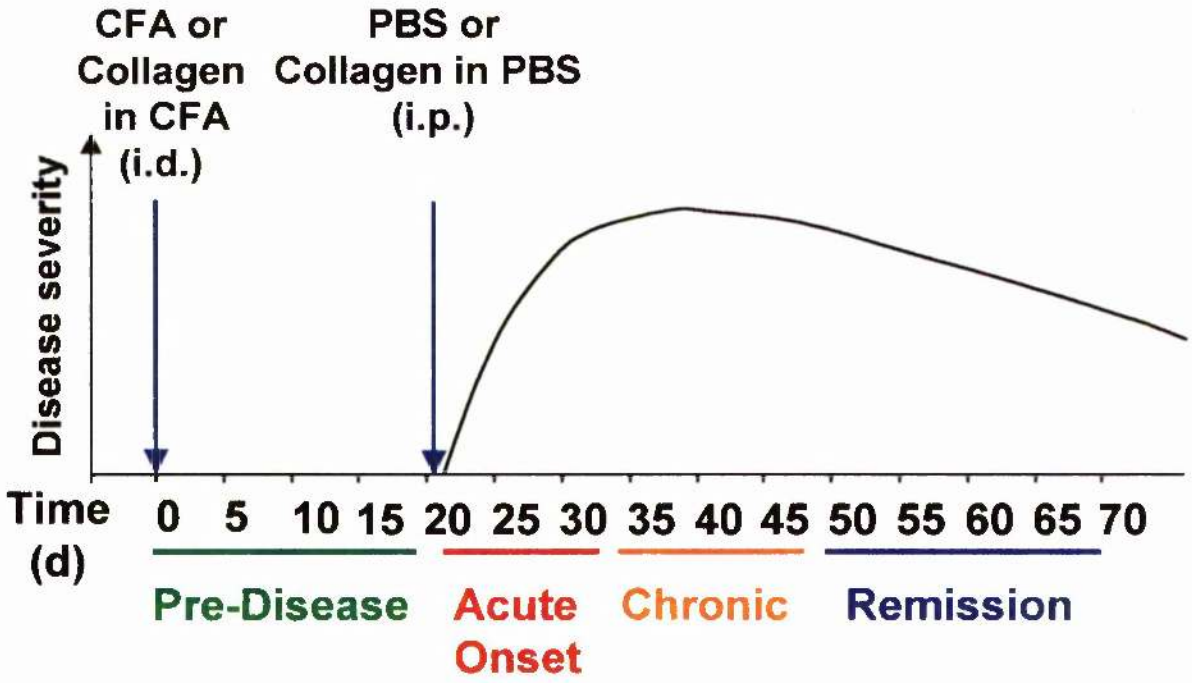
[2]Use: WB = Western Blotting, IP = Immunoprecipitation, F = FACS, S = Stimulation (*in vitro*), PD = Pull Down, Pure = Purification

Table 2.7 Other Reagents

Description	Common Name	
3,3'-dihexyloxacarbocyanine iodide	$DiOC_6$ (3)	Mol. Probes
5-/6-carboxyfluorescindiacetate, succinimidyl ester	CFSE	Calbiochem
Acrylamide/Bis Solution, 29:1	Acrylamide	Bio-Rad
AmpliTaq Gold DNA Polymerase	Taq	PE Biosystems
Bovine type II collagen	Collagen	Sigma-Aldrich
Carboxy-fluorescein diacetate succinimidyl ester	CFSE	Mol. Probes
Dowex-1-Chloride (1 x 8-400)	Dowex	Sigma-Aldrich
Enhanced ChemiLuminescence Kit	ECL	Amersham
Freund's complete adjuvant	CFA	Difco
Foetal Calf Serum	FCS	Gibco-BRL
Glutamine	Glutamine	Gibco-BRL
MEM Non-essential amino acids	Amino acids	Gibco-BRL
MicroBCA or Coomassie Protein Assay	Protein Assay	Pierce
Oligo-d(T)16	Oligo-d(T)	Roche
Penicillin/Streptomycin	Pen/Strep	Gibco-BRL
Propidium Iodide	PI	Calbiochem
RPMI-1640 culture media	RPMI-1640	Gibco-BRL
Sodium Pyruvate	Sodium Pyruvate	Gibco-BRL
SP-Sepharose Fast Flow	Sepharose	Amersham
Superscript Preamplification System	SuperscriptII	Gibco-BRL
[6-^{3}H]-thymidine (5 Ci/mmol)	[3H]- thymidine	Amersham
[^{3}H]-Inositol-1,3,4-phosphate	[3H]-IP_3	NEN
[^{3}H]-Inositol-1,3,4,5,-tetrakisphosphate [inositol-1-3H(N)]	[^{3}H]-IP_4	NEN
D-myo-Inositol-1,3,4,5 tetrakisphosphate.octapotassium	IP_4	Alexis
Uniscint BD (High Salt)	Scintillation fluid	National Diagnostics

Figure 2.1 Protocol for induction of Collagen-induced Arthritis (CIA) in DBA/1 mice.

↑ = Induction of CIA. Male DBA/1 mice (3 per group) were immunised intradermally at the tail base with bovine type II collagen (CII, 200 μg) emulsified in CFA (100μl) or CFA alone on day 0. An intraperitoneal collagen challenge (CII, 200 μg in PBS) was carried out on day 21. A gradual onset of arthritis was observed 7-10 days after the intraperitoneal challenge and animals were observed for up to 5 weeks for the development of arthritis. Dr. Bernard Leung undertook all clinical measurements and histological assessments of the collagen-induced arthritis model. Data of individual measurements from the groups were collected from the time of the intraperitoneal CII challenge on day 21 until the termination of the study on day 47. Graphs are representative of the mean articular index and disease incidence rates of the CFA/CII group only. Data are expressed as the mean ± S.E.M from a single experiment, representative of at least three other independent experiments.


CFA or
Collagen
in CFA
(i.d.)
PBS or
Collagen in PBS
(i.p.)
Disease severity
Time
(d)
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Pre-Disease
Acute
Onset
Chronic
Remission

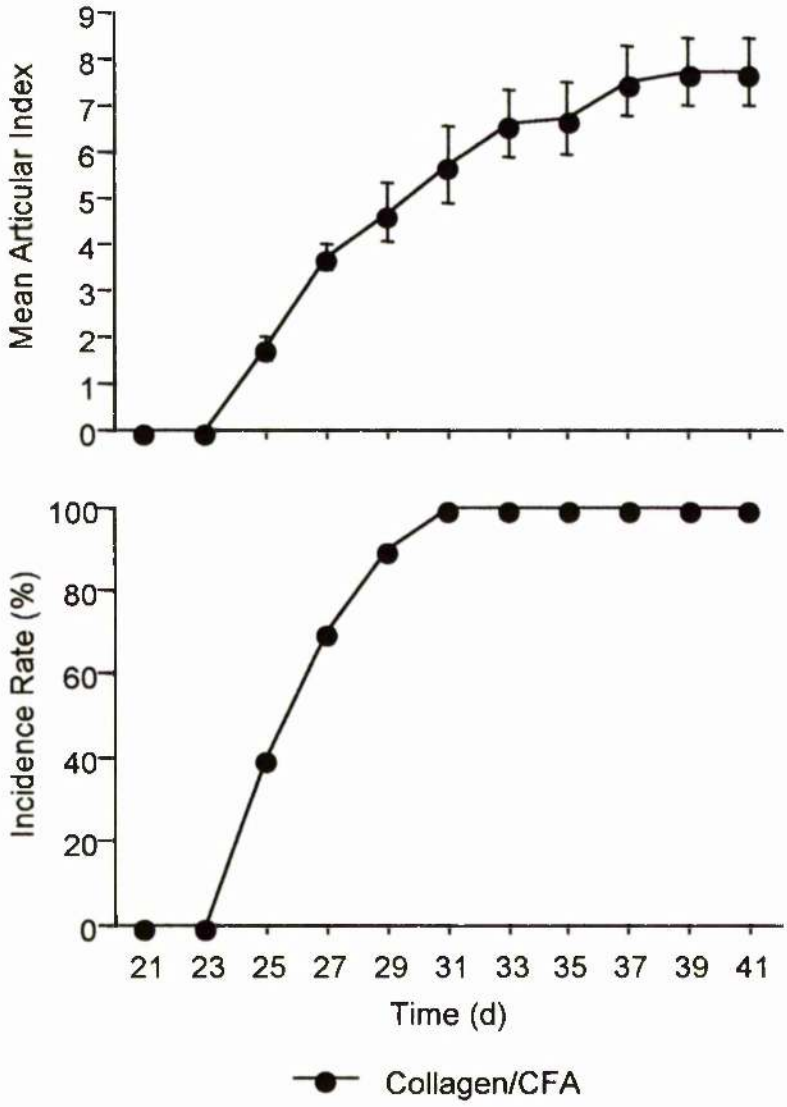
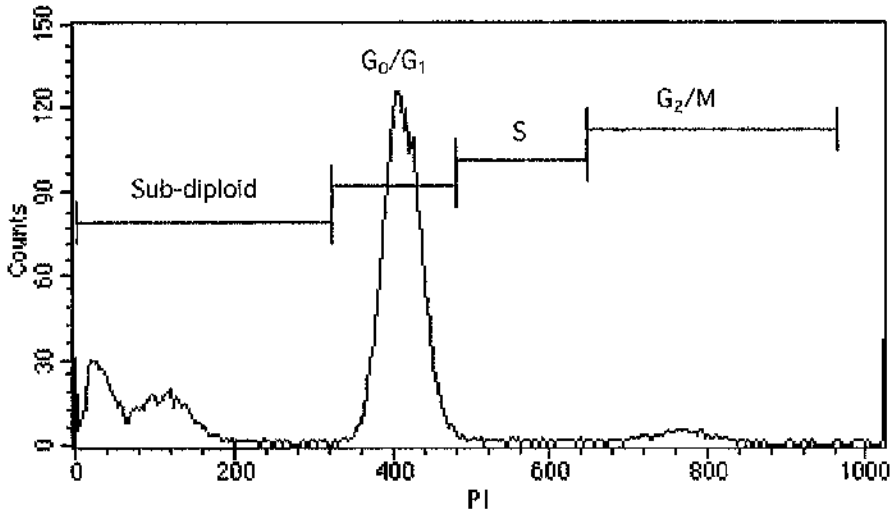

Mean Articular Index
0 1 2 3 4 5 6 7 8 9
Incidence Rate (%)
0 20 40 60 80 100
21 23 25 27 29 31 33 35 37 39 41
Time (d)
Collagen/CFA

Figure 2.2 FACS histogram of DNA content analysis of murine splenic B cells.

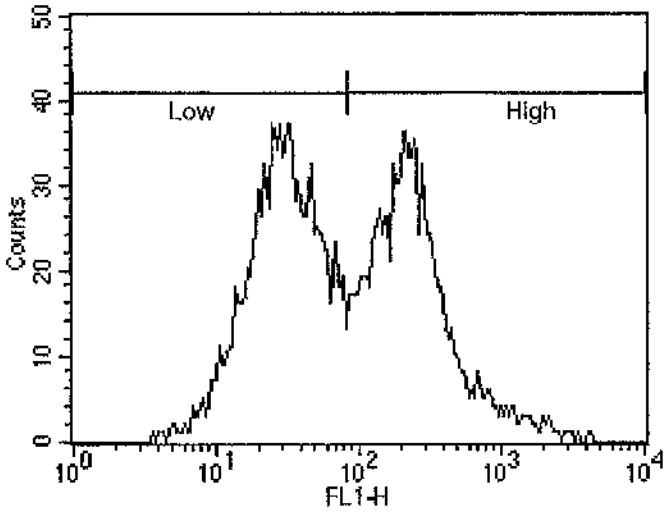
A histogram plot of splenic B cells from Balb/c mice (1×10^6) stained with propidium iodide (250 μg/ml) for DNA content analysis. Histogram markers determine what percentage of cells are in each stage of the cell cycle. The G_1 peak (2N DNA) is set at 400 fluorescence units on the FL-3 x-axis and the G_2/M (4N DNA) calculated accordingly. Sub-diploid DNA or apoptotic cells are marked as those below the 2N peak. Whilst cells in the S phase are determined as those between the 2N and 4N peaks.



Marker	Left, Right	Events	% Gated	% Total	Mean
All	0, 1023	10000	100.00	100.00	354.00
Sub-diploid	3, 325	2425	24.25	24.25	81.22
G_0/G_1	325, 483	6852	68.52	68.52	412.36
S	483, 651	133	1.33	1.33	547.98
G_2/M	651, 966	408	4.08	4.08	765.24

Figure 2.3 FACS histogram of the mitochondrial membrane potential of murine splenic B cells.

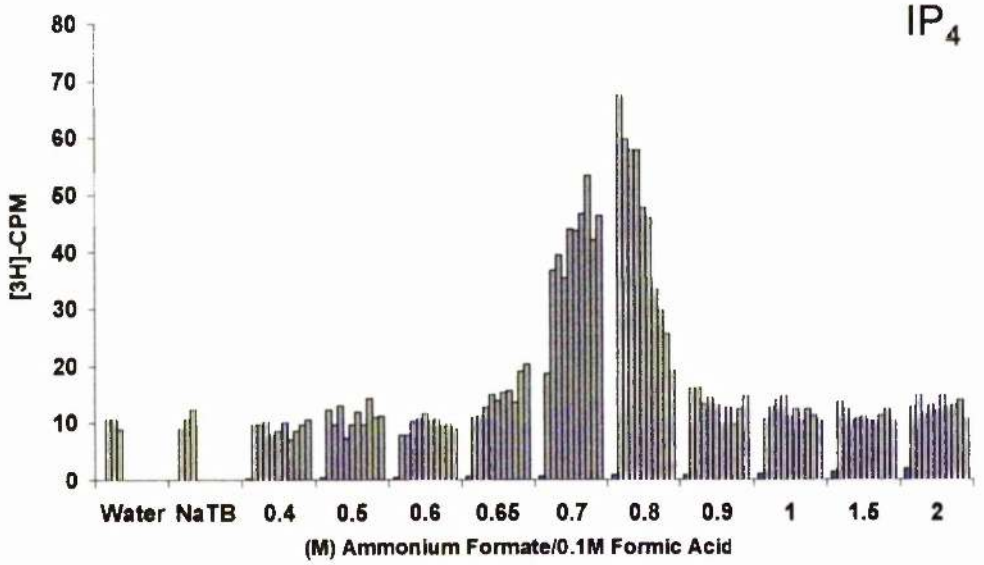
A histogram plot of splenic B cells from Balb/c mice (1 x 10^6) stained with $DiOC_6(3)$ (2.5 μM) for analysis of mitochondrial membrane potential. Histogram markers determine what proportion of cells are represented by low and high $DiOC_6(3)$ fluorescence on the FL-1 x-axis. Cells with low $DiOC_6(3)$ fluorescence are thought to represent the apoptotic population with increased membrane potential and permeability.



Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	10000	100.00	100.00	167.26
Low	1, 83	5293	52.93	52.93	33.56
High	83, 9910	4725	47.25	47.25	316.70

Figure 2.4 Characterisation of Dowex-formate 1x 8-400.

Elution profiles of [^{3}H]-IP_4 and [^{3}H]-IP_3 standards run through Dowex-formate 1 x 8-400 columns. Loaded [^{3}H]-standards are eluted in 1 ml fractions of increasing concentrations of Ammonium formate/0.1 M formic acid. Eluted loads are assessed for [^{3}H]-incorporation by liquid scintillation counting.


IP4
[3H]-CPM
80
70
60
50
40
30
20
10
0
Water NaTB 0.4 0.5 0.6 0.65 0.7 0.8 0.9 1 1.5 2
(M) Ammonium Formate/0.1M Formic Acid

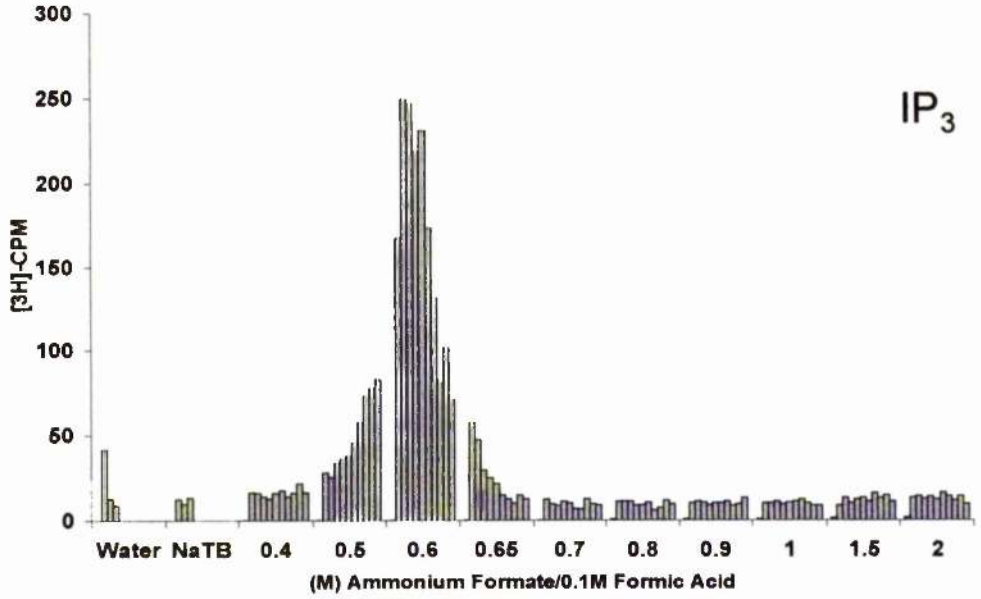

IP3
[3H]-CPM
300
250
200
150
100
50
0
Water NaTB 0.4 0.5 0.6 0.65 0.7 0.8 0.9 1 1.5 2
(M) Ammonium Formate/0.1M Formic Acid

Figure 2.5 TaqMan® real-time RT-PCR.

The TaqMan® oligonucleotide probe contains a 5' reporter dye (R) and a 3' quencher (Q). Whilst the probe is intact, the reporter fluorescence is suppressed by the close proximity of the quencher. As the PCR reaction proceeds, the 5'-3' nuclease activity of the polymerase enzyme cleaves the probe. The reporter and quencher become separated, resulting in reporter fluorescence. The 3' end of the probe is blocked to prevent extension of the probe during PCR.

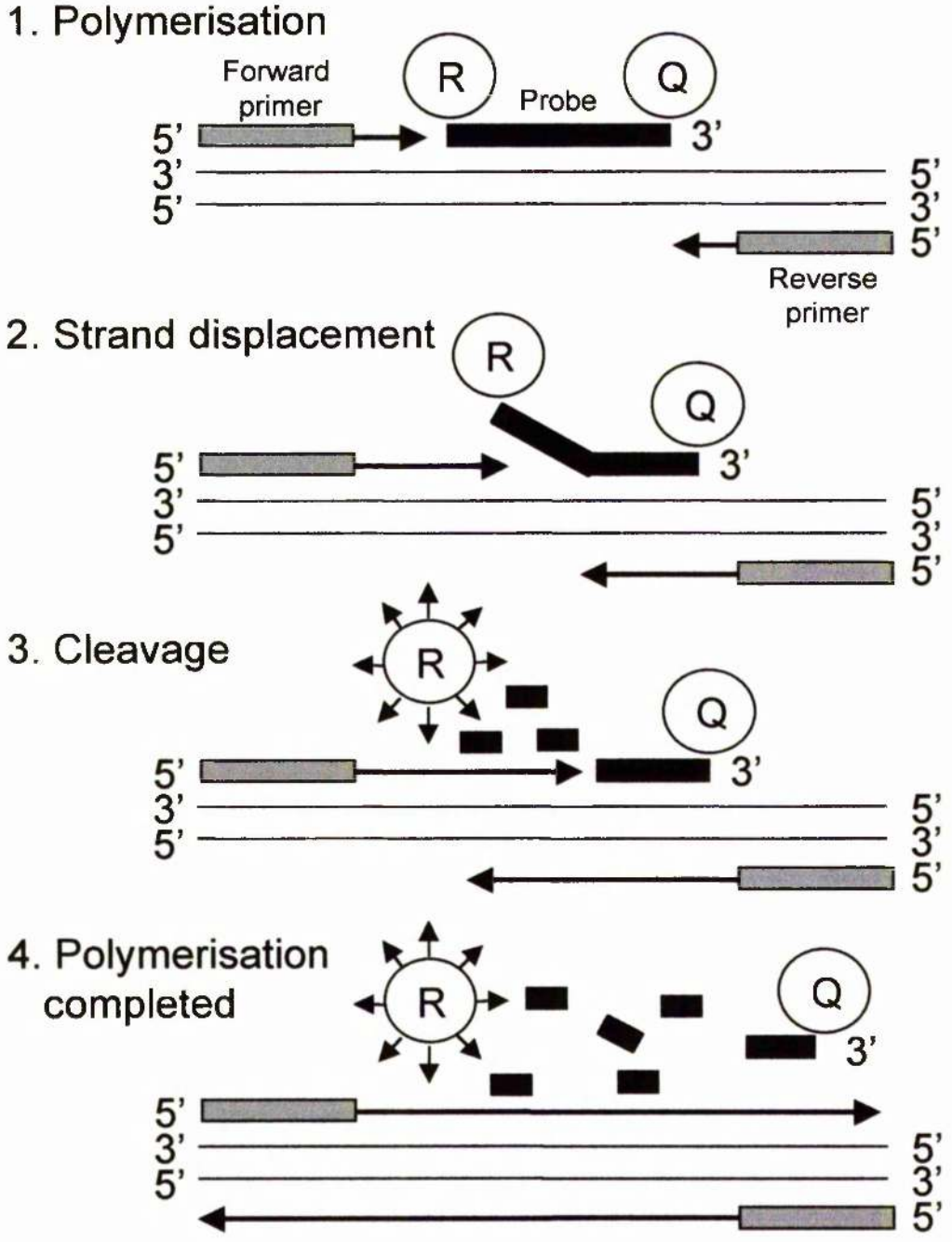

1. Polymerisation
Forward primer
R
Probe
Q
5'
3'
5'
3'
5'
5'
3'
5'
Reverse primer
2. Strand displacement
R
Q
3'
5'
3'
5'
5'
3'
5'
3. Cleavage
R
Q
3'
5'
3'
5'
5'
3'
5'
4. Polymerisation completed
R
Q
3'
5'
3'
5'
5'
3'
5'

Figure 2.6 TaqMan® amplification plot

The TaqMan® amplification plot depicts how the fluorescence emission (normalised Reporter, Rn) varies with PCR cycle (0-45). During the first rounds of PCR amplification, the fluorescence emission is below the limit of detection. Detection occurs as the signal increases in direct proportion to the amount of specific amplified product. As the reaction continues the ratio of polymerase enzyme to PCR product decreases and hence the amount of PCR product ceases to increase exponentially. Eventually the Rn reaches a plateau. The Ct (threshold cycle) is determined during the exponential phase of amplification (Ct indicated by the black line). A typical triplicate sample (F4-F6) is shown with a Ct value of 0.013.

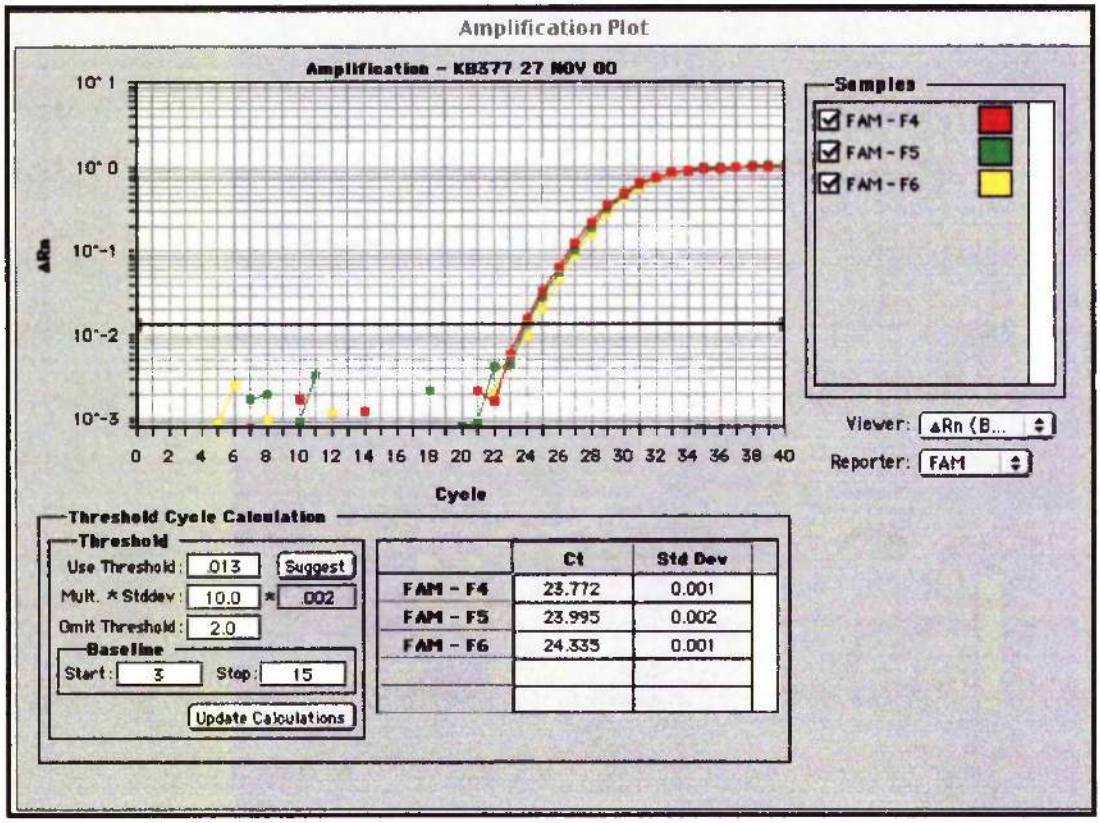
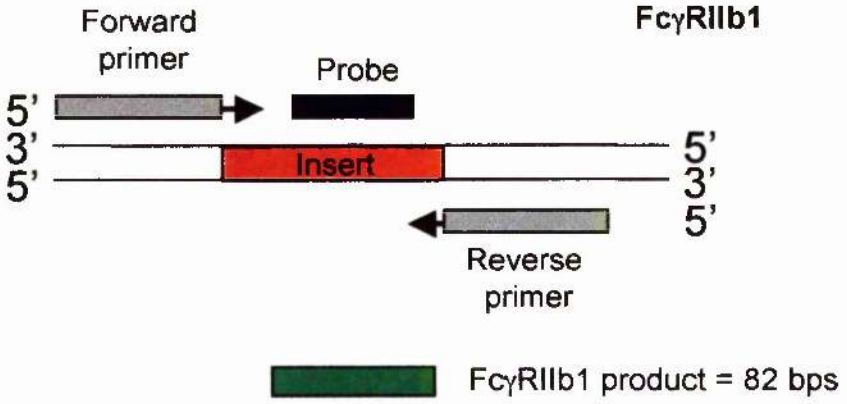
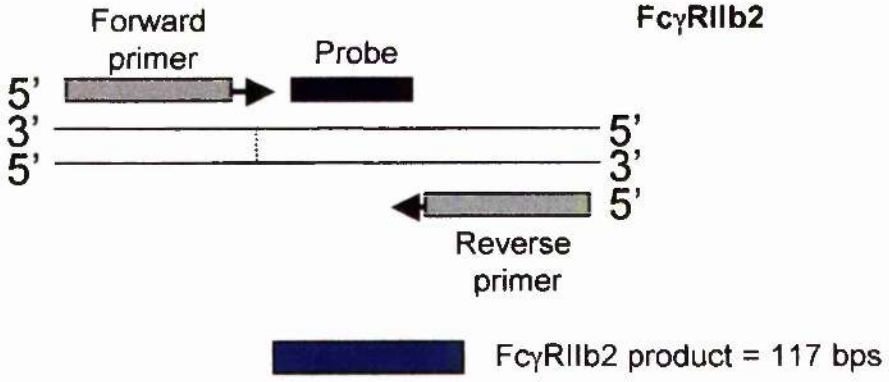

Amplification Plot
Amplification - KB377 27 NOV 00
ΔRn
Cycle
Samples
FAM - F4
FAM - F5
FAM - F6
Viewer: ΔRn (B...
Reporter: FAM
Threshold Cycle Calculation
Threshold
Use Threshold: .013
Suggest
Mult. * Stddev: 10.0 * .002
Omit Threshold: 2.0
Baseline
Start: 3
Step: 15
Update Calculations
Ct
Std Dev
FAM - F4 23.772 0.001
FAM - F5 23.995 0.002
FAM - F6 24.335 0.001

Figure 2.7 TaqMan® amplification of the FcγRIIb isoforms

To amplify the FcγRIIb1 isoform (**A**) the probe and primer set were designed to amplify a FcγRIIb1 insert specific sequence of 82 base pairs. To amplify the FcγRIIb2 isoform (**B**) the forward FcγRIIb2 primer was designed to have four base pairs overlapping the FcγRIIb1 specific insert junction to prevent the primers from amplifying if the probe bound the wrong isotype sequence. It is essential for TaqMan® PCR amplification that the 3' sequence is a perfect match, so four incorrect base pair matches will prevent spurious amplification.


Forward primer
Probe
FcγRIIb1
5'
3'
5'
Insert
5'
3'
5'
Reverse primer
FcγRIIb1 product = 82 bps


Forward primer
Probe
FcγRIIb2
5'
3'
5'
5'
3'
5'
Reverse primer
FcγRIIb2 product = 117 bps

Chapter 3 - Molecular mechanisms of FcγRIIb signalling

3.1 Introduction

B cell regulation of the immune response to antigen requires the maintenance of appropriate levels of signalling through the B cell antigen receptor (sIg/BCR). To maintain this control, B cells employ a variety of positive and negative co-receptors such as CD19, CD22, CD72 and FcγRIIb that modulate the signal that is transduced by the BCR (section **1.2.5**). Signals generated by these molecules appear to alter the signalling threshold of the BCR either by facilitating the positive signalling or by downmodulating its function (Doody *et al.*, 1995; Sato *et al.*, 1996; Cornall *et al.*, 1998). The essential requirement for a balance of activatory and inhibitory pathways has been highlighted by observations that a loss of suitable inhibitory signalling can lead to inappropriate stimulation and activation of B cells (O'Keefe *et al.*, 1996; Clynes *et al.*, 1999; Dijstelbloem *et al.*, 2001). Indeed, aberrant B cell activation can result in a variety of chronic inflammatory processes, including autoimmune disease.

By binding the Fc domain (Fc) of antibodies, Fc receptors (FcRs) provide a critical link between the humoral and cellular arms of the immune system by the targeting of antigen-antibody complexes to effector cells and priming of an immune response (section **1.3**). B cells express only one class of Fc receptor, the low affinity IgG receptor, FcγRIIb (Daeron, 1997a). Co-ligation of the BCR with FcγRIIb, by means of IgG-containing antigen-antibody complexes, negatively regulates the BCR response to antigen (section **1.4**). Although the mechanisms underlying the negative regulation of BCR by FcγRIIb are poorly understood, several of the well characterised cellular responses of BCR stimulation: protein tyrosine phosphorylation, phosphoinositide hydrolysis, calcium influx, cellular proliferation and immunoglobulin secretion are all inhibited following co-engagement. By the induction of growth arrest and/or apoptosis, aggregation of FcγRIIb provides a valuable physiological negative feedback mechanism for the inhibition of B cell activation. Indeed, this feedback mechanism may be an active determinant in the selection of B cells by signals generated by the interaction of immune complexes. Moreover, FcγRIIb

signalling may be of importance for preventing the entry of autoreactive B cells into the periphery, in addition to the negative selection of immature B cells, by promoting the deletion of low-affinity, self-reactive B cells in the germinal centres and by the induction of IgG-mediated peripheral B cell tolerance.

In common with many inhibitory immunoreceptors, coupling of FcγRIIb to downstream signalling pathways is dependent upon the clustering of a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) (Muta *et al.*, 1994, Amigorena *et al.*, 1992a). This unique thirteen amino acid motif possesses a consensus sequence I/VxYxxL/V that constitutes the minimal structural requirement for the ITIM. In an analogous fashion to that observed with the Ig-α/Ig-β ITAMs, aggregation of FcγRIIb by immune complexes results in the phosphorylation of an essential tyrosine residue within the ITIM by Src-family kinases. Phosphorylation of the ITIM provides a docking site for the recruitment of Src-homology domain 2 (SH2) containing phosphatases that act to dephosphorylate key signalling components downstream of the BCR, thereby inhibiting normal B cell responses.

3.1.1 FcγRIIb modulation of BCR activated signalling pathways

FcγRIIb appears to inhibit B cell activation by modulating key BCR-activated signalling pathways involving the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns-(4,5)P_2) by phospholipase C-γ (PLC-γ), the generation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns-(3,4,5)P_3) by phosphatidylinositol 3-kinase (PI-3-Kinase) and the activation of the Ras/MAPKinase pathway. Signals emanating from these pathways all converge at the level of nuclear signalling where they regulate transcription factors and ultimately dictate the fate of the B cell. In order to determine the mechanisms of FcγRIIb-induced growth arrest and/or apoptosis in B cells, it is necessary to re-examine the components of these BCR signalling pathways and how they regulate B cell growth and survival.

Activated by a range of extracellular stimuli, the mitogen-activated protein (MAP) kinases are able to mediate a wide range of cellular functions from

proliferation and activation to growth arrest and cell death (**Figure 1.8**). MAPK signalling cascades, consisting of an upstream serine/threonine kinase (MAPKKK), a middle dual-specificity kinase (MAPKK) and a downstream serine/threonine kinase (MAPK) (Dhanasekaran and Premkumar-Reddy, 1998) are common to many cell types. Activation of the extracellular signal-regulated kinase (Erk) MAPK pathway has been shown to act on a wide range of downstream molecules and transcription factors controlling growth and differentiation. In B cells, initiation of this signalling cascade requires recruitment of the adaptor molecule Shc to the phosphorylated cytoplasmic tail of the activated receptor. The phosphorylation of Shc promotes its interaction with the adaptor molecule, Grb2, which is already complexed to the Ras guanine nucleotide exchange factor (GEF), SOS , in an SH2-domain dependent manner. SOS then promotes conversion of Ras from its GDP-bound inactive form into its active GTP-bound form (Li *et al.,* 1993; Henning and Cantrell, 1996). Active Ras then recruits the serine/threonine kinase Raf-1 (ErkMAPKKK) to the plasma membrane. Phosphorylated Raf-1 is then able to activate the dual-specificity kinases MEK1 (MAPKinase Kinase 1) and MEK2 (MAPKinase Kinase 2) through the phosphorylation of serine residues 217/218 and 221, respectively. MEK1 and MEK2 then activate Erk1 (p44) and Erk2 (p42) MAPK by phosphorylating specific threonine (202) and tyrosine (204) residues within a characteristic TPY motif. Activated Erk1/2 MAPK then phosphorylates and activates a number of cytoplasmic effectors, such as cytosolic phospholipase A_2 ($cPLA_2$) and/or translocates to the nucleus where it phosphorylates a variety of transcription factors regulating gene expression, such as Elk-1 and Stat3. The 90 kDa Ribosomal S6 Kinase (p90RSK) is also phosphorylated by active ErkMAPK and regulates gene expression through the phosphorylation of the Creb and c-fos transcription factors.

The elevation of cytosolic calcium levels is a major downstream event of B cell activation via the BCR. Intracellular levels can be increased both by the release of calcium from intracellular stores and by calcium influx. The generation of the second messenger, $Ins(1,4,5)P_3$, by PLC-γ has been shown to be essential for the release of calcium by binding to $InsP_3$ receptors on intracellular store

membranes (Parekh and Penner, 1997). This, in turn, induces calcium influx through cytoplasmic membrane calcium channels, termed I_{CRAC} (calcium release activated current). In co-operation with the second PLC-γ product, diacylglycerol (DAG), calcium regulates several key B cell signalling events including protein kinase C (PKC) activation and the nuclear localisation and activity of the transcription factor NF-κB (Berridge, 1993). The activation of PKC isoforms is one of the earliest events in the cascade leading to a variety of cellular responses such as gene expression and proliferation (Nishizuka, 1992; Dekker and Parker, 1994). Whilst both PKC activation and calcium mobilisation seem to be required for the induction of genes such as *c-fos*, induction of *egr-1* and *c-myc* appear to require only PKC activation. In contrast, *Ets-1* phosphorylation requires calcium but is independent of PKC activation. PKC activation is also thought to play a role in the regulation of the MAPK pathways, by exerting regulatory control on the activation of ErkMAPK at multiple levels (Schonwesser *et al.*, 1998).

PI-3-Kinase activity and the generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, are known to be important for BCR-mediated B cell proliferation, differentiation and survival (Campbell, 1999). Recent studies have identified that the generation of PtdIns(3,4,5)P_3 by PI-3-Kinase can activate the survival-promoting factor Akt/PKB via the recruitment of the protein-serine/threonine kinases, PDK1 and PDK2, to the plasma membrane. PDK1 and PDK2 activate Akt via phosphorylation of Thr^{308} and Ser^{473}, respectively, within its activation loop. Further phosphorylation at its C-terminal facilitates the activation of Akt. Akt then promotes cell survival by phosphorylating and compromising multiple targets of the cell-death pathway. These include the pro-apoptotic Bcl-2 family member BAD (del Peso *et al.*, 1997) and the cell-death pathway enzyme caspase-9 (Cardone *et al.*, 1998) (**Figure 1.19**). In addition to promoting cell survival, Akt may regulate cell proliferation by phosphorylation of other target molecules. For example, Akt phosphorylation of the pro-apoptotic serine/threonine kinase, glycogen synthase kinase-3 (GSK-3), results in GSK-3 inhibition and promotes cell survival. Recent studies indicating that GSK-3 promotes Cyclin D proteolysis provide a mechanism for such survival signalling

as by inhibiting GSK-3 Akt signalling contributes to Cyclin D accumulation and cell cycle entry. Furthermore, GSK-3 has also been proposed to regulate sub-cellular localisation of the calcium dependent transcription factor. NF-ATc (Gold *et al.*,1999; Cross *et al.*,1995). By inhibiting GSK-3, Akt prevents the export of NF-ATc from the nucleus in response to calcium thus promoting transcription essential for cell survival, growth and proliferation. According to recent reports, Akt may also promote survival by negatively regulating the Ras-Raf-MAPKinase pathway. At first sight this proposal may appear counter-intuitive as this pathway has traditionally been associated with promoting growth and proliferation (Zimmerman and Moelling, 1999). In the human breast cancer cell line, MCF-7, however, prolonged Raf activation has been shown to lead to growth arrest. Thus, by phosphorylating and inactivating Raf at Ser^{259}, Akt shifts the cellular response from growth arrest to proliferation.

3.1.2 Mechanisms of FcγRIIb negative regulation

Following BCR-FcγRIIb co-ligation *in vitro*, the phosphorylated ITIM of FcγRIIb recruits the tyrosine phosphatases, SHP-1 and SHP-2 and an SH2 domain containing 5'-inositol phosphatase, SHIP-1 (section **1.4**). Studies with dominant negative SHIP mutants and knockout models have confirmed that SHIP-1, rather than SHP-1, is preferentially recruited to FcγRIIb following BCR-FcγRIIb co-ligation *in vivo* (Gupta *et al.*, 1997; Liu *et al.*, 1998; Huber *et al.*, 1998). By hydrolysing $Ins(1,3,4,5)P_4$ and $PtdIns(3,4,5)P_3$ to $Ins(1,3,4)P_3$ and $PtdIns(3,4)P_2$ respectively, SHIP is able to uncouple the BCR from a specific subset of pathways involving PLC-γ and the Tec family kinases, such as phosphoinositide hydrolysis, extracellular calcium influx and cellular proliferation.

SHIP-1 mediated hydrolysis of $PtdIns(3,4,5)P_3$ prevents the recruitment of PLC-γ and Btk to the plasma membrane and thus abrogates the pathways downstream of their activation, in particular the sustained mobilisation of calcium. In addition, $Ins(1,3,4,5)P_4$ has been proposed to participate in calcium signalling thus SHIP may inhibit calcium mobilisation not only by hydrolysing $Ins(1,3,4,5)P_4$ to produce a form of $InsP_3$ ($Ins(1,3,4)P_3$) unable to bind to receptors ($InsP_3R$) in the endoplasmic reticulum (Bolland *et al.*, 1998;

Scharenberg, 1998) but also by blocking entry of extracellular calcium (Luckhoff and Clapham, 1992).

The conversion of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 by SHIP-1 has also been reported to result in the partial inhibition of the survival factor Akt/PKB (Jacob *et al.*, 1999; Aman *et al.*, 1998). However, since PtdIns(3,4)P_2 has also been shown to be involved in the positive regulation of Akt activity, the observed inhibition of Akt may rather reflect upstream deregulation by the alternative inositol phosphatase, PTEN. Whilst SHIP has been shown to dephosphorylate the 5' position of PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P4, PTEN is able to dephosphorylate the 3' position. PTEN or MMAC (*p*hosphatase and *t*ensin homologue deleted on chromosome *ten* or mutated in multiple advanced cancer) (Li *et al.*, 1997) was originally identified as a tumour suppresser gene implicated in the progression of a variety of human cancers. Further studies demonstrated that this 55-kDa protein contained a signature catalytic motif common to members of the protein tyrosine phosphatase (PTP) family. This was confirmed *in vitro*, where PTEN displayed enzymatic activity towards both protein and lipid substrates. Substantial evidence suggests that PTEN acts primarily to dephosphorylate the phosphoinositide PtdIns(3,4,5)P_3 and possibly PtdIns(3,4)P_2 and thus acts to directly antagonise the actions of PI-3-Kinase. Both lipid substrates are commonly associated with mediating positive cell signals resulting in cell proliferation and survival.

Consistent with this, several papers have now established a link between the PI-3-K/Akt pathway and PTEN. Indeed, a study by Stambolic *et al.*, 1998 confirmed that negative regulation of Akt/PKB by PTEN is critical for normal apoptotic signalling induced by a variety of apoptotic stimuli. Furthermore, PTEN deficiency leads to enhanced phosphorylation and activation of Akt/PKB confirming that PTEN can inhibit Akt-dependent survival signals induced in response to PI-3-kinase activation. These studies suggest that, like SHIP, PTEN may act *in vivo* as a negative regulator of cell proliferation and activation. Indeed, knockout models support a role for PTEN in maintaining lymphocyte homeostasis and immune tolerance, as PTEN insufficiency results in T cell

lymphoma, hyperplasia and the development of lethal autoimmune disorders (Suzuki *et al.*, 2001).

In addition to its phosphatase activity, SHIP has a number of putative tyrosine phosphorylation sites and a proline rich domain, which could enable protein adapter interactions. In particular, via its association with the adapter molecule, Shc, SHIP-1 is thought to abrogate normal recruitment and activation of the GTPase Ras. FcγRIIb activated SHIP-1 is thought to mediate inhibition of the Ras/MAP kinase pathway by competing with the Grb2/SOS complex for Shc binding. The sequestration of Shc by SHIP-1 is believed to prevent the recruitment and activation of the GTPase, Ras. In addition, SHIP-1 may act to terminate Ras/MAPK activation via its interaction with p62Dok, a RasGAP binding protein (Tamir *et al.*, 2000a). RasGAP acts to convert the active form of Ras-GTP into an inactive GDP form, thus preventing activation of the Raf-MAPK pathway.

However, the molecular mechanisms of SHIP-1 inhibition appear to alter depending on the activation status of the cells (Brauweiler *et al.*, 2001). Unlike resting B cells, B cell blasts from SHIP-1 deficient mice are still able to inhibit calcium mobilisation, Akt and ErkMAPK activation following FcγRIIb co-ligation. The discovery of an alternative SHIP isoform, SHIP-2 that is upregulated in activated B cells may provide an alternative inhibitory mechanism for FcγRIIb on memory and plasma cells and explain why activated cells are able to convert PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 in a SHIP-1 independent manner.

Chimeric FcγRIIb experiments and SHP-1 knockout models have demonstrated that SHP-1 is dispensable for FcγRIIb-mediated inhibition of B cells, since FcγRIIb is still able to inhibit BCR-mediated proliferative events. However, a modulatory role for SHP-1 in FcγRIIb-mediated B cell inhibition has not been completely discounted. The strongest evidence in support of SHP-1 involvement comes from the studies in the SHP-1 deficient motheaten (*me*) and motheaten viable (me^v) mice (previously described in section **1.4**) as FcγRIIb-mediated inhibition of BCR-induced proliferation in mature B cells is impaired in

these mice (Nadler *et al.*, 1997). This suggests that SHP-1 is not (completely) redundant in all FcγRIIb-mediated signalling events and that it is required for FcγRIIb to be fully functional. Thus, it is possible that SHP-1 and SHIP differentially but synergistically target distinct signalling pathways in B cells.

Specific targets of SHP-1 in FcγRIIb signalling have not been characterised but the number of potential targets is quite large and varied. A proposed substrate, however, is CD19, which is dephosphorylated during FcγRIIb-mediated negative signalling (Hippen *et al.*, 1997). One function of CD19 is to promote BCR-mediated activation of PLC-γ by the recruitment of PI-3-kinase. The dephosphorylation of CD19 by SHP-1 could therefore result in an abrogation of decreased calcium influx. Other possible targets of SHP-1 include the Src-family PTKs, dephosphorylation of which would disrupt initiation of all signalling pathways. Indeed, studies in a BCR-expressing myeloma cell line demonstrated that SHP-1 mediates the negative regulatory effects of CD72 by dephosphorylating the BCR accessory molecules, Ig-α/Ig-β, the PTK Syk and the recruitment of BLNK (Adachi *et al.*, 2001).

3.2 Aims and objectives of this study

Previous studies on FcγRIIb signalling have concentrated on how BCR-FcγRIIb co-ligation inhibits early B cell activation. However, recent studies have indicated that FcγRIIb ligation can lead to distinct cellular responses of growth arrest and/or apoptosis in B cells, dependent on the levels and type of aggregation. This finding has important implications for the selection and modulation of B cell activity by immunocomplexes during B cell development.

Therefore, the aims of this study were to investigate the signalling mechanisms responsible for FcγRIIb inhibition of B cell antigen receptor (BCR) mediated proliferative signalling in primary, mature B cells. In particular, it was planned to address,

- the differing signals involved in immune complex-induced growth arrest and/or commitment to apoptosis of resting B cells.

- the signals responsible for FcγRIIb mediated uncoupling of BCR signalling resulting in growth arrest.

In particular, potential differences in early signalling relating to SHIP, SHP-1 and PTEN regulation of PI-3-Kinase, PLC-γ and MAPK pathways in resting and activated cells were investigated in order to dissect differential effects of immune complexes on resting cells and those underlying negative feedback inhibition of BCR-driven proliferation and antibody production.

In addition, investigations into how such differential FcγRIIb-mediated signals result in sustained B cell growth arrest or lead to apoptosis have been limited. Therefore, part of this study was to examine whether FcγRIIb signalling modulates the BCR-activated cell cycle machinery by examining the role and kinetics of BCR-driven nuclear signalling components in the presence and absence of inhibitory signalling via FcγRIIb.

3.3 Results

3.3.1 FcγRIIb ligation abrogates antigen receptor mediated B cell proliferation

It is known that ligation of the BCR with $F(ab')_2$ fragments of anti-mouse Ig antibodies leads to B cell proliferation whilst BCR-FcγRIIb co-ligation using intact anti-Ig antibodies inhibits BCR-mediated DNA synthesis (Klaus *et al.*, 1984 and 1985; section **1.4**). Using DNA synthesis as a measure of proliferation, **Figure 3.1A** shows that stimulation of murine splenic B cells via the BCR ($F(ab')_2$), results in a dose-dependent increase in DNA synthesis. However, no DNA synthesis was observed following ligation of FcγRIIb with the intact form of the antibody alone, over a range of similar concentrations (**Figure 3.1B**). BCR-FcγRIIb co-ligation by a combination of both $F(ab')_2$ and intact anti-Ig antibodies together results in a dose-dependent decrease in BCR-induced DNA synthesis (**Figure 3.1C**) and provides an experimental model for negative feedback inhibition of ongoing B cell responses. Maximal inhibition of $F(ab')_2$ induced DNA synthesis was achieved using intact anti-Ig antibodies at a concentration of 75 μg/ml i.e. at a concentration which is equimolar to that of the $F(ab')_2$ concentration required for maximum DNA synthesis (**Figure 3.1** and results not shown).

To confirm that co-ligation of BCR-FcγRIIb is required for the inhibitory effect of FcγRIIb on BCR-mediated proliferation, an anti- FcγRII/III antibody (2.4G2) was used to block BCR-FcγRIIb co-ligation (**Figure 3.1D**). Consistent with published observations (Unkeless, 1979), co-stimulation of B cells via the BCR and FcγRIIb, in the presence of 2.4G2, resulted in almost total restoration of BCR-induced levels of DNA synthesis. Together these results confirm that FcγRIIb co-ligation can reduce or prevent BCR-mediated proliferation of murine B cells, supporting a role for this receptor in negative feedback regulation of B cells.

To confirm that FcγRIIb-mediated inhibition of splenic B cells was independent of the activation status of the cells the effect of FcγRIIb ligation on populations of resting and activated mature B cells was investigated. Activated splenic B

cells were separated from the resting population by differential density centrifugation using Percoll. The results shown in **Figure 3.2A** confirm that FcγRIIb is able to induced growth arrest in B cells that are already actively proliferating, similar to that shown by resting splenic B cells **Figure 3.2B**.

3.3.2 Ligation of FcγRIIb on splenic B cells induces apoptosis.

The above experiments confirmed that BCR-FcγRIIb co-ligation was able to induce growth-arrest in splenic B cells, a process that could be prevented by an anti-FcγRII/III blocking antibody. To investigate whether the FcγRIIb–mediated growth-arrest observed in the DNA synthesis assay was truly due to growth arrest or actually a consequence of apoptosis, sub-diploid DNA content levels were determined by FACS analysis of propidium iodide incorporation. The results shown in **Figure 3.3** confirm the ability of BCR stimulation to promote B cell entry into S and G_2/M phases of the cell cycle, leading to increased levels of proliferation and DNA synthesis seen in **Figure 3.1.** In contrast, BCR-FcγRIIb co-ligation resulted in an increased percentage of B cells undergoing cell cycle arrest (G_0/G_1) and apoptosis (sub-diploid) above control level. Indeed, sub-diploid levels are enhanced in B cells stimulated with intact anti-Ig antibody alone.

These results suggested that FcγRIIb-ligation is indeed able to induce responses of cell cycle arrest and apoptosis in splenic B cells. The remainder of the study will therefore investigate the signalling mechanisms responsible for these differential responses and will begin with an examination of FcγRIIb-induced growth-arrest.

3.3.3 Differential tyrosine phosphorylation during positive and negative cell signalling.

As discussed in the introduction, one of the earliest events detected following BCR ligation is protein tyrosine phosphorylation and activation of key downstream effector proteins. FcγRIIb-mediated inhibition of BCR-induced proliferation may act to modulate the phosphorylation of these proteins and thus prevent downstream signalling events. To investigate the possible target

recruited proteins following FcγRIIb ligation, splenic B cells were stimulated via the BCR with $F(ab')_2$ fragments of anti-Ig antibody or via FcγRIIb with the intact form of the antibody, or a combination of the two, for the times indicated The presence of phosphotyrosine containing proteins was detected by Western blotting, using the monoclonal anti-phosphotyrosine antibody (4G10).

In unstimulated B cells few proteins are tyrosine phosphorylated but following BCR stimulation several proteins become tyrosine phosphorylated (**Figure 3.4A**). Phosphorylation peaks at 1 minute post stimulation and is reduced by 30 minutes. Ligation of FcγRIIb induces the phosphorylation of a distinct group of proteins. The approximate molecular weights of these target proteins were 145, 70 and 65 kDa. Again, the strongest signals are transient, appearing 1 minute post stimulation and returning towards basal levels by 30 minutes post stimulation. Certain proteins are tyrosine phosphorylated during both positive and negative signalling. Indeed, the phosphorylation of these proteins seems to be stronger following FcγRIIb co-ligation. Co-stimulation via the BCR and FcγRIIb using $F(ab')_2$ and intact anti-Ig antibodies together, leads to a similar profile of tyrosine phosphorylation to that seen following stimulation with intact antibodies alone. As the recruitment and phosphorylation of the phosphatases SHP-1 (65 kDa), SHP-2 (70 kDa) and the 5'-inositol-phosphatase SHIP (145 kDa) by FcγRIIb, is well documented *in vitro*, it was not surprising that the identity of these bands was confirmed by immunoprecipitation and Western Blot analysis using the anti-phosphotyrosine antibody, 4G10 (**Figure 3.4B**).

Interestingly, these results indicate that SHIP is also recruited and activated following stimulation via the BCR, disputing earlier studies that demonstrated an exclusive role for SHIP in FcγRIIb-mediated negative signalling. Indeed, SHIP is transiently phosphorylated following BCR aggregation, with peak phosphorylation occurring after 1 minute. However, whilst following similar kinetics, the levels of SHIP phosphorylation induced by BCR ligation are lower than that observed following FcγRIIb co-ligation (**Figure 3.4B**).

In agreement with the kinetics of SHIP tyrosine phosphorylation following BCR-FcγRIIb co-ligation, measurement of *in vitro* SHIP inositol 5' phosphatase

activity revealed a rapid enhancement of phosphatase activity, resulting in an increase of Ins(1,3,4)P_3 levels (**Figure 3.5**). Maximal SHIP activity was shown to peak at 5 minutes post-FcγRIIb co-ligation, returning towards basal levels by 15 minutes. In contrast, stimulation via the BCR alone resulted in limited inositol phosphatase activity by anti-SHIP immune complexes. Thus, the level of tyrosine phosphorylation of SHIP seems to correlate with its inositol phosphatase activity *in vitro.*

3.3.4 FcγRIIb co-ligation induces the formation of SHIP complexes with downstream signalling molecules.

The above results highlighted the possibility that SHIP is differentially activated in B cells following BCR and/or FcγRIIb ligation. To investigate the possibility that SHIP may associate with different downstream regulatory proteins under the different stimulatory conditions, anti-SHIP immune precipitates were Western blotted for associated proteins (**Figure 3.6**). Following BCR or FcγRIIb stimulation, SHIP was shown to transiently associate with the adaptor protein, Shc. The comparable time course of SHIP tyrosine phosphorylation (**Figure 3.4B**) with Shc association suggests that this interaction is tyrosine phosphorylation dependent. These results are in agreement with recent published kinetic data for the recruitment and phosphorylation of SHIP by FcγRIIb resulting in enhanced affinity of SHIP for Shc (Tridandapani *et al.*, 1999). Consistent with the SHIP tyrosine phosphorylation profile following BCR ligation (**Figure 3.4B**), Shc-SHIP complexes are also detectable in BCR stimulated cells. Recent studies have confirmed that tyrosine phosphorylation of Shc, in response to BCR stimulation, is SHIP-dependent thus describing a novel role for SHIP in BCR signalling (Ingham *et al.*, 1999).

In agreement with a recent study (Harmer and DeFranco, 1999), SHIP immune precipitates also revealed the presence of Grb2, another adaptor protein normally associated with Shc and SOS in a tri-molecular complex following BCR ligation. Grb2 was shown to be associated with SHIP, following B cell stimulation via the BCR and/or FcγRIIb however levels of Grb2 association were

reduced under conditions modelling negative feedback inhibition of BCR-signalling. Grb2 is thought to stabilise SHIP-Shc complexes, presumably by simultaneously binding via its SH3 domains to SHIP and via its SH2 domain to Shc. The demonstration by Harmer and DeFranco, 1999 that SHIP is unable to associate with Shc in Grb-2 deficient B cells highlighted the importance of this interaction.

The formation of SHIP complexes with the protein phosphatases SHP-1 and SHP-2 is intriguing and may indicate a possible modulatory role of SHIP on downstream pathways associated with these molecules. Indeed, a study of FcγRIIb function using SHIP, SHP-1 and SHP-2 binding decoys demonstrated that although SHIP is the predominant intracellular ligand for the phosphorylated FcγRIIb ITIM, the SHP-2 decoy exhibited some ability to bind to FcγRIIb and block its function (Nakamura, K. *et al.*, 2000). SHIP recruited SHP-2 may therefore act to switch off further FcγRIIb signals.

3.3.5 Association of Shc with SHIP but not SHP-1 following FcγRIIb co-ligation

SHIP has been demonstrated to be essential for the inhibitory role of FcγRIIb in B cells (Ono *et al.*, 1996; Chacko *et al.*, 1996; Liu *et al.*, 1998). The consensus that the sequestration of the adaptor protein Shc by SHIP is thought to lead to a reduction in Ras activation and subsequent inhibition of the Ras/MAP kinase pathway is still not fully established, however. SHIP-$1^{-/-}$ splenic B cells, display enhanced MAPK activation in response to BCR ligation or BCR-FcγRIIb co-ligation (Liu *et al.*, 1998). However, these results are not consistent with those of Ingham *et al.*,1999, which demonstrate that BCR-mediated tyrosine phosphorylation of Shc and hence, activation of RasMAPK, is SHIP dependent. Similarly, the SHIP-Shc sequestration model is not consistent with the results reported here showing that mitogenic BCR signalling also promotes strong SHIP-Shc complex formation. These results imply that further modulation of SHIP activity is required to produce the differential responses following BCR or FcγRIIb ligation.

The formation of SHIP-Shc and SHIP-SHP-1 complexes (**Figure 3.6**) suggested that SHP-1 might modulate FcγRIIb signalling by forming a SHP-1-SHIP-Shc complex. To investigate whether SHP-1 could associate with Shc, possibly via SH2 domain interactions, anti-SHP-1 immune precipitates were Western blotted with an anti-Shc antibody (**Figure 3.7A**). Despite the strong expression of SHP-1, no association between SHP-1 and Shc was demonstrated under any stimulation conditions. In addition, anti-Shc immune precipitates failed to demonstrate any associated SHP-1 phosphatase activity (**Figure 3.7B**). These results suggest that complexes containing SHP-1 and Shc are unlikely to be formed following FcγRIIb ligation and that FcγRIIb activated SHP-1 does not mediate its inhibitory effects via inhibition of the RasMAPK pathway by recruitment of Shc. However, it does not exclude the formation of SHIP-SHP-1 complexes with the BCR and the potential for SHP-1 to contribute to Ras inhibition by dephosphorylating the ITAMs of the BCR accessory molecules, Ig-α/Ig-β, or their associated PTKs.

3.3.6 SHP-1 is activated following BCR-FcγRIIb co-ligation and dephosphorylates Lyn

SHP-1 is known to be associated with the BCR in resting B cells and is thought to maintain the resting status of the unstimulated BCR by dephosphorylating the ITAMs on the BCR-accessory molecules, Ig-α and Ig-β (section **1.2.2**). In contrast, the PTK, Lyn is thought to initiate both BCR and FcγRIIb activation by phosphorylating the cytoplasmic tails of their respective ITAMs or ITIMs. Thus, FcγRIIb may inhibit BCR-coupling to PTK-dependent events by recruiting SHP-1 to counteract Lyn mediated phosphorylation.

To investigate this possibility, SHP-1 activity was assessed in both SHP-1 (**Figure 3.8A**) and Lyn containing (**Figure 3.8B**) immune complexes over a 30 minute time period of BCR or BCR-FcγRIIb co-ligation. BCR stimulated SHP-1 activity was maximal at 1 minute post-stimulation, possibly reflecting residual inhibition prior to activation and the transient requirement for phosphatase activity in reinitiation and perpetuation of PTK signals in activated B cells.

Importantly, FcγRIIb co-ligation induced a sustained increase in SHP-1 activity over the equivalent time period.

Analysis of the SHP-1 activity associated with Lyn demonstrated a reduced association following BCR stimulation that reverted to basal level by 5 minutes (**Figure 3.8B**). These results reflect the transient downregulation of SHP-1 mediated inhibition of Lyn activity following BCR ligation, which is followed by the reinitiation of phosphatase activity to return the BCR signalling complex to a resting state. In contrast, Lyn-SHP-1 association was enhanced in FcγRIIb stimulated B cells at 5 minutes returning to basal levels by 30 minutes. In agreement with this, SHP-1 was found to co-immunoprecipitate with Lyn in control unstimulated cells, supporting the proposal that SHP-1 acts to keep the BCR in a resting state (**Figure 3.8C**). Interestingly, Lyn association with SHP-1 was maximal in BCR stimulated cells at 1 minute post-stimulation and decreased steadily over the time period of BCR stimulation FcγRIIb ligation also resulted in decreased association of Lyn with SHP-1 over time. Taken together, these results might suggest that Lyn-association is required for phosphorylation and activation of SHP-1, as dissociation correlates with a decrease in SHP-1 activity in BCR-stimulated cells. This latter point, however, suggests that in FcγRIIb-stimulated cells, SHP-1 activity is somehow maintained in the absence of Lyn, perhaps by an, as yet, unidentified alternative PTK. Unlike SHIP and SHP-1, FcγRIIb signalling was shown not to modulate SHP-2 phosphatase activity (results not shown) therefore questioning the role of this protein tyrosine phosphatase activity in the inhibitory SHIP-1 complexes in **Figure 3.6**.

3.3.7 FcγRIIb inhibits Erk/MAPK phosphorylation

The BCR is coupled to the ErkMAPK pathway via PTK recruitment of the adaptor molecule Shc and subsequent activation of the GTPase, Ras (Campbell, 1999; Henning and Cantrell, 1998). As shown, FcγRIIb-ligation results in the activation of the phosphatases SHIP-1 (**Figure 3.5**) and SHP-1 (**Figure 3.8**) which are known to dephosphorylate and deactivate key BCR-

coupled signalling molecules and thus interrupt normal proliferative signalling. In particular, SHIP, may act as a negative regulator of the ErkMAPK pathway by the sequestration of Shc and via its interaction with p62Dok, a RasGAP binding protein (Tamir *et al.*, 2000a). Both of these mechanisms are thought to lead to the inactivation of Ras and downstream MAPK effector molecules. FcγRIIb ligation also leads to enhanced Lyn associated SHP-1 activity, which may lead to an inactivation of all B cell pathways downstream of this primary PTK (**Figure 3.8**). However, it has been shown that tyrosine phosphorylation of the FcγRIIb ITIM, following coligation with the BCR, is almost exclusively catalysed by Lyn in murine B cells (Nishizumi *et al.*, 1998). Thus, FcγRIIb may promote a negative feedback mechanism for its own activation to switch off further negative signalling.

To investigate the inhibitory role of FcγRIIb ligation on BCR-induced MAPK activation, the effects of stimulation via the BCR and FcγRIIb on ErkMAPK phosphorylation was investigated. Western blotting of whole cell lysates show that unstimulated splenic B cells exhibit little or no Erk phosphorylation, reflecting the resting state of the cells (**Figure 3.9**). Consistent with a BCR-mediated proliferative response, stimulation via the BCR results in a rapid phosphorylation of ErkMAPK, which is sustained for at least 30 minutes. In contrast, stimulation via FcγRIIb results in a transient phosphorylation of ErkMAPK, which is rapidly terminated by 30 minutes. Furthermore, FcγRIIb stimulation was able to abrogate the tyrosine phosphorylation of ErkMAPK induced by mitogenic signalling via the BCR. Thus, the induction of growth-arrest and apoptosis by FcγRIIb ligation appears to correlate with the uncoupling of the BCR from early ErkMAPK phosphorylation.

3.3.8 Sustained FcγRIIb inhibition of Erk/MAPK phosphorylation is independent of Ras activation.

The above data demonstrated that FcγRIIb might act to prevent B cell activation through the termination of early ErkMAPK signals. However, the exact mechanism by which this is achieved is still unknown. Studies demonstrating that SHIP-Shc interactions are also important for normal BCR-mediated signals

have questioned the initial theories that the sequestration of Shc by SHIP and inhibition of subsequent interactions with the GTPase, Ras, are essential for FcγRIIb-mediated inhibition of RasMAPK. This reasoning suggests that the inhibition of ErkMAPK could be due to a negative regulatory effect on a mediator downstream of Shc.

Raf-1, a mitogen-activated protein kinase, is the main effector recruited by GTP-bound Ras in order to activate the MAPK pathway (Avruch *et al.*, 1994). To determine if the FcγRIIb-mediated inactivation of ErkMAPK was due to inactivation of Ras and an inability to recruit Raf-1, the kinetics of Ras activation were investigated under similar conditions of BCR and/or FcγRIIb ligation. The study utilised the availability of a glutathione-coupled Ras-RBD substrate, which binds selectively to active-GTP-bound Ras and not inactive-GDP-bound Ras. Thus, the levels of activated Ras following FcγRIIb-ligation can be assayed for by the selective pull-down of active-Ras from whole-cell lysates. Interestingly, unstimulated cells expressed a basal level of Ras activity, despite the lack of downstream ErkMAPK activation (**Figure 3.10A).** This suggests that B cells must initiate additional inhibitory mechanisms to prevent excessive ErkMAPKinase activation during normal, unstimulated, proliferation. However, as Ras has been shown to have several additional downstream effectors, such basal Ras activity could also represent a signalling requirement for the survival of resting B cells .

Ras activation was not shown to be consistent with the kinetics of BCR mediated ErkMAPK activation, confirming reports that BCR-mediated ErkMAPK activation is not exclusively Ras-dependent. For example, BCR-coupled Raf-1 activation via DAG-sensitive PKC isoforms has been reported. Importantly, however, although co-stimulation via FcγRIIb lead to a transient decrease in Ras activation, consistent with the kinetics of SHIP-Shc complex formation (**Figure 3.6**) and downstream inactivation of ErkMAPK (**Figure 3.9**), this period of downregulation was followed by a dramatic upregulation of Ras activation at 30 minutes, which may reflect a downregulation in the formation of SHIP-Shc complexes, as shown in **Figure 3.6**. Despite the re-activation of Ras, a

concomitant upregulation in ErkMAPK activity is not shown at 30 minutes (**Figure 3.9**). These results suggest that whilst SHIP sequestration of Shc may lead to short-term inactivation of the Ras/ErkMAPK pathway alternative inhibitory signals must be induced at later time points to uncouple the BCR from ErkMAPK under conditions in which Ras is hyper-activated.

Additional studies were carried out to determine whether long term downregulation of ErkMAPK activation was due to the sequestration of active Raf-1 by the alternative GTPases Rap-1 or Ral. Rap-1 activity has not been extensively studied in B cells, however, its role as a negative regulator of T cell mediated ErkMAPK activation has been studied (Henning and Cantrell, 1998). Results indicated that neither of these molecules was activated in either basal, BCR or FcγRIIb stimulated splenic B cells (results not shown). In addition to the sequestration of Shc, SHIP is proposed to inhibit Ras activation via the recruitment of the RasGAP binding protein p62Dok (Tamir *et al.*, 2000a). Thus, whilst Ras may be activated at later time points, p62Dok could promote its rapid inactivation. However, no expression of p62Dok in Ras immune complexes could be detected at these later time points (results not shown).

3.3.9 FcγRIIb co-ligation modulates BCR-mediated MEK activation.

The MAPKinase kinases (MEKs), MEK1 and MEK2, are responsible for the regulation and activation of ErkMAPK by the dual phosphorylation of tyrosine and threonine residues within a characteristic threonine-X-tyrosine activation motif of ErkMAPK. Similarly, each family of the MAPKinase group is activated by specific MEKs allowing independent activation and regulation following cell stimulation (**Figure 1.8**). Ras activated Raf-1 is able to activate the dual-specificity kinases MEK1 and MEK2. Thus to determine if the sustained FcγRIIb-mediated inactivation of ErkMAPK was due to inactivation of MEK, the kinase activity in MEK immune precipitates following BCR and FcγRIIb ligation was investigated.

Figure 3.10B shows that MEK activity is increased in BCR stimulated cells with kinetics that are consistent both with ErkMAPK activity and activation by Ras.

Although BCR-FcγRIIb co-stimulated B cells demonstrate MEK activity, the levels of activation are dramatically reduced in comparison with BCR-stimulated samples. Thus, sustained ErkMAPK inactivation following FcγRIIb-ligation is occurring in a Ras independent manner but FcγRIIb-mediated inhibitory mechanisms may operate downstream or at the level of MEK activation.

The inhibition of ErkMAPK activation can occur through a variety of negative feedback mechanisms, several of which rely on molecules that compete as substrates for the Ras/MAPK signalling kinases. Amongst these is the recently discovered Raf-kinase inhibitor protein (RKIP), which prevents MEK activation by Raf-1 (Yeung *et al.*, 1999). In order to determine whether RKIP could be responsible for sustained FcγRIIb-mediated suppression of ErkMAPK, Raf-1 immunoprecipitates were Western blotted with RKIP antiserum (a kind gift from W. Kolch, Beatson Institute for Cancer Research, Glasgow). Unfortunately, no conclusive results could be obtained concerning the association of RKIP with Raf-1 following BCR or FcγRIIb-ligation in splenic B cells (results not shown). However, as evidence is emerging that RKIP may not be expressed in lymphocytes (Walter Kolch, personal communication) these results do not exclude a role for a RKIP-homologue in the negative regulation of ErkMAPK by FcγRIIb.

3.3.10 FcγRIIb modulates Erk/MAPK activation by recruiting the Erk/MAPK phosphatase Pac-1.

A number of proteins such as Pac-1 and PP2A have recently been identified that act to terminate sustained MAPKinase activity (Robinson and Cobb, 1997). Furthermore, Pac-1 has been shown to be involved in the regulation of antigen-receptor directed ErkMAPK activation in lymphocytes (Ward *et al.*, 1994). Therefore, these molecules may play essential roles in FcγRIIb-mediated mechanisms of ErkMAPK down-regulation in splenic B cells, in addition to modulating other BCR-mediated responses.

To investigate whether ErkMAPK associated protein tyrosine phosphatases play a role in FcγRIIb-mediated suppression of ErkMAPK phosphorylation, phosphatase activity was studied in ErkMAPK immune precipitates following BCR and/or FcγRIIb ligation. **Figure 3.11A** demonstrates that phosphatase activity following BCR stimulation is reduced compared to basal levels, with minimal activity at 5 minutes post-stimulation. BCR stimulated phosphatase activity is also shown to be biphasic in nature, possibly reflecting the requirement to down modulate ErkMAPK activity following BCR ligation. In contrast, FcγRIIb co-ligation results in a rapid and increase in ErkMAPK associated phosphatase activity, which peaks at 5 minutes and remains above basal level for the entire period of co-stimulation. Thus, FcγRIIb-mediated inhibition of early and sustained BCR-induced ErkMAPK activation, may be due to the rapid recruitment of protein tyrosine phosphatases.

Pac-1 was originally identified as a nuclear product of an early-response gene in activated T-cells (Rohan *et al.*, 1993). It is an inducible nuclear phosphatase that functions to terminate the activation of Erk, JNK and p38 MAPKinases through the targeting of specific tyrosine and threonine phosphorylation sites (Ward *et al.*, 1994). Recombinant Pac-1 has also been shown to inhibit MAPKinase activity stimulated by epidermal growth factor, phorbol myristyl acetate, or TCR ligation (Ward *et al.*, 1994). Studies have also indicated a role for Pac-1 in B cells, demonstrating that expression of Pac-1 mRNA is suppressed in resting mature B cells (Grumont *et al.*, 1996). However, levels are upregulated following mitogenic stimulation with anti-Ig, anti-CD40 antibodies and LPS. These results suggest that Pac-1 may also be involved in the regulation of proliferative B cell responses.

To investigate a possible association between ErkMAPK and Pac-1 in splenic B cells, whole cell lysates from BCR and/or FcγRIIb stimulated B cells were immuno-precipitated with an ErkMAPK antibody. Samples were then subjected to Western blot analysis with an antibody recognising Pac-1. Little or no detectable association between Pac-1 and ErkMAPK was found in unstimulated or BCR stimulated cells, indicating that the majority of the phosphatase activity

employed to regulate ErkMAPK phosphorylation under these conditions in splenic B cells is not Pac-1 (**Figure 3.11B**). However, in agreement with published observations by our lab (Deehan *et al.*, 2001) Western blot analysis revealed an association with ErkMAPK and Pac-1 under conditions of negative regulation. The association appears rapidly after just 1 minute co-stimulation via FcγRIIb and the signal is sustained for at least 30 minutes. The kinetics of association were mirrored in western blots of total Pac-1 in whole cell lysates.

The serine/threonine protein phosphatase, PP2A, is present in most tissues and cell types, and has been implicated in the regulation of cell cycle progression, DNA replication, transcription, and translation. Importantly, PP2A has been shown to both negatively and positively regulate the Ras/MAPK cascade by dephosphorylating factors that function at different steps in the cascade (Wassarman *et al.*, 1996). However, no association of PP2A could be demonstrated in ErkMAPK immune precipitates following stimulation via the BCR or FcγRIIb. Consistent with this finding, no modulation of PP2A phosphatase activity could be found in either BCR and/or FcγRIIb-stimulated B cells (results not shown).

Measurement of *in vitro* Pac-1 phosphatase activity in anti-Pac-1 immune complexes revealed a similar profile of activation to ErkMAPK associated phosphatase activity (results not shown). FcγRIIb co-ligation resulted in an enhancement of Pac-1 activity at the earliest time points, which subsequently decreased and then remained above BCR induced levels for the remaining stimulation period. Moreover, analysis of these anti-Pac-1 immune precipitates showed association of ErkMAPK with Pac-1 (30 minutes) under conditions of negative but not BCR signalling (results not shown).

The rapid upregulation of ErkMAPK-associated Pac-1 expression and activity following BCR-FcγRIIb co-ligation made it unlikely that this reflected *de novo* gene induction or even protein synthesis. This prompted an investigation into the localisation of Pac-1 protein expression within the cell, to determine whether existing Pac-1 was being recruited from the nucleus. However, Western blot

analysis of nuclear and cytoskeleton fractions, failed to demonstrate re-localisation of Pac-1 expression following co-stimulation via the BCR or FcγRIIb (results not shown) suggesting that the observed upregulation reflects rapid translation of pre-existing mRNA.

3.3.11 FcγRIIb inhibits BCR-induced activation of the PI-3-Kinase/Akt signalling pathway by recruitment of the inositol phosphatases, SHIP and PTEN, *in vitro*.

FcγRIIb signalling is also thought to contribute to B cell growth-arrest and apoptosis through SHIP-mediated reduction of BCR-induced PtdIns(3,4,5)P_3 levels. Consistent with this hypothesis, recent findings have shown that a reduction in PtdIns(3,4,5)P_3 levels disrupts PH domain-phosphoinositol lipid interactions and prevents the association of Btk and PLC-γ with the plasma membrane (Bolland *et al.*, 1998).

BCR mediated generation of PtdIns(3,4,5)P_3 is also known to be essential for the activation of the survival-promoting factor, Akt/PKB. Akt promotes cell survival by phosphorylating and compromising multiple targets of the cell-death pathway, including the pro-apoptotic Bcl-2 family member BAD and the cell-death pathway enzyme, caspase-9. Thus, SHIP activity may contribute to the induction of apoptosis by converting PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 resulting in the inhibition of the activation/recruitment of the survival factor Akt/PKB. Western blot analysis for the expression and activation of Akt, following FcγRIIb ligation on splenic B cells, proved unsuccessful. Thus, to determine Akt activity in splenic B cells, Akt-mediated phosphorylation of the pro-apoptotic serine/threonine kinase, glycogen synthase kinase-3 (GSK-3) was investigated. Measurement of Akt activity was determined in anti-Akt immune complexes from BCR and FcγRIIb stimulated B cells, by an *in vitro* kinase assay using GSK-3 as a substrate. As Akt is known to phosphorylate GSK-3 at Ser21 of GSK-3α and Ser9 of GSK-3β, GSK-3 activation was determined by Western blot analysis using a phospho-GSK-3α/β (Ser21/9) antibody.

BCR stimulation of mature splenic B cells results in the upregulation of Akt pro-survival activity, as demonstrated by the phosphorylation of GSK-3 (**Figure 3.12**). Activation of GSK-3 is biphasic and maximal activity is shown at 30 minutes post-BCR stimulation. In agreement with others, (Jacob, *et al.*, 1999; Aman, *et al.*, 1998) BCR and FcγRIIb co-stimulation results in the downregulation of Akt activity from 5 minutes, consistent with the kinetics of SHIP activation.

Interestingly, the product of SHIP activity, PtdIns(3,4)P_2, has also been shown to be involved in the positive regulation of Akt (Downward, 1998). Thus, the inhibition of Akt demonstrated may not reflect SHIP activity but rather be due to upstream deregulation by an alternative inositol phosphatase. PTEN has recently been identified as a protein and lipid phosphatase (reviewed by Cantley and Neel, 1999), which is able to negatively regulate Akt/PKB by dephosphorylating both PtdIns(3,4,5)P_3 (Stambolic, *et al.*, 1998) and PtdIns(3,4)P_2 (Haas-Kogan, *et al.*, 1998). Furthermore, the negative regulation of Akt/PKB by PTEN is known to be critical for normal apoptotic signalling induced by a variety of apoptotic stimuli. To investigate the involvement of PTEN in FcγRIIb-induced B cell apoptosis, PTEN expression and protein/lipid phosphatase activity was investigated in splenic B cells, following BCR and/or FcγRIIb ligation.

PTEN expression levels in whole cell lysates of BCR or FcγRIIb co-stimulated splenic B cells were determined by Western blot analysis **(Figure 3.13A)**. Surprisingly, PTEN levels in FcγRIIb co-stimulated cells were reduced compared to resting B cells. However, PTEN expression was not shown to be modulated following BCR stimulation thus, the PTEN identified in resting and BCR stimulated cells may represent inactive PTEN. To investigate the activation status of PTEN under the same stimulation conditions, *in vitro* protein phosphatase activity was assessed in anti-PTEN immune complexes following stimulation of the BCR or co-ligation with FcγRIIb. Consistent with the Western blot analysis, levels of PTEN protein phosphatase activity were similar in both resting and BCR stimulated cells (**Figure 3.13B**). However, co-stimulation with

FcγRIIb resulted in enhanced levels of activity, which were maximal at ten minutes, suggesting that the PTEN protein identified in the Western blot analysis represents an inactive form of PTEN.

To determine whether the lipid phosphatase activity of PTEN was similarly activated following FcγRIIb ligation, inositol 3' phosphatase activity was assessed in anti-PTEN immune complexes. **Figure 3.14** shows that at early time points, FcγRIIb co-ligation results in similar levels of inositol phosphatase activity, compared with BCR ligation alone. However, enhanced levels of Ins(1,4,5)P_3 are produced at 30 minutes, following FcγRIIb co-ligation. These results suggest that PTEN is activated in splenic B cells under conditions of negative regulation by FcγRIIb. The kinetics of PTEN activation are slower than those demonstrated by SHIP and thus may act downstream of SHIP, to terminate ongoing BCR stimulation of the PI-3-kinase/Akt pathway. Furthermore, sustained PTEN activation may account for FcγRIIb induction of an apoptotic response in splenic B cells by the negative regulation of Akt/PKB and the prevention of a cell survival signal. Indeed, the kinetics of PTEN activity are more consistent with the negative regulation of GSK-3 than those of SHIP activity.

Thus, to investigate whether PTEN activity was sustained in FcγRIIb co-stimulated B cells, inositol phosphatase activity was assessed in PTEN immune complexes over 24 hours. **Figure 3.15A** shows that PTEN inositol phosphatase activity is enhanced in BCR-FcγRIIb co-stimulated B cells for up to four hours, compared to B cells stimulated via the BCR alone, and is maximal at 2 hours. The profile of activation correlated with the tyrosine phosphorylation and hence activation of PTEN, as indicated by Western blot analysis with 4G10 (**Figure 3.15B**). However, tyrosine phosphorylation of PTEN was also observed following BCR stimulation from 8 hours and this was reflected in increased levels of PTEN activity after 24 hours. Thus, PTEN activation may also provide a regulatory mechanism for BCR proliferative signalling. Overall, these results support a potential role for PTEN in the sustained inhibition of the PI-3-kinase/Akt pathway by FcγRIIb.

3.3.12 FcγRIIb induction of growth-arrest and apoptosis in splenic B cells is independent of JNK/p38 activation.

The results demonstrated in this study have supported a role for FcγRIIb in the induction of B cell growth arrest and apoptosis via the inhibition of BCR-mediated proliferative signals, such as ErkMAPK. However, FcγRIIb may also contribute to B cell apoptosis by promoting B cell neglect, via the prevention of BCR signalling, or by active apoptotic signalling mechanisms. Studies of BCR-mediated cell death in immature B cell lines Graves, *et al.*, 1996 have implicated a role for the stress activated JNK and p38 MAPKinases in active apoptotic mechanisms. Indeed, studies have suggested that the balance of activities between ErkMAPKinase and the stress-activated MAPKinases may be an important factor in determining cell fate. Thus, whilst FcγRIIb acts to downregulate the ErkMAPK proliferative pathway, upregulation and expression of the pro-apoptotic, stress-activated MAPKinases, JNK and p38 could also occur and result in the promotion of commitment of apoptosis.

Western blot analysis of dually phosphorylated activated and total JNK and p38 MAPK in splenic B cell lysates and immune precipitates proved to be unsuccessful. The activation of JNK and p38 was thus examined indirectly by investigating the activation of the downstream targets of these kinases, c-Jun and ATF-2, respectively. **Figure 3.16** demonstrates that basal levels of c-Jun activation are increased in cells stimulated via the BCR and/or FcγRIIb. Indeed, phosphorylation of c-Jun is enhanced in B cells stimulated via the BCR for 10 minutes, compared to the levels observed following co-ligation of the BCR and FcγRIIb. Thus, JNK activation does not appear to be a pro-apoptotic mechanism induced exclusively by FcγRIIb ligation. In contrast, as no p38/SAPK activation of ATF-2 could be detected in basal or BCR and/or FcγRIIb stimulated splenic B cells, an active role for p38/SAPK in FcγRIIb-mediated negative signalling can not as yet be ruled out. Additional studies were carried out to examine the activation of Rac and Cdc42, which have been proposed to be upstream activators of JNK. These results indicated that neither of these molecules were activated in either basal, BCR and/or FcγRIIb stimulated splenic B cells (results not shown).

3.3.13 FcγRIIb modulates the expression of key cell-cycle regulator proteins

The tumour suppressor protein, retinoblastoma protein (Rb), controls progression through the late G_1 restriction point of the cell cycle by binding to and sequestrating a family of transcription factors, called the E2-F family (section **1.2.4**). When bound to E2-F, hypo-phosphorylated Rb represses transcription of E2-F response genes that are important for S-phase entry. Differential phosphorylation of Rb has been shown to modulate its function both *in vitro* and *in vivo* and *in vitro* hyper-phosphorylation and deactivation of Rb is mediated by Cdk4/6 and Cdk2-Cyclin complexes (Zarkowska and Mittnacht, 1997). Importantly, both immature and mature B cells show BCR-induced increases in Cyclin D and Cdk4 levels, which are essential for early G_1 phase progress and transition through the G_1 restriction point. This may be due to ErkMAPK dependent activation of the Cyclin D gene via the transcription of MAPK responsive genes. However, in some systems high levels of Cdk activity have also been demonstrated to be sufficient for the induction of apoptosis (Harvey, 1998)

Since FcγRIIb signalling has been demonstrated to inhibit B cell G_1-S progression following co-ligation with the BCR (reviewed by Anderson and Sinclair, 1998) the effect of FcγRIIb recruitment on the expression of key cell-cycle regulator proteins was investigated. The relative expression levels of Cdk4 and Rb were investigated in whole cell lysates of BCR and BCR-FcγRIIb stimulated splenic B cells. **Figure 3.17A** shows that BCR stimulation for 1 hour, results in a downregulation in Cdk4 expression levels followed by an increase over time. A similar pattern of Cdk4 suppression and upregulation is shown following FcγRIIb co-ligation with the BCR. However, whilst longer BCR stimulation results in higher levels of Cdk4 expression, the levels induced are still below basal. The lack of significant modulation of Cdk4 levels following stimulation may be due to the short stimulation times chosen and an investigation of later times in the cell cycle may highlight differential expression or activation of Cdk4 by FcγRIIb and the BCR. Indeed, recent studies in murine B cells over 48 hours, have shown that FcγRIIb ligation does not inhibit the

expression of Cdk4 but acts to block the assembly and stabilisation of Cyclin D-Cdk4/6 complexes (Tanguay *et al.*, 1999).

G_1 Cyclin dependent kinases (G_1-Cdks) have been implicated in the phosphorylation of Rb (Zarkowska and Mittnacht, 1997). Cyclin D-Cdk4/6 complexes contribute only partially to the activation of Rb; Cyclin E-Cdk2 complexes are also required. Whilst Cdk4 did not appear to be modulated following FcγRIIb ligation, FcγRIIb may act on the regulation of Rb via interaction with other cell-cycle regulatory proteins. Thus, to avoid such complications the phosphorylation status of Rb was investigated using a phospho-Rb specific antibody in cells stimulated for up to 72 hours. The existence of multiple forms of Rb observed during Western blotting is indicative of Rb phosphorylation; with hyper-phosphorylated Rb exhibiting a greater apparent molecular weight in comparison to hypo- or unphosphorylated Rb. **Figure 3.17B** shows that Rb is differentially phosphorylated in splenic B cells following BCR and/or FcγRIIb stimulation. Correlating with entry into the S and G_2/M phases of the cell cycle, BCR stimulation over 72 hours results in the hyper-phosphorylation of Rb, as indicated by the predominance of the higher molecular weight form. In contrast, co-ligation of FcγRIIb results in a Rb phosphorylation profile similar to that of basal cells, with a large percentage of cells demonstrating hypo-phosphorylated Rb. Thus, FcγRIIb actively inhibits entry in the proliferative phases of the cell cycle by preventing the hyper-phosphorylation and deactivation of Rb.

Following mitogenic stimulation of cells, two events controlling the G_1/S transition are thought to occur, firstly Cyclin D levels are increased and secondly, the levels of p27, a Cyclin-dependent kinases inhibitor (CKI), are reduced (Sherr and Roberts, 1999). Together with another CKI, p21, p27 inhibits Cyclin-E/Cdk2 complexes whilst acting as an assembly factor for Cyclin D and Cdk4/6 (Cheng, 1999). By binding to the Cyclin/Cdk complexes, these CKIs lead to the inhibition of Cdk activity and thus prevent the phosphorylation of Rb and cell cycle progression. Whilst p27 is constitutively expressed, p21 levels are induced during periods of stress by p53.

The tumour suppresser protein, p53, plays a major role in the cellular response to DNA damage and other genomic aberrations. Importantly, the activation of p53 by MAPK mediated phosphorylation on multiple residues, can either lead to cell cycle arrest and DNA repair or apoptosis (Meek, 1994; Levine, 1997). p53 is normally held in an inactive complex with a negative regulator, MDM2. MDM2 inhibits the accumulation of p53 by targeting it for ubiquination and proteasomal degradation (Chehab, 1999). DNA damage or apoptotic signals, induce the phosphorylation of p53 at residues serine 15 and serine 20. These phosphorylation events impair the MDM2-p53 interaction thus promoting both the accumulation and functional activation of p53.

To investigate whether p53 was functionally activated by FcγRIIb ligation, whole cell lysates from BCR and/or FcγRIIb co-stimulated splenic B cells were Western blotted for the expression of phospho-p53 Ser^{15}. **Figure 3.17C** clearly demonstrates that FcγRIIb induces the phosphorylation of p53 on the critical serine residue over 72 hours, compared to control or BCR stimulated cells. Thus, the activation of p53 may represent an important pro-apoptotic mechanism initiated by FcγRIIb following ligation on splenic B cells. Further attempts to investigate the role of these key cell-cycle regulator proteins in FcγRIIb-mediated negative regulation, by intracellular staining, proved unsuccessful due to problems of cross-reactivity between the stimulating antibodies and primary fluorescence-conjugated FACS antibodies.

3.3.14 FcγRIIb modulates the expression of nuclear transcriptional proteins

Nuclear factor κB (NF-κB) transcriptionally activates genes that promote immunity and cell survival (reviewed by Ghosh *et al.,* 1998). Activation of NF-κB is induced by an IκB kinase (IKK) complex that phosphorylates and promotes dissociation of IκB from NF-κB, which then translocates to the nucleus. Conversely, NF-κB activation is inhibited by an IκBα complex. Interestingly, PTEN has been shown to inhibit cytokine induced NF-κB activation (Koul *et al.,* 2001). PTEN did not appear to interfere with the degradation of IκB or with the

nuclear translocation of p65/Rel, normally obligatory for NF-κB activation. This suggests that additional PI-3-Kinase-mediated signals may be required for the ability of NF-κB to bind to DNA and to transactivate genes.

Given that BCR-FcγRIIb co-ligation modulates PTEN activity it was decided to investigate whether FcγRIIb-mediated B cell inhibition reflected modulation of NF-κB. The activation status of NF-κB was assessed following FcγRIIb-ligation by electrophoretic mobility shift assay (EMSA). As shown in **Figure 3.18**, BCR stimulation of splenic B cells leads to an increase in nuclear NF-κB activity after 4 hours which decreases by eight hours. Co-ligation of BCR and FcγRIIb results in a marked increase in NF-κB activity over four hours that is rapidly downregulated. In contrast, under conditions of FcγRIIb-mediated negative feedback inhibition of mitogenic BCR signalling, co-ligation of the BCR with FcγRIIb results in minimal NF-κB activity. Importantly, the EMSA demonstrates the formation of distinct NF-κB/DNA complexes following BCR and/or FcγRIIb ligation. Several groups have shown that the predominant form of NF-κB in B cells is the p50-c-Rel heterodimer (Buhl and Cambier, 1999); in particular c-Rel was shown to be essential for B cell proliferation after BCR engagement. Attempts to determine whether FcγRIIb signalling modulates the relative expression levels of c-Rel and the p50 subunit in the nucleus of splenic B cells were hampered by poor protein recovery from stimulated cells.

E2-F responsive elements are present in the promoters of cell cycle-related genes **(REF)**. Hypo-phosphorylated Rb protein binds to the E2-F gene product, inhibiting E2-F activity and inducing G_1 growth arrest. Recent studies have also demonstrated that p53 is able to bind to E2-F and inhibit E2-F transcriptional activity, suggesting that transcription of the gene can also be independently repressed by p53 (Ookawa *et al.*, 2001). Since FcγRIIb ligation on splenic B cells is shown to modulate the phosphorylation profile of both Rb and p53 the binding activity of E2-F, following BCR and/or FcγRIIb ligation, was assessed by EMSA. Unfortunately, conclusive results could not be obtained concerning the activation of E2-F following BCR or FcγRIIb-ligation in splenic B cells (results

not shown). However, since Rb phosphorylation is modulated by FcγRIIb ligation, a role for E2-F in the inhibition of cell cycle progression by FcγRIIb can not be excluded.

3.3.15 FcγRIIb-mediated mechanisms of B cell inhibition result in a decrease in mitochondrial membrane potential.

Several studies have highlighted an important role for mitochondrial integrity in determining commitment to apoptosis (section **1.2.4**). Association of apoptosis-regulatory proteins from the Bcl-2 and caspase families with the mitochondria largely determine the fate of the cell. Thus, in many cases of apoptotic cell death, the decision is dependent upon mitochondrial events. Depolarisation of the mitochondrial membrane potential is one of the main internal mitochondrial changes that can occur during the early initiation stages of apoptosis. A decrease in mitochondrial membrane potential can be assayed for by the incorporation of the lipophilic dye $DiOC_6(3)$ (ZamZami *et al.*, 1995). $DiOC_6(3)$ possesses a net negative charge, permitting its accumulation in the mitochondrial matrix along an electrochemical gradient. Cells with a high mitochondrial membrane potential retain the dye and possess high fluorescence intensity, as assessed by FACS analysis. The initiation of apoptotic mechanisms may lead to a loss of mitochondrial membrane potential and result in the subsequent loss of dye retention by the mitochondria. Thus, cells possessing a low $DiOC_6(3)$ fluorescence intensity are considered to be committed to apoptosis.

To determine whether mitochondrial membrane potential was perturbed following FcγRIIb ligation, primary B cells stimulated via the BCR and/or FcγRIIb were assessed for loss of $DiOC_6(3)$ fluorescence over 72 hours. In agreement with the analysis of DNA content, stimulation via co-ligation of the BCR and FcγRIIb either on resting cells or cells mitogenically stimulated via the BCR, resulted in an increased percentage of B cells with a low $DiOC_6(3)$ fluorescence intensity (**Figure 3.19**). Interestingly, co-ligation of FcγRIIb in cells mitogenically stimulated via the BCR, conditions that have previously been reported to promote survival despite growth-arrest, demonstrated enhanced depolarisation

of the mitochondrial membrane potential. These results therefore confirm that FcγRIIb ligation on B cells can initiate mechanisms that lead to the disruption of mitochondrial stability.

3.3.16 Effects of BCR and FcγRIIb co-ligation on the mRNA expression levels of FcγRIIb1 and FcγRIIb2 in splenic B cells.

FcγRIIb is expressed on B cells as two differentially spliced isoforms, FcγRIIb1 and FcγRIIb2. These isoforms appear to be identical except for an amino acid insert in the cytoplasmic domain of FcγRIIb1, which abolishes the endocytic capacity of the receptor. Thus, FcγRIIb2 could be the isoform predominantly utilised by B cells for antigen uptake and processing whilst modulation of FcγRIIb1 expression may dictate the threshold for BCR signalling. Indeed, B cell expression levels of the two isoforms are thought to be modulated in response to activation by crosslinking of the BCR or culture with cytokines (Gergely, *et al.*, 1994). Therefore, it may be possible that differences in the relative expression levels of the two isoforms of FcγRIIb, may influence T cell-mediated help and thus the regulation of B cell activation by immune-complexes.

Various attempts to determine the relative FcγRIIb expression levels on splenic B cells by FACS analysis or immunoprecipitation with the pan-FcγRII monoclonal antibody, 2.4G2, proved unsuccessful. Due to the identical nature of the extracellular domains, antibodies raised against these FcγRIIb structures are unable to discriminate between the two isoforms. Thus, to identify the two FcγRIIb isoforms and quantify the relative expression levels, RT-PCR was performed to identify the isoforms at the level of mRNA message. Total RNA was extracted from BCR and/or FcγRIIb stimulated splenic B cells, reversed transcribed and the relative expression levels of the isoform transcripts were determined by quantitative TaqMan analysis.

Figure 3.20 demonstrates that the levels of FcγRIIb1 mRNA detected were higher than that of FcγRIIb2, throughout the period of stimulation and independent of the stimulus. Stimulation of the BCR alone resulted in an

increase in the levels of both FcγRIIb1 and FcγRIIb2 mRNA. Similarly, co-ligation of BCR and FcγRIIb on resting cells increased the message levels of both isoforms. However, co-ligation of the receptors, under conditions of mitogenic BCR signalling, results in minimal enhancement above basal levels and predominantly a downregulation in expression over 30 minutes. By maintaining expression of both isoforms, a B cell can remain responsive to mitogenic stimulation by antigen and co-stimulatory T cell help, whilst still being able to undergo negative regulation following further interactions with immune complexes. In contrast a selective downregulation in FcγRIIb2 expression on normal B cells following co-ligation of the receptors, under conditions of mitogenic BCR signalling, may result in the abrogation of antigen uptake, processing and presentation. A downregulation in antigen presentation will prevent T cell mediated help and promote B cell anergy and survival or commitment to apoptosis.

3.4 Discussion

Signalling via the FcγRIIb receptor subsequently decreases B cell responsiveness and provides a potent mechanism for the downregulation of humoral immunity. Indeed, it is thought that deregulation of FcγRIIb receptor signalling may contribute to the pathology of certain autoimmune diseases (see **Chapter 4**; Nakamura, A. *et al.,* 2000; Dijstelbloem *et al.,* 2001; Ravetch and Bolland, 2001). Unfortunately our understanding of the mechanisms underlying such Fcγllb-mediated suppression of BCR signalling are not well defined. Experimental models have developed, however, which potentially allow disection of the key regulatory events. For example, stimulation of the BCR with $F(ab')_2$ fragments of anti-Ig antibodies results in B cell activation and proliferative responses. In contrast, by using intact anti-Ig antibodies to mimic physiological cognate antibody-antigen complexes, it is possible to co-ligate the BCR with FcγRIIb, conditions which do not induce DNA synthesis in murine B cells, Moreover, co-ligation of the BCR and FcγRIIb inhibits the response to mitogenic $F(ab')_2$ antibodies. Indeed, by stimulating murine B cells with a combination of $F(ab')_2$ fragments and intact antibody, the intact antibodies produce a dominant negative signal. Thus, using these different methods of antibody stimulation, we have investigated the functional uncoupling of the BCR by FcγRIIb in primary, mature B cells.

As stated above, our understanding of the mechanisms underlying such Fcγllb-mediated suppression of BCR signalling are not well defined. However, it is well established that following FcγRIIb co-ligation with the BCR the SH2-domain containing inositol 5'-phosphatase, SHIP is recruited to the phosphorylated ITIM of FcγRIIb . SHIP is thought to act in part by preventing the activation of Btk and PLC-γ by $PtdIns(3,4,5)P_3$, resulting in the inhibition of sustained influx of extracellular calcium required for growth.

In addition, SHIP is proposed to prevent the induction of the BCR-coupled Ras/MAPKinase cascade via interactions with the adaptor molecule Shc and the RasGAP binding protein, p62Dok (Tamir *et al.*, 2000a). In addition to the

involvement of SHIP, we now demonstrate that FcγRIIb uncouples the BCR from the Ras/MAPKinase pathway through the inhibition of MEK and the rapid recruitment and activation of the dual-specific nuclear MAPK phosphatase, Pac-1 (**Figure 3.21**).

Furthermore, it was thought that SHIP activity may contribute to the FcγRIIb-mediated induction of apoptosis by inhibiting the BCR-coupled activation/recruitment of the survival factor Akt/PKB. However, we now demonstrate the activation of a second inositol phosphatase, PTEN, which is known to negatively regulate Akt/PKB. PTEN activation following BCR-FcγRIIb co-ligation in B cells could potentially contribute to the inactivation of the PI-3-K/Akt pathway by FcγRIIb (**Figure 3.22**) suggesting that, like SHIP, PTEN may act *in vivo* as a negative regulator of B cell survival and proliferation. Thus, this study effectively demonstrates that FcγRIIb co-ligation acts to switch off both BCR-mediated proliferative and survival pathways resulting in the induction of B cell growth-arrest and/or commitment to apoptosis.

Recent studies have demonstrated that aggregation of FcγRIIb alone, via non-cognate antigen-antibody complexes, generates a pro-apoptotic signal that is mediated via Btk (Bolland and Ravetch, 1998). Rather surprisingly, this pro-apoptotic signal is blocked by the recruitment of SHIP to FcγRIIb following BCR co-ligation. This novel FcγRIIb-mediated mechanism has been proposed to act as a means of maintaining B cell peripheral tolerance in the germinal centre, by promoting apoptosis of B cells with low-affinity, potentially cross-reactive antigen-receptors. Antigen (in the form of immune complexes) that interacts with FcγRIIb alone will result in commitment to apoptosis whilst BCR-FcγRIIb co-ligation promotes survival yet growth-arrest. Interestingly, we demonstrate that BCR-FcγRIIb co-ligation is also capable of inducing apoptosis in B cells. Thus, inactivation of B cells may represent the integration of signals generated by the interaction of immunocomplexes with the BCR and FcγRIIb through pathways modulated by SHIP.

The binding of antigen-antibody complexes by FcγRIIb may also result in a feedback mechanism that regulates the production of the inhibitory receptor. An investigation of the relative mRNA expression levels of FcγRIIb1 and FcγRIIb2 following BCR stimulation demonstrated increased message levels of both isoforms consistent with the idea that activation primes the cell for cognate negative feedback regulation. However, FcγRIIb co-ligation, resulting in negative feedback regulation of ongoing B cell responses, resulted in an overall downregulation of isoform expression. Importantly, this downregulation of FcγRIIb production could result in the abrogation of antigen uptake, processing and presentation to helper T cells. Moreover, a downregulation in antigen presentation will prevent T cell mediated help which can overcome negative feedback inhibition and promote B cell anergy and survival or commitment to apoptosis.

We have confirmed that FcγRIIb ligation modulates the recruitment and activation of three SH2-domain containing phosphatases, SHIP, SHP-1 and SHP-2, *in vitro*. Unlike SHP-1 and SHP-2, SHIP was previously thought to be phosphorylated and activated exclusively under negative signalling conditions. However, in agreement with recent studies by Ingham *et al.*, 1999 we now show that phosphotyrosine-dependent Shc-SHIP complexes are also detectable in BCR stimulated cells, questioning the role of SHIP-Shc complexes in regulating BCR-coupling to MAPK. For example, SHIP has been proposed to sequester Shc (Damen *et al.*, 1996; Tridandapani *et al.*, 1999) and hence result in an ability to inhibit MAPKinase activation. However, Shc has been shown to be dispensable for BCR coupling to the RasMAPK pathway in chicken DT40 B cells (Hashimoto *et al.*, 1998), although many other groups have demonstrated an essential role for Shc in coupling the BCR to ErkMAPK in mammalian B cells (Campbell, 1999; Kelly and Chan, 2000). In addition, further doubt about the role of SHIP-Shc complexes is provided by studies revealing the existence of Shc-independent pathways for Grb2-SOS recruitment by the BCR, involving BLNK (reviewed by Kelly and Chan, 2000). This has lead to the proposal that Shc may regulate the level of activation of Ras via Grb2-SOS complexes. Interestingly, Tridandapani *et al.* 1999 have discovered that two separate pools

of SHIP may exist: one bound to FcγRIIb and one bound to Shc. Kinetic studies revealed a rapid association of SHIP with FcγRIIb following ligation but a slower and more transient association with Shc. Subcellular localisation of these signaling molecules is regulated by the phosphotyrosine-SH2 domain interactions, thus dephosphorylation may result in the re-direction of Shc and SHIP within the cell, and consequently, in the modulation of their activity.

In addition to the p52/46 Shc isoforms recruited by SHIP, recent studies have identified a third Shc isoform, p66Shc, which may play a role in promoting apoptosis. Homozygous mutation of p66Shc in mice was shown both to prolong the life span and to prevent oxidative-stress induced apoptosis (Migliaccio *et al.*, 1996). Despite its tyrosine phosphorylation by active RTKs, p66Shc is not involved in Ras activation (Migliaccio *et al.*, 1996). The ability of SHIP to recruit p66Shc is unknown but an association may implicate a novel mechanism by which SHIP might be linked mechanistically to growth arrest and apoptosis.

The formation of SHIP complexes with the protein phosphatases SHP-1 and SHP-2 indicates a further role for SHIP in assembling regulatory complexes. Indeed, recent studies have identified a Grb2-associated binder 1 docking protein (Gab1) which binds to the SH2 domains of SHP-2, SHIP and the p85 subunit of PI-3-Kinase and may regulate their activity (Koncz *et al.*, 2001). For example activation of SHP-2, following binding to the FcγRIIb-SHIP complex, results in partial dephosphorylation of Gab1, resulting in the release of PtdIns(3,4,5)P_3 and the inhibition of downstream activation pathways from the BCR.

Although following ligation of FcγRIIb, Shc and SHP-1 containing SHIP complexes were formed, we found no evidence to support the disruption of Ras/MAPKinase signalling by SHP-1 via the recruitment of Shc. However, SHP-1 was shown to be activated following FcγRIIb co-ligation and appeared to be modulated by association with the PTK, Lyn. Thus, SHIP and SHP-1 do not appear to have redundant functions in negative signalling with each targeting distinct signalling intermediates and pathways. So far, and in line with previous

studies these results support a more dominant and central role for SHIP in FcγRIIb-mediated inhibitory signalling than SHP-1. Investigation into the expression and activation of the newly identified isoforms of SHIP, which may reside in separate activation 'pools' may help to confirm the primary importance of this activity in FcγRIIb signalling. Studies by March *et al.*, 2000 have already demonstrated that a second isoform, SHIP-2 can substitute for SHIP in FcγRIIb-mediated inhibitory signalling in B cells. However, Brauweiler *et al.*, 2000a and 2000b have shown that the different SHIP isoforms mediate distinct molecular mechanisms depending on the activation status of the B cells. They revealed that whilst resting cells express only SHIP, LPS stimulated B cell blasts also express SHIP-2. Moreover, that both isoforms can mediate downstream biological consequences of FcγRIIb signalling, including inhibition of the proliferative response. Thus, recruitment of a second isoform of SHIP by activated cells represents a novel mechanism by which FcγRIIb can inhibit BCR-mediated proliferative responses. It would therefore be interesting to investigate whether this isoform is preferentially recruited following co-ligation of the BCR and FcγRIIb in our experimental model for negative feedback inhibition of ongoing B cell responses

The demonstration that sustained FcγRIIb inhibition of ErkMAPK phosphorylation is independent of Ras implies that much of the early ErkMAPK phosphorylation by the BCR in mature B cells may occur via cross-talk from other signalling pathways, such as PKC activation of Raf-1. Nevertheless, the modulation of ErkMAPK by Pac-1 represents an important direct signal induced by FcγRIIb to terminate ongoing ErkMAPK activity. Since, Pac-1 is thought to be a nuclear phosphatase we were initially surprised at its rapid association with ErkMAPK following FcγRIIb co-ligation. This apparent upregulation of Pac-1 expression and activity following FcγRIIb co-ligation was initially thought to represent an initial priming event further upstream, whereupon Pac-1 rapidly associates with ErkMAPK in the cytosol following an accumulation of cytoskeletal or nuclear Pac-1 that is lost in the preparation of whole cell lysates. However, analysis of sub-cellular fractions demonstrated this not to be the case and hence, its expression may represent pre-existing mRNA that is rapidly

translated following FcγRIIb co-ligation and investigations of Pac-1 mRNA expression levels using TaqMan quantitative RT-PCR analysis may confirm this theory. However, it may also reflect a simple enhancement of association above the levels of detection permitted by the commercially available antibodies. The finding that Pac-1 did not associate with ErkMAPK under conditions of BCR-mediated signalling may be consistent with this latter proposal as Pac-1 has been demonstrated to be rapidly upregulated in lymphocytes to regulate mitogen-driven MAPKinase signalling. Thus, lack of detection may simply reflect low levels of detection or alternatively that another phosphatase is preferentially recruited to terminate ErkMAPK signals following BCR-stimulation. We are currently investigating cellular fractions to identify where Pac-1 is located upon association with ErkMAPK, following FcγRIIb ligation. Thus, co-ligation of FcγRIIb under conditions of negative feedback inhibition of ongoing B cell responses may act to inhibit early BCR-mediated activation of ErkMAPK through the recruitment of SHIP whilst Pac-1 is activated to terminate any residual ongoing responses (**Figure 3.21**).

Whilst co-ligation of FcγRIIb acts to downregulate BCR-mediated proliferative pathways, recent studies have confirmed the ability of FcγRIIb to signal independently of BCR co-ligation and directly mediate an apoptotic response (Pearse *et al.*, 1999). An investigation of the DNA content of B cells following FcγRIIb ligation confirmed this observation over 72 hours. FcγRIIb induction of apoptotic mechanisms has been proposed to be due to a failure to recruit SHIP, as demonstrated by a deletion of SHIP or mutation of FcγRIIb (Pearse *et al.*, 1999). A lack of SHIP recruitment prevents the membrane association of Btk, resulting in an inhibition of calcium influx (Bolland *et al.*, 1998). This novel FcγRIIb-mediated mechanism has been proposed to act as a means of maintaining B cell peripheral tolerance in the germinal centre, by promoting apoptosis of B cells with low-affinity, potentially cross-reactive antigen-receptors. Antigen that interacts with FcγRIIb alone will result in B cell apoptosis whilst antigen co-engagement of FcγRIIb with the BCR will promote survival. Interestingly, SHP-1-mediated inhibitory signalling blocks this apoptotic mechanism, whilst SHIP recruitment attenuates it (Ono *et al.*, 1997). These

studies highlight the differential roles for SHIP in mediating FcγRIIb induced B cell inhibition while implicating novel mechanisms for the newly identified SHIP isoforms.

Most recently, it has been shown that BCR ligation and CD19 activation induces activation of the pro-survival factor Akt and promotes B cells survival (Pogue *et al.*, 2000; Otero *et al.*, 2001). Co-ligation of the BCR with FcγRIIb has been proposed to result in the termination of this signal (Aman *et al.*, 1998; Carver *et al.*, 2000). Due to the requirement of SHIP, termination of Akt activity is more consistent with FcγRIIb-mediated growth-arrest than apoptosis. However, these results are inconsistent with recent studies that demonstrate that the product of SHIP activity, PtdIns(3,4)P_2, may also be involved in the positive regulation of Akt (Downward, 1998). Thus, the inhibition of Akt demonstrated may not reflect SHIP activity but rather be due to upstream deregulation by an alternative inositol phosphatase. We now show that FcγRIIb inhibition of BCR-activated Akt might be due to the recruitment of the inositol 3'-phosphatase, PTEN. Through the conversion of PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2, PTEN could impair BCR mediated activation of Akt by counteracting the activities of PI-3-Kinase (**Figure 3.22**). Interestingly, PTEN activity has been shown in T cells to induce an increased rate of apoptosis, whilst inhibiting basal and TCR-induced ErkMAPK phosphorylation (Wang *et al.*, 2000). This could be explained following evidence indicating that PI-3-Kinase signalling can upregulate the ErkMAPKinase pathway at the level of Raf-1. Thus, inhibition of PI-3-K activity by PTEN could lead to the suppression of ErkMAPK activation and cell proliferation. This mechanism may also explain the observation that PTEN appears to differentially suppress cell growth in different tumours. PTEN lipid phosphatase activity promotes G_1 cell cycle arrest in glioblastoma cells (Furnari *et al.*, 1998) but induces commitment to apoptosis in carcinomas (Myers *et al.*, 1998). The kinetics of PTEN activation by FcγRIIb are slower than that demonstrated by SHIP. Thus, PTEN may act sequentially to terminate any ongoing PI-3-Kinase activity following the downregulation of transient SHIP activity.

Interestingly, PTEN has also been demonstrated to inhibit TNF-mediated activation of NF-κB by preventing the activation of Akt and the IKK complex (Gustin *et al.*, 2001). BCR-mediated activation of the transcription factor, NF-κB, has been shown to be important for B cell activation and development by promoting the expression of survival genes and regulating the expression of several anti-apoptotic molecules (Ghosh *et al.*, 1998). Indeed, inhibition of NF-κB activity with protease inhibitors, which prevents BCR mediated IκB degradation, has been shown to induce apoptosis in a number of B cell lines (Baichwal and Baeuerle, 1997). Indeed, FcγRIIb co-ligation with the BCR resulted in the formation of differential NF-κB/protein complexes that were rapidly downregulated. Thus, FcγRIIb ligation may alter the ability of NF-κB/protein complexes to bind to DNA and therefore modulate the expression of survival genes. One candidate NF-κB protein is c-Rel, which is vital for maintaining c-myc levels and preventing cell death (Baichwal and Baeuerle, 1997). Further investigation of the NF-κB/protein complexes using band-shift assays will help characterise the proteins involved and possibly determine whether FcγRIIb-mediated abrogation of NF-κB activation is mediated via PTEN.

Recent studies in bone marrow derived mast cells (BMMCs) have shown that crosslinking of FcγRIIb with the stem cell factor receptor (SCFR), Kit induces cell cycle arrest and SHIP-1 dependent inhibition of p38 and JNK (Malbec *et al.*, 2001). The demonstrated inhibition of the stress activated MAPKinases is likely to be due to the recruitment of SHIP-1 as the subsequent degradation of PtdIns(3,4,5)P_3 is thought to prevent the membrane recruitment of the exchange factor Vav and thus inhibit downstream SAPK activation. However, in the absence of SHIP recruitment, FcγRIIb ligation mediates apoptosis via Btk-dependent activation of JNK (Kawakami *et al.*, 1997; Bolland *et al.*, 1998). In contrast, our studies revealed that FcγRIIb induction of splenic B cell inhibition appears to be independent of JNK and p38. The differential triggering of JNK by FcγRIIb may represent differences in the experimental systems utilised and

warrants further investigation. A role for p38 in FcγRIIb-mediated B cell inhibition has yet to be determined.

Malbec *et al.*, 2001 and others have shown that FcγRIIb-mediated growth-arrest leads to the modulation of cell-cycle regulatory proteins. In BMMCs, FcγRIIb-mediated inhibition of Kit-dependent cell proliferation abrogated activation of Cyclins D2, D3 and A, thus preventing the BMMCs from entering the cell cycle (Malbec *et al.*, 2001). In contrast, studies in mature B cells have shown that BCR-FcγRIIb co-ligation does not affect BCR-induced Cyclin D expression (Tanguay *et al.*, 1999). Since Cyclin D-Cdk4/6 complexes are essential for the G_1-S transition, we postulated that FcγRIIb might abrogate Cdk4 expression and in this way achieve its inhibitory effects on cell progression. However, Cdk4 expression was shown not to be modulated following FcγRIIb stimulation. Thus, FcγRIIb appears to mediate its effects on the cell cycle independently of Cdk4 expression.

Both Cyclin D-Cdk4/6 complexes and Cyclin E-Cdk2 complexes contribute to the phosphorylation of Rb following BCR stimulation (Cheng *et al.*, 1999). Thus to determine whether FcγRIIb inhibited cell cycle progression at the level of Rb, the phosphorylation status of Rb was investigated following FcγRIIb ligation. Indeed, FcγRIIb ligation was shown to result in a Rb phosphorylation profile similar to that of quiescent cells. B cells stimulated to progress to S phase following signalling via the BCR exhibited an increase in Rb phosphorylation, whereas signals derived from FcγRIIb partially suppressed BCR-induced Rb phosphorylation (**Figure 3.23**). Further investigations of whether modulation of Rb phosphorylation by FcγRIIb impacts on downstream interactions with E2-F, will help elucidate the mechanisms by which FcγRIIb induces cell-cycle arrest in B cells.

Interactions between the tumour suppressor protein, p53 and phospho-Rb have been suggested due to the ability of forced phospho-Rb expression to relieve MDM2-mediated repression and restore DNA binding of the transcription factor

Sp-1 (Johnson-Pais *et al.,* 2001). These results suggest a model in which the opposing activities of MDM2 and phospho-Rb regulate Sp1 DNA-binding and transcriptional activity. p53 plays a central role in cellular responses to stress and damage by inducing cell cycle arrest and DNA repair or apoptosis (Meek, 1994; Levine *et al.,* 1997). The mechanisms involved in the activation of p53 responsive genes are still elusive but phosphorylation of Ser^{15} on p53 is considered to be the minimal requirement for its transactivation as it impairs the ability of MDM2 to bind p53, promoting both the accumulation and functional activation of p53 (Meek, 1994). In this study, we show that co-ligation of BCR and FcγRIIb, results in the rapid phosphorylation of p53 on Ser^{15}. Thus, FcγRIIb-mediated B cell inhibition may promote conditions of growth arrest by modulating the phosphorylation and hence transactivation of p53. A reduction in the phosphorylation levels of Ser^{15}, observed from 24 hours, could be due to the partial reassociation of MDM2. However, attempts to demonstrate the reassociation of MDM2 with p53, following long term FcγRIIb co-ligation, proved unsuccessful. Interestingly, the Cdk inhibitor, p19Arf, blocks the nucleo-cytoplasmic shuttling of MDM2, which normally degrades p53 (Tao and Levine, 1999). Thus, inactivation of p19Arf would subsequently lead to reassociation of MDM2 with p53 and result in p53 inactivation. It would therefore be interesting to investigate whether the reduction in Ser^{15}phosphorylation levels was due to the inactivation of p19Arf following long term FcγRIIb co-ligation or the downregulation of the ATM/ATR kinases which promote phosphorylation of p53 on serine 15.

The loss of mitochondrial function and integrity is proposed to be an initial indication of commitment to apoptosis. Mitochondrial function may also be modulated by p53, which induces the expression of both Bcl-2 and Bax, leading to a rise in oxidative species and degradation of mitochondrial components (Miyashita, *et al.,* 1994). By controlling levels of pro- and anti-apoptotic members of the Bcl-2 family, p53 may exert another level of control in determining cell fate. A role for mitochondria in FcγRIIb-mediated B cell apoptosis has not previously been described. Therefore, the mitochondrial membrane potential of splenic B cells following FcγRIIb ligation was

investigated. Indeed, FcγRIIb ligation was shown to induce a decrease in mitochondrial membrane potential, consistent with the commitment to apoptosis.

Overall, these results suggest that FcγRIIb is capable of inducing a rapid and effective inhibition of BCR coupled signalling pathways, in particular the Ras/MAPKinase and PI-3-Kinase/Akt pathways. FcγRIIb rapidly terminates early BCR-coupled signals whilst recruiting further downstream inhibitory mediators, such as PTEN and Pac-1, to terminate any residual BCR-induced activity. Synergistically these events result in the modulation of BCR-activated cell cycle machinery and nuclear signalling components resulting in B cell anergy, growth arrest and/or apoptosis.

Figure 3.1 FcγRIIb co-ligation inhibits BCR-mediated B cell proliferation, which can be restored in the presence of a blocking anti-FcγRII/III antibody.

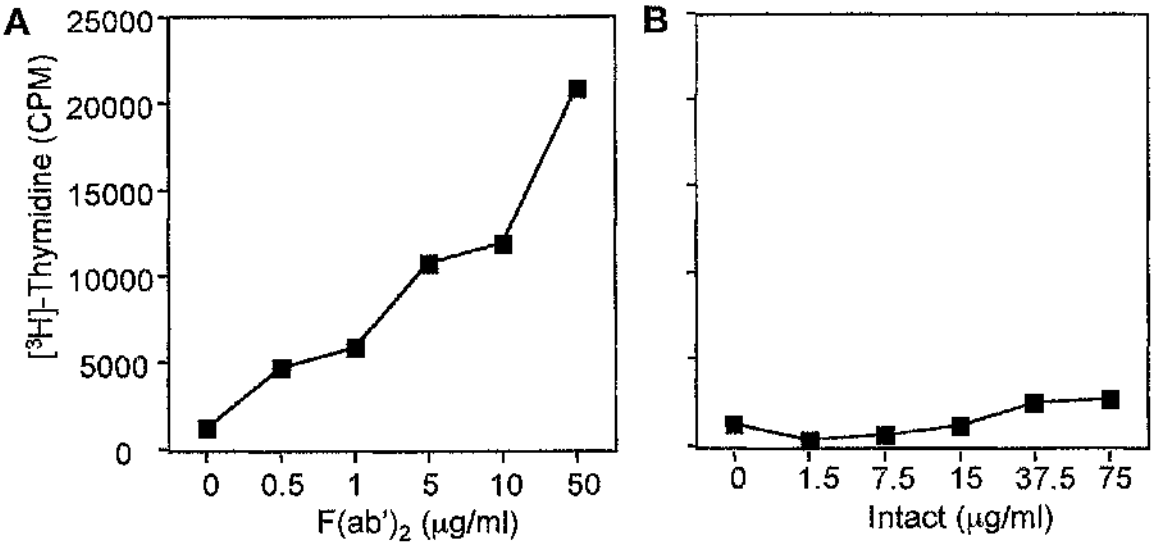
Purified splenic B cells from Balb/c mice (2 x 10^5 cells/well) were stimulated for 48 hours in the presence of the indicated antibodies. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment, representative of at least two other independent experiments.

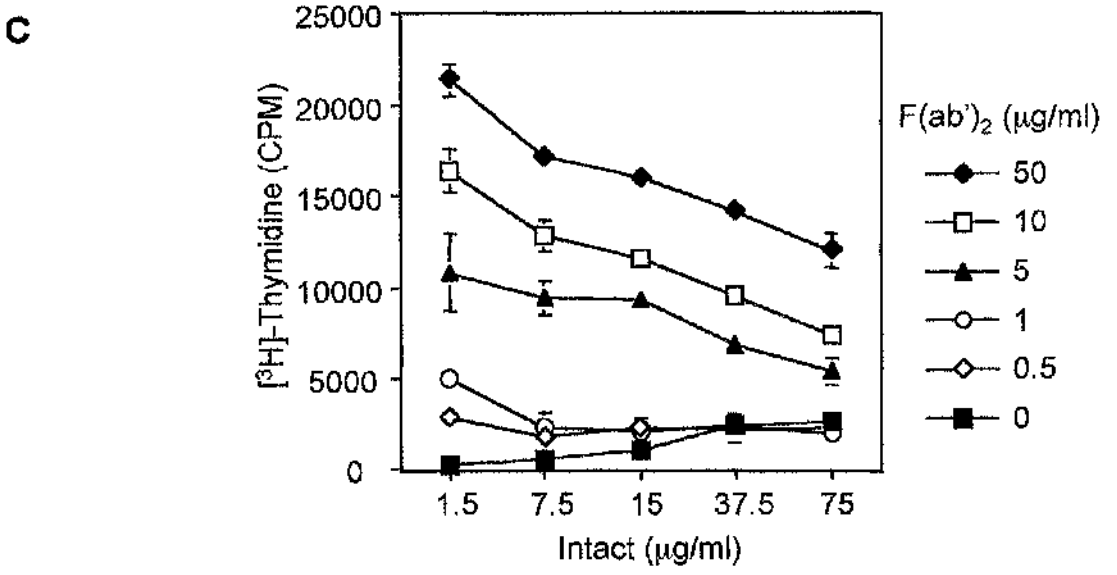
(A) Cells stimulated with increasing concentrations of F(ab')$_2$ fragments of goat anti-mouse IgM (μ-chain specific).

(B) Cells stimulated with increasing concentrations of intact rabbit anti-mouse IgG/IgM.

(C) Cells stimulated with increasing concentrations of F(ab')$_2$ fragments of goat anti-mouse IgM (μ-chain specific) in combination with increasing concentrations of intact rabbit anti-mouse IgG/IgM.

(D) Cells stimulated with 50 μg/ml F(ab')$_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM or 50 μg/ml anti-FcγRII/III (2.4G2) antibody either alone, or in combination, as indicated.


A
25000
20000
15000
10000
5000
0
[3H]-Thymidine (CPM)
0 0.5 1 5 10 50
F(ab')2 (μg/ml)
B
0 1.5 7.5 15 37.5 75
Intact (μg/ml)


C
25000
20000
15000
10000
5000
0
[3H]-Thymidine (CPM)
1.5 7.5 15 37.5 75
Intact (μg/ml)
F(ab')2 (μg/ml)
50
10
5
1
0.5
0

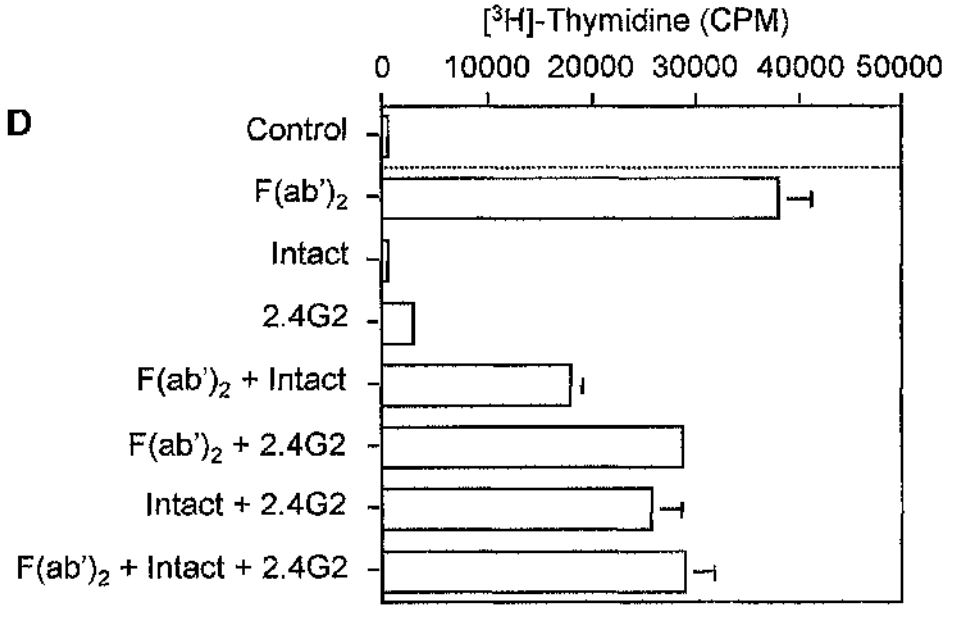

D
[3H]-Thymidine (CPM)
0 10000 20000 30000 40000 50000
Control
F(ab')2
Intact
2.4G2
F(ab')2 + Intact
F(ab')2 + 2.4G2
Intact + 2.4G2
F(ab')2 + Intact + 2.4G2

Figure 3.2 Effect of FcγRIIb and BCR co-ligation on the BCR-mediated proliferative response of resting and activated splenic B cells.

Activated **(A)** and resting **(B)** purified splenic B cells from Balb/c mice were isolated by Percoll density centrifugation and stimulated (2×10^5 cells/well) for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements, from a single experiment, representative of at least two other independent experiments.

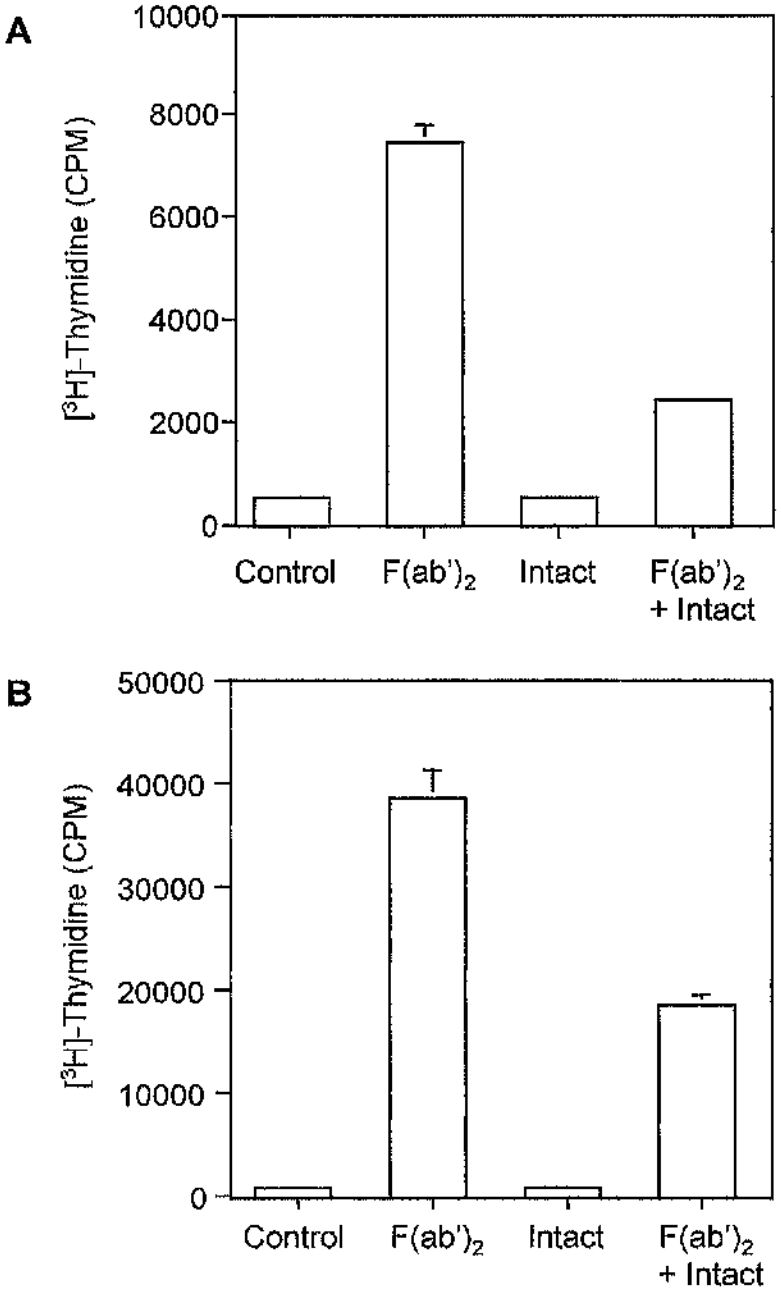

A
10000
8000
6000
4000
2000
0
[3H]-Thymidine (CPM)
Control
F(ab')2
Intact
F(ab')2 + Intact
B
50000
40000
30000
20000
10000
0
[3H]-Thymidine (CPM)
Control
F(ab')2
Intact
F(ab')2 + Intact

Figure 3.3 Effects of BCR and FcγRIIb co-ligation on the DNA content of splenic B cells from Balb/c mice.

Purified splenic B cells from Balb/c mice (1×10^6 cells) were subjected to DNA content analysis post-sacrifice or were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total cells analysed from a single experiment, representative of at least two other independent experiments.

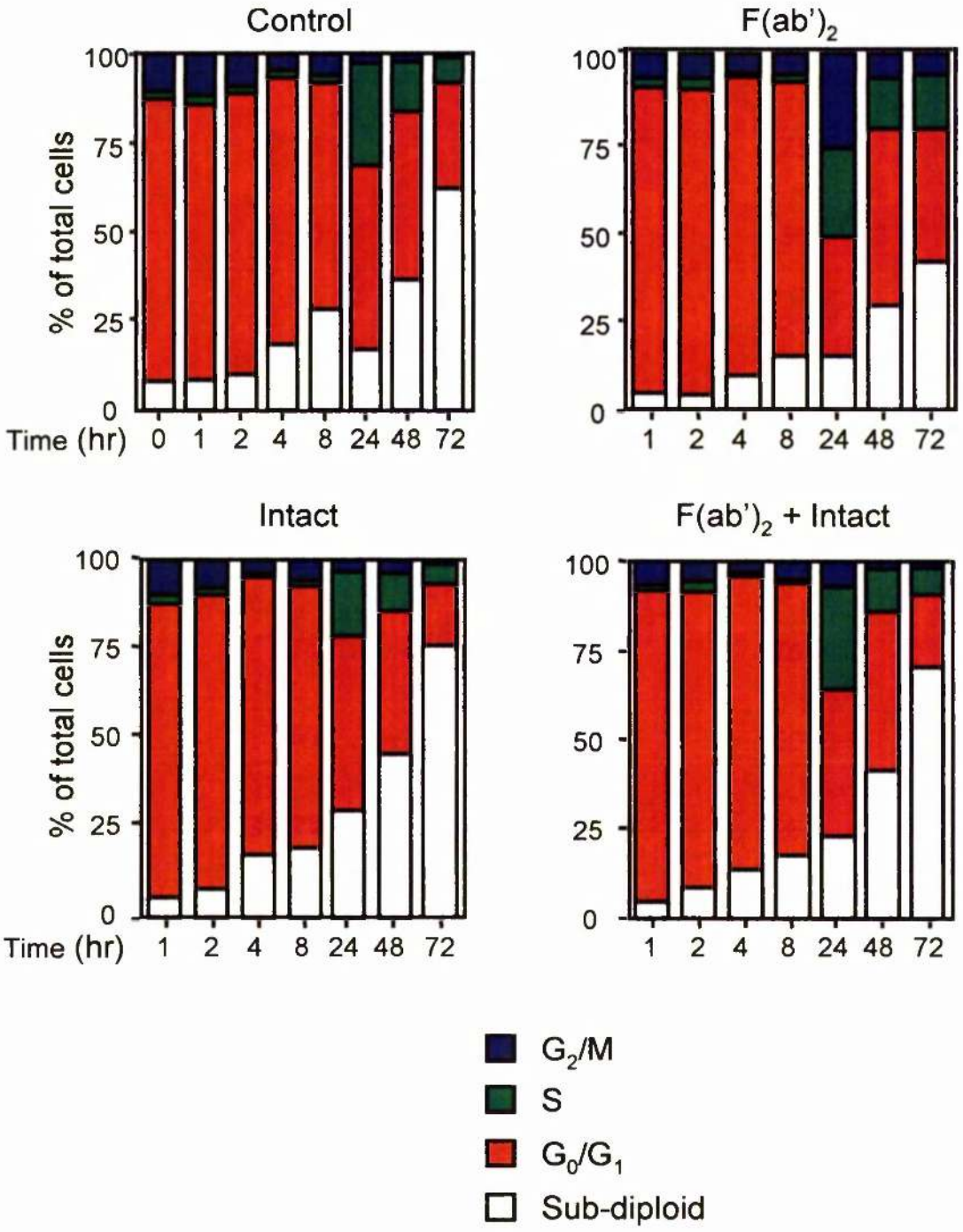

Control
F(ab')2
Intact
F(ab')2 + Intact
% of total cells
100
75
50
25
0
Time (hr)
0 1 2 4 8 24 48 72
1 2 4 8 24 48 72
G2/M
S
G0/G1
Sub-diploid

Figure 3.4 Effect of BCR and FcγRIIb co-ligation on tyrosine phosphorylation of splenic B cell proteins.

Purified splenic B cells from Balb/c mice (1 x 10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Data are from a single experiment, representative of at least two other independent experiments.

(A) Tyrosine phosphorylation was assessed by Western blot analysis of whole cell lysates (50 μg/lane) with the anti-phosphotyrosine monoclonal antibody, 4G10.

(B) Tyrosine phosphorylation in anti-SHIP, SHP-1 and SHP-2 immune complexes was assessed by Western blot analysis with the anti-phosphotyrosine monoclonal antibody, 4G10. To confirm equal loading of samples, blots were stripped and reprobed with the immune precipitating antibody.

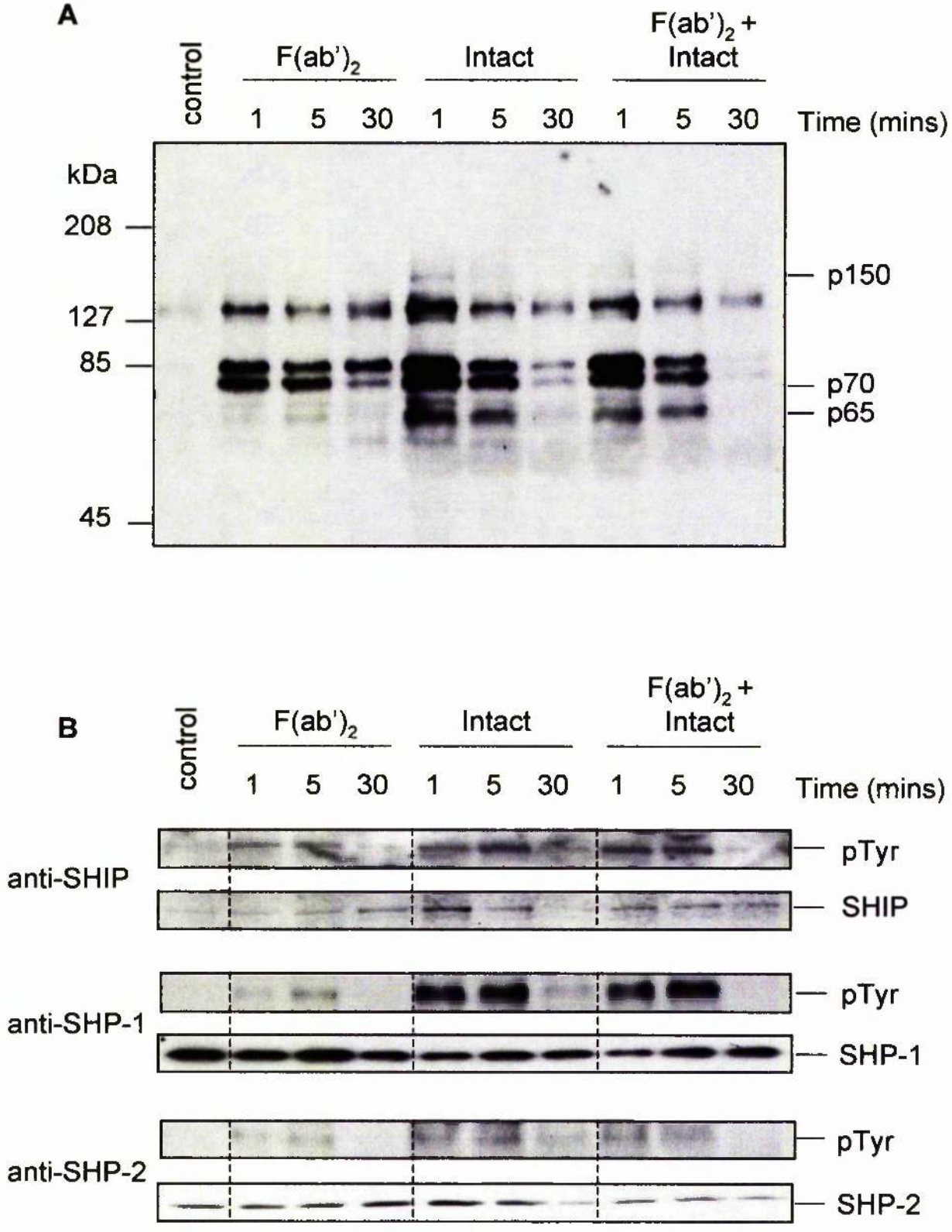

A
control
F(ab')2
Intact
F(ab')2 + Intact
1 5 30 1 5 30 1 5 30
Time (mins)
kDa
208
127
85
45
p150
p70
p65
B
control
F(ab')2
Intact
F(ab')2 + Intact
1 5 30 1 5 30 1 5 30
Time (mins)
anti-SHIP
pTyr
SHIP
anti-SHP-1
pTyr
SHP-1
anti-SHP-2
pTyr
SHP-2

Figure 3.5 Effect of BCR and FcγRIIb co-ligation on the 5'-inositol phosphatase activity of SHIP.

Purified splenic B cells from Balb/c mice (1.5×10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control.

Inositol phosphatase activity in anti-SHIP immune precipitates was assessed by the hydrolysis of $[^3H]$-$InsP_4$ and the subsequent production of $[^3H]$-$InsP_3$, as described in Materials and Methods. Data are the mean ± standard deviation of triplicate measurements from a single experiment, representative of at least three other independent experiments.

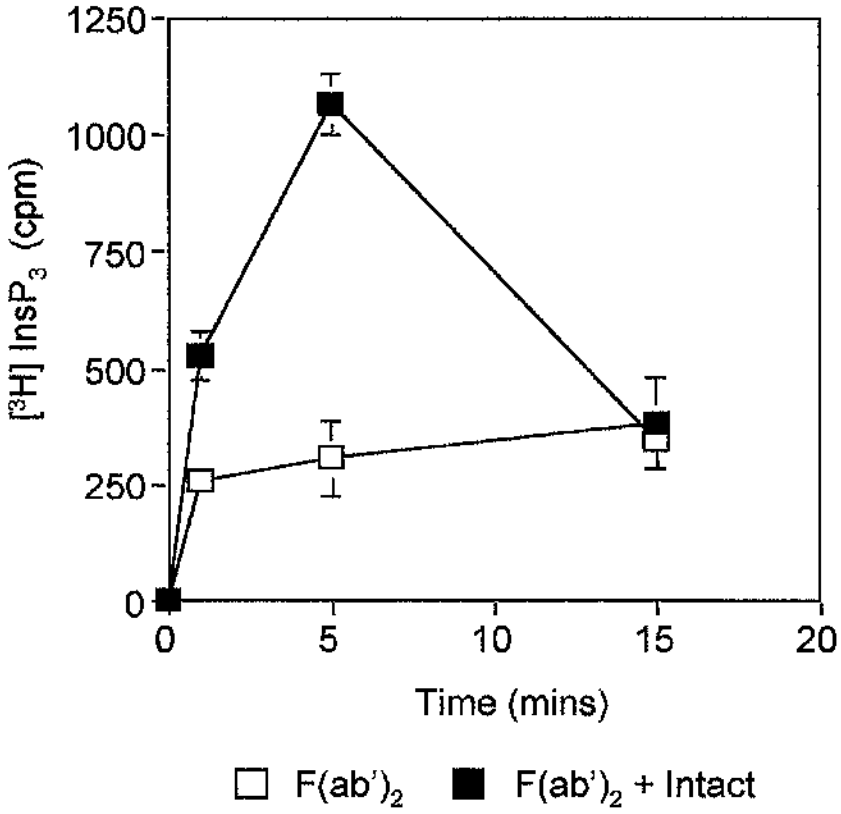

1250
1000
750
500
250
0
[3H] InsP3 (cpm)
0
5
10
15
20
Time (mins)
F(ab')2
F(ab')2 + Intact

Figure 3.6 Effect of BCR and FcγRIIb co-ligation on the formation of SHIP-complexes.

Purified splenic B cells from Balb/c mice (1.5×10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Anti-SHIP immune precipitates were subjected to Western blot analysis with anti-Shc, anti-SHP-1, anti-SHP-2, anti-Grb2 and anti-SHIP antibodies as indicated. Data are from a single experiment, representative of at least one other independent experiment.

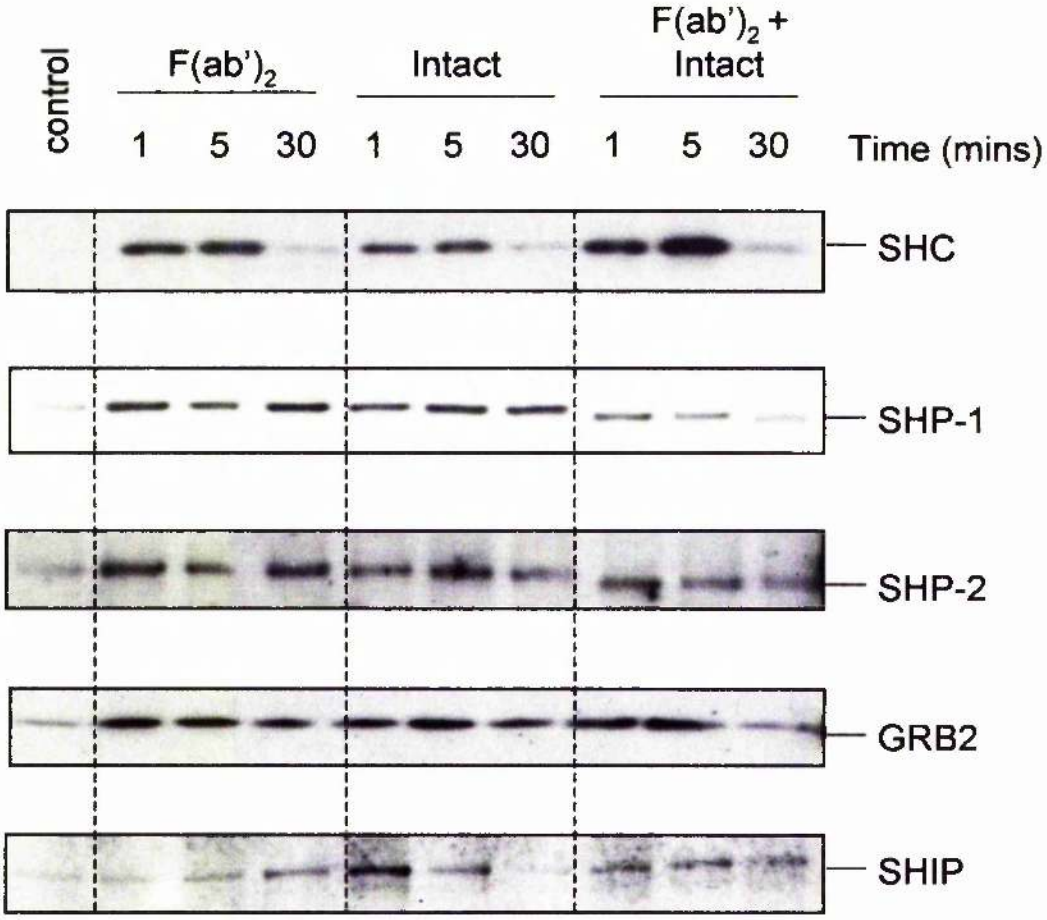
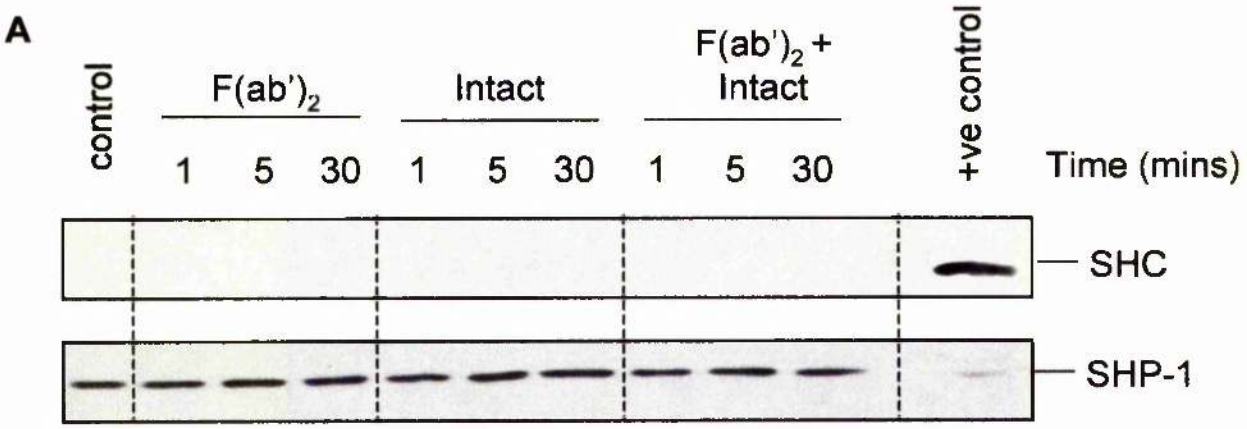

control
F(ab')2
Intact
F(ab')2 + Intact
1 5 30 1 5 30 1 5 30
Time (mins)
SHC
SHP-1
SHP-2
GRB2
SHIP

Figure 3.7 Effect of BCR and FcγRIIb co-ligation on Shc associated SHP-1 phosphatase activity.

Purified splenic B cells from Balb/c mice (1 x 10^7 cells) were stimulated in the presence of 50 μg/ml F(ab')$_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control.

(A) Anti-SHP-1 immune precipitates were subjected to Western blot analysis with anti-Shc antibody (upper panel), stripped and reprobed with anti-SHP-1 antibody (lower panel).

(B) Anti-Shc immune precipitates were assayed for associated SHP-1 phosphatase activity by the hydrolysis of a SHP-1 specific phosphopeptide, as detailed in Materials and Methods. Data are expressed from a single experiment, representative of at least two other independent experiments


A
control
F(ab')2
Intact
F(ab')2 + Intact
+ve control
1 5 30 1 5 30 1 5 30
Time (mins)
SHC
SHP-1

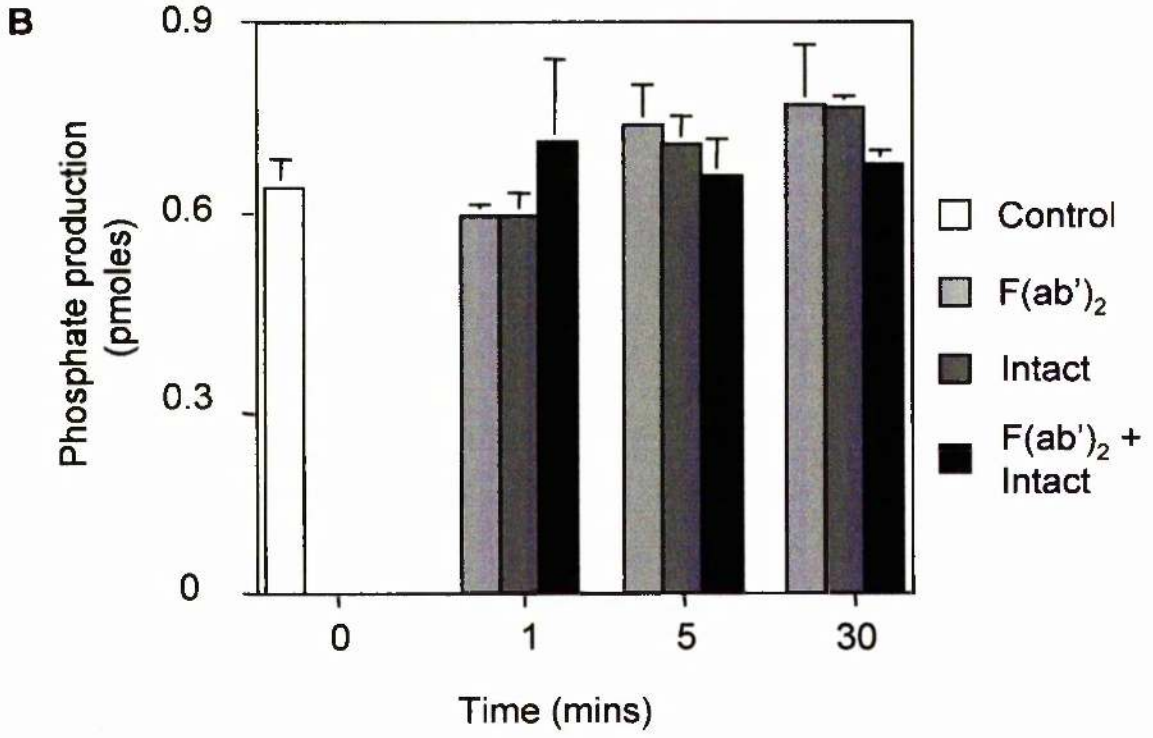

B
Phosphate production (pmoles)
0.9
0.6
0.3
0
0 1 5 30
Time (mins)
Control
F(ab')2
Intact
F(ab')2 + Intact

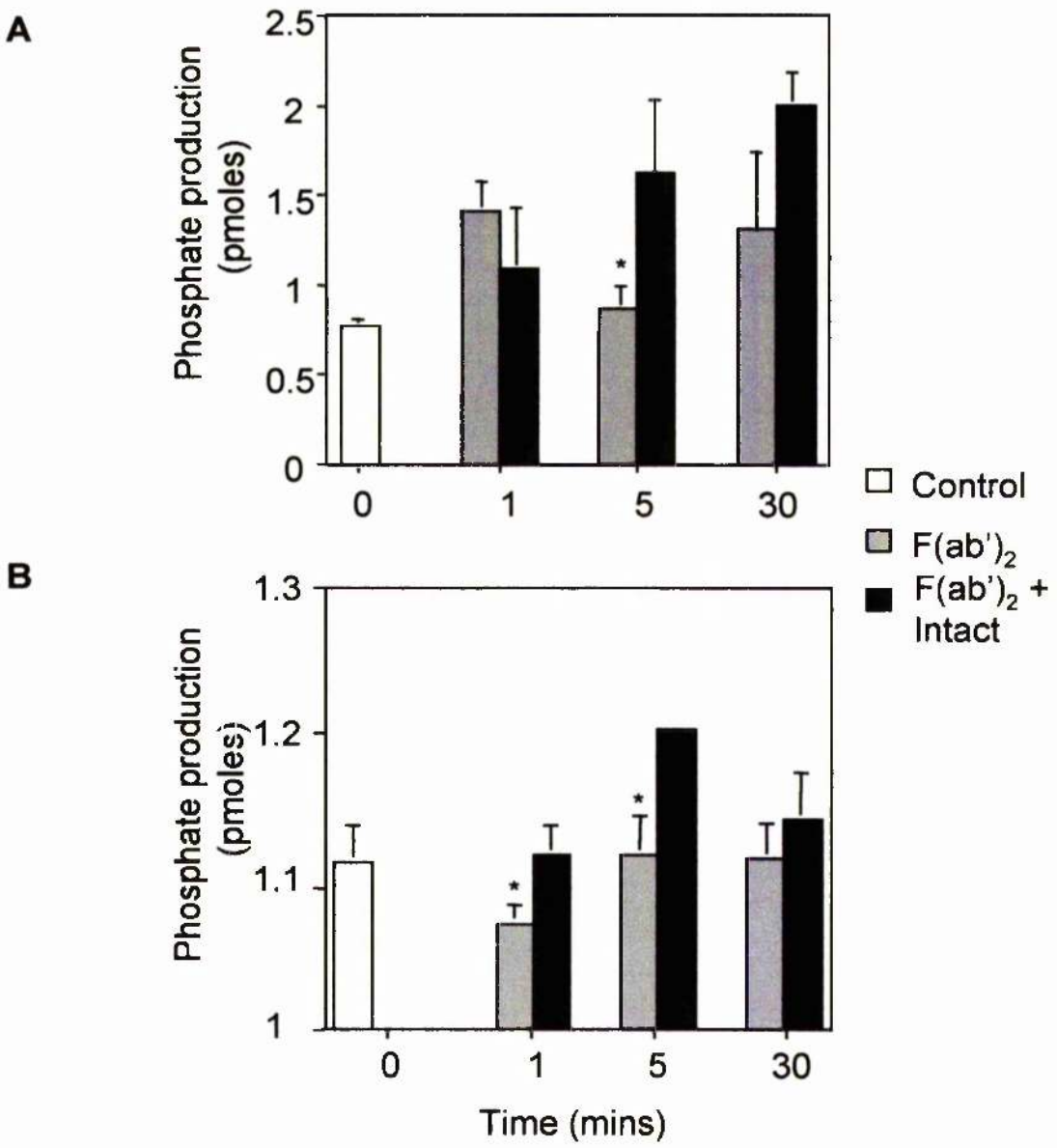
Figure 3.8 Effect of BCR and FcγRIIb co-ligation on Lyn associated SHP-1 phosphatase activity.

Purified splenic B cells from Balb/c mice (1×10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control.

(A) Anti-SHP-1 immune precipitates were assayed for associated SHP-1 phosphatase activity by the hydrolysis of a SHP-1 specific phosphopeptide, as detailed in Materials and Methods. Data are expressed as the mean ± standard deviation from triplicate measurements of a single experiment and representative of at least one other independent experiment. *$p<0.05$, paired Student T-test.

(B) Anti-Lyn immune precipitates were assayed for associated SHP-1 phosphatase activity by the hydrolysis of a SHP-1 specific phosphopeptide, as detailed in Materials and Methods. Data are expressed as the mean ± standard deviation from triplicate measurements of a single experiment and representative of at least one other independent experiment. *$p<0.05$, paired Student T-test.

(C) Anti-SHP-1 immune precipitates were subjected to Western blot analysis with anti-Lyn antibody (upper panel), stripped and reprobed with anti-SHP-1 antibody (lower panel).The numbers beneath the blots represent the relative band densities of Lyn as assessed by densitometry, adjusted with respect to SHP-1 loading and compared to the band densities obtained for the basal control and arbitrarily set as 1.


A
Phosphate production (pmoles)
2.5
2
1.5
1
0.5
0
0
1
5
30
Control
F(ab')2
F(ab')2 + Intact
B
Phosphate production (pmoles)
1.3
1.2
1.1
1
0
1
5
30
Time (mins)

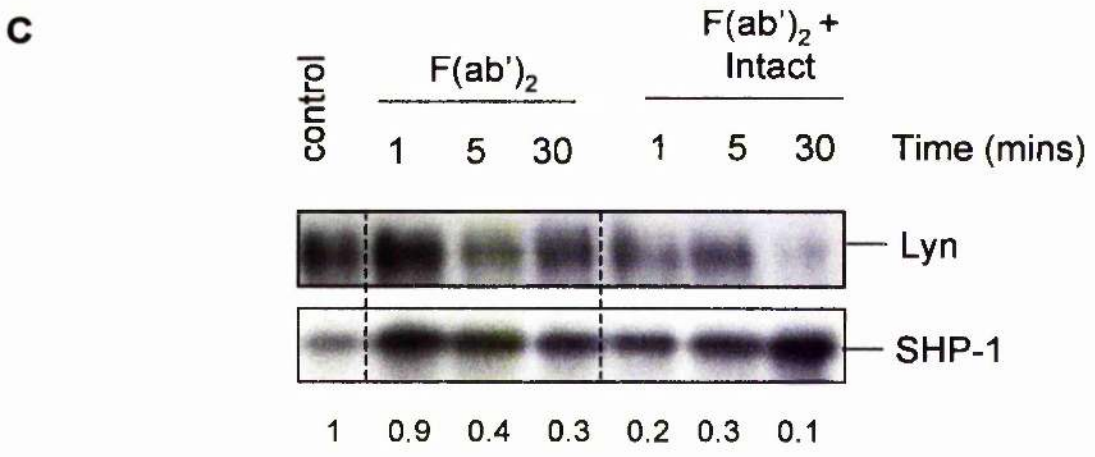

C
control
F(ab')2
F(ab')2 + Intact
1
5
30
1
5
30
Time (mins)
Lyn
SHP-1
1
0.9
0.4
0.3
0.2
0.3
0.1

Figure 3.9 Effect of BCR and FcγRIIb co-ligation on the phosphorylation of ErkMAPK.

Purified splenic B cells from Balb/c mice (1 x 10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Whole cell lysates were subjected to Western blot analysis with phospho-specific ErkMAPK antibody (upper panel), stripped and reprobed with total ErkMAPK antibody (lower panel).

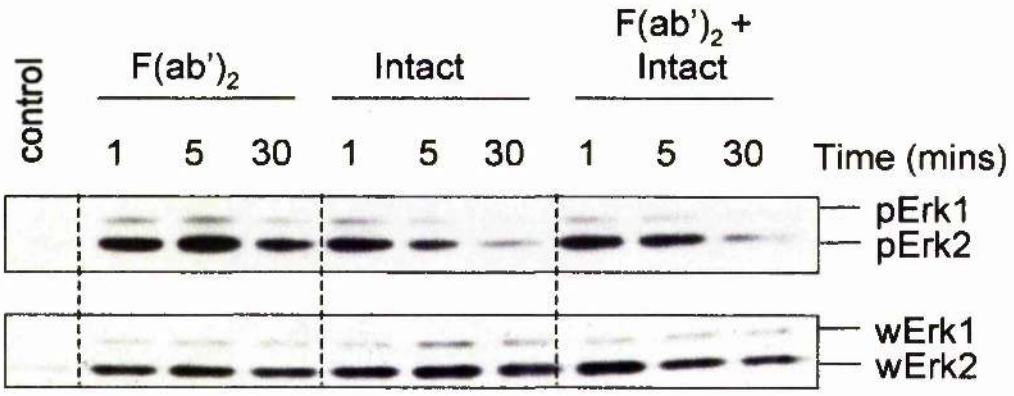

control
F(ab')2
Intact
F(ab')2 + Intact
1
5
30
1
5
30
1
5
30
Time (mins)
pErk1
pErk2
wErk1
wErk2

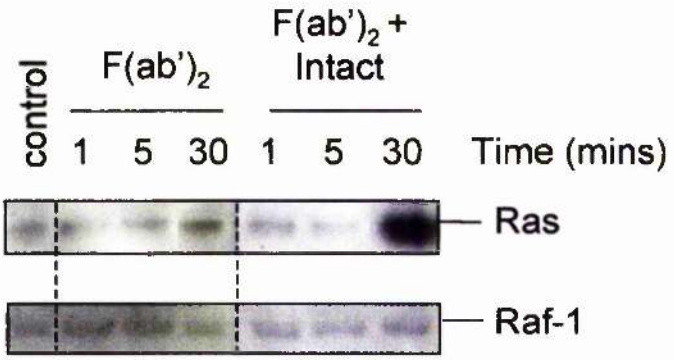
Figure 3.10 Effect of BCR and FcγRIIb co-ligation on Ras and MEK activation.

Purified splenic B cells from Balb/c mice (3×10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM either alone, or in combination, with 75 μg/ml rabbit anti-mouse IgG/IgM intact antibody for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Data are from a single experiment, representative of at least one other independent experiment.

(A) Ras activity was assayed for by the phosphorylation of a Raf-1-RBD-agarose conjugate, as described in Materials and Methods. Raf-1-RBD-agarose conjugate pull-downs were subjected to Western blot analysis with an anti-Ras antibody (upper panel), stripped and reprobed with an anti-Raf-1 antibody (lower panel).

(B) MEK kinase activity was assayed for by the phosphorylation of a p42-MAPKinase GST-fusion protein, as described in Materials and Methods. MEKp42-MAPKinase GST-fusion protein pull-downs were subjected to Western blot analysis with an anti-phospho-ErkMAPKantibody (upper panel), stripped and reprobed with an anti-MEK antibody (lower panel).

A


control
F(ab')2
F(ab')2 + Intact
1 5 30 1 5 30 Time (mins)
Ras
Raf-1

B

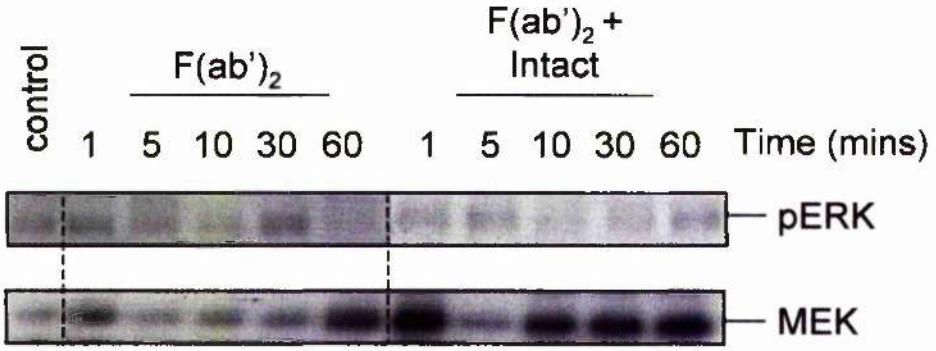
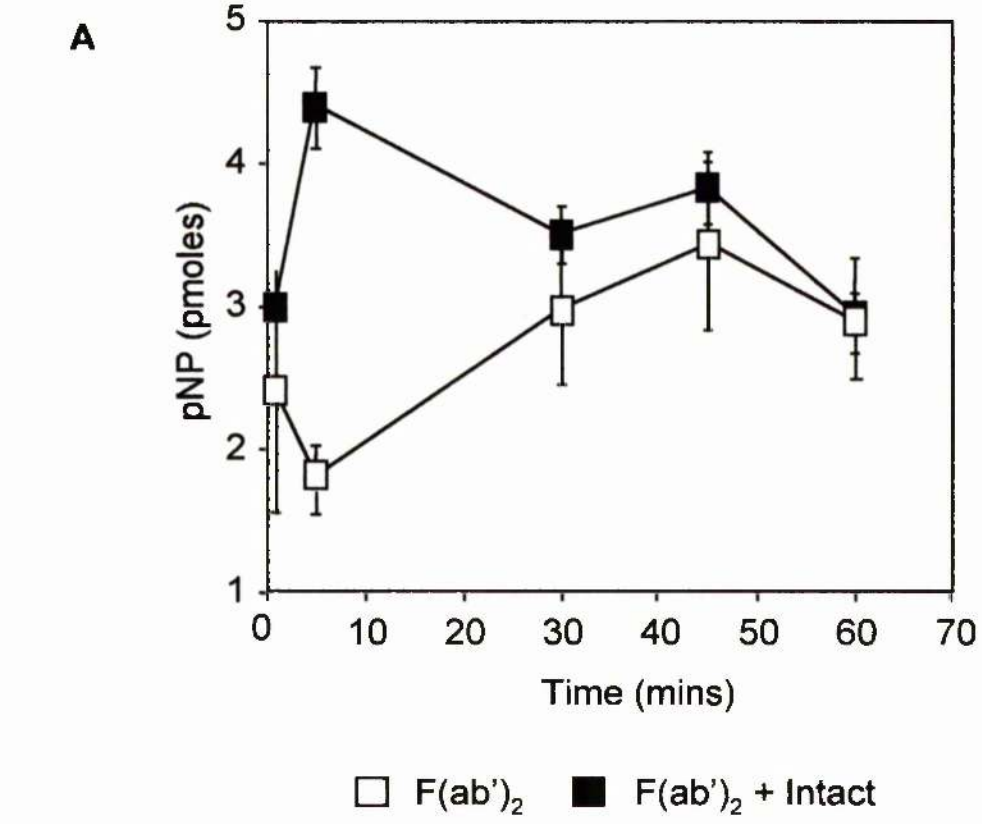

control
F(ab')2
F(ab')2 + Intact
1 5 10 30 60 1 5 10 30 60 Time (mins)
pERK
MEK

Figure 3.11 Effect of BCR and FcγRIIb co-ligation on ErkMAPK-associated phosphatase activity.

Purified splenic B cells from Balb/c mice (1 x 10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control.

(A) Phosphatase activity was assayed for in anti-ErkMAPK immune precipitates by the hydrolysis of pNPP, as detailed in Materials and Methods. Data are expressed as the mean ± standard deviation from triplicate measurements of a single experiment and representative of at least one other independent experiment. **Basal control level** - 3.3 ± 0.2.

(B) Anti-ErkMAPK immune precipitates were subjected to Western blot analysis with Pac-1 antibody (upper panel), and PP2A antibody (middle panel). Whole cell lysates were subjected to Western blot analysis with Pac-1 antibody (lower panel).


A
5
4
3
2
1
pNP (pmoles)
0
10
20
30
40
50
60
70
Time (mins)
F(ab')2
F(ab')2 + Intact

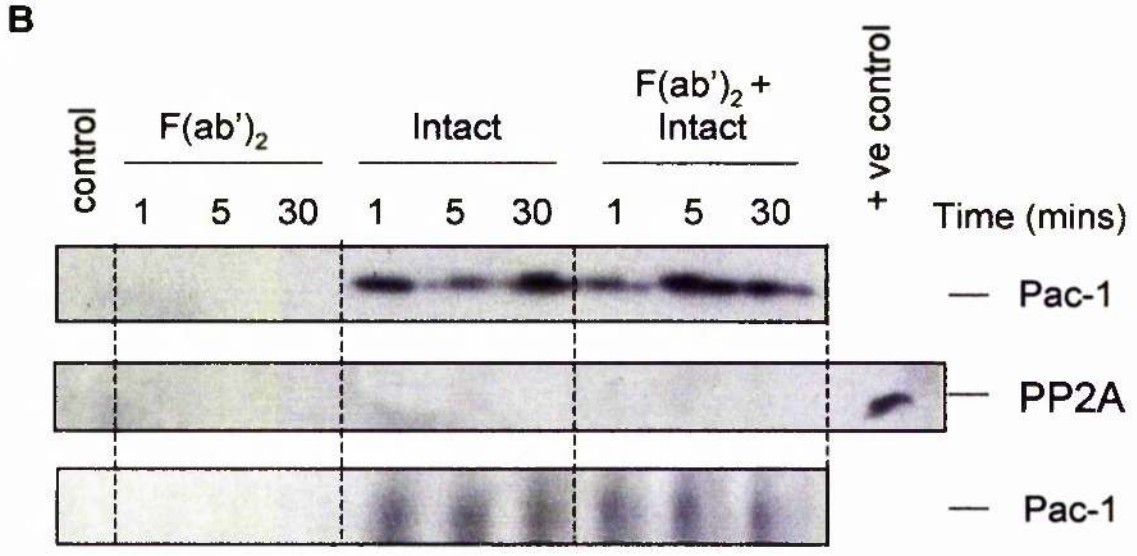

B
control
F(ab')2
Intact
F(ab')2 + Intact
+ ve control
1
5
30
1
5
30
1
5
30
Time (mins)
Pac-1
PP2A
Pac-1

Figure 3.12 Effect of BCR and FcγRIIb co-ligation on Akt kinase activity.

Purified splenic B cells from Balb/c mice (2 x 10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml rabbit anti-mouse IgG/IgM intact antibody either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Data are from a single experiment, representative of at least one other independent experiment.

Akt kinase activity was assayed for by the phosphorylation of a GSK-3α/β fusion protein, as described in Materials and Methods. Akt/GSK-3α/β fusion protein pull-downs were subjected to Western blot analysis with anti-phospho-GSK-3α/β antibody, stripped and reprobed with anti-Akt antibody.

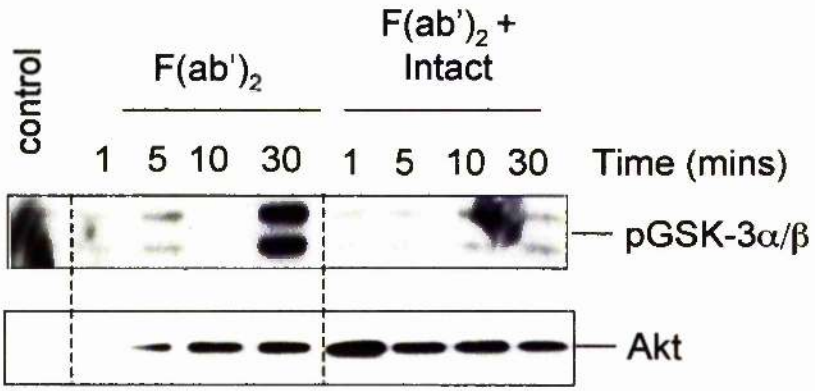

control
F(ab')2
F(ab')2 +
Intact
1 5 10 30 1 5 10 30 Time (mins)
pGSK-3α/β
Akt

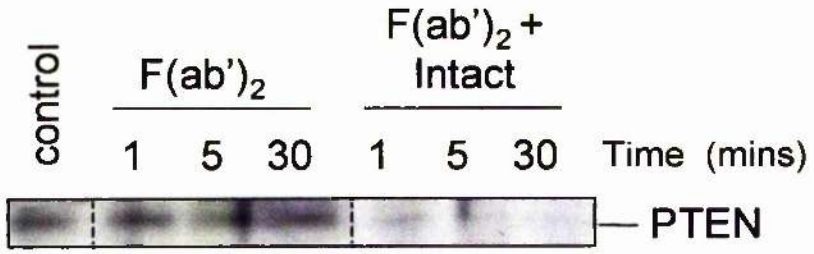
Figure 3.13 Effect of BCR and FcγRIIb co-ligation on PTEN protein phosphatase activity.

Purified splenic B cells from Balb/c mice (1.5×10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control.

(A) Whole cell lysates (50 μg/lane) were subjected to Western blot analysis with an anti-PTEN antibody.

(B) Protein phosphatase activity was assayed for in anti-PTEN immune complexes by the hydrolysis of pNPP, as detailed in Materials and Methods. Data are expressed as the mean ± standard deviation from triplicate measurements of a single experiment and representative of at least two other independent experiments.

A


control
F(ab')2
F(ab')2 + Intact
1 5 30 1 5 30 Time (mins)
PTEN

B

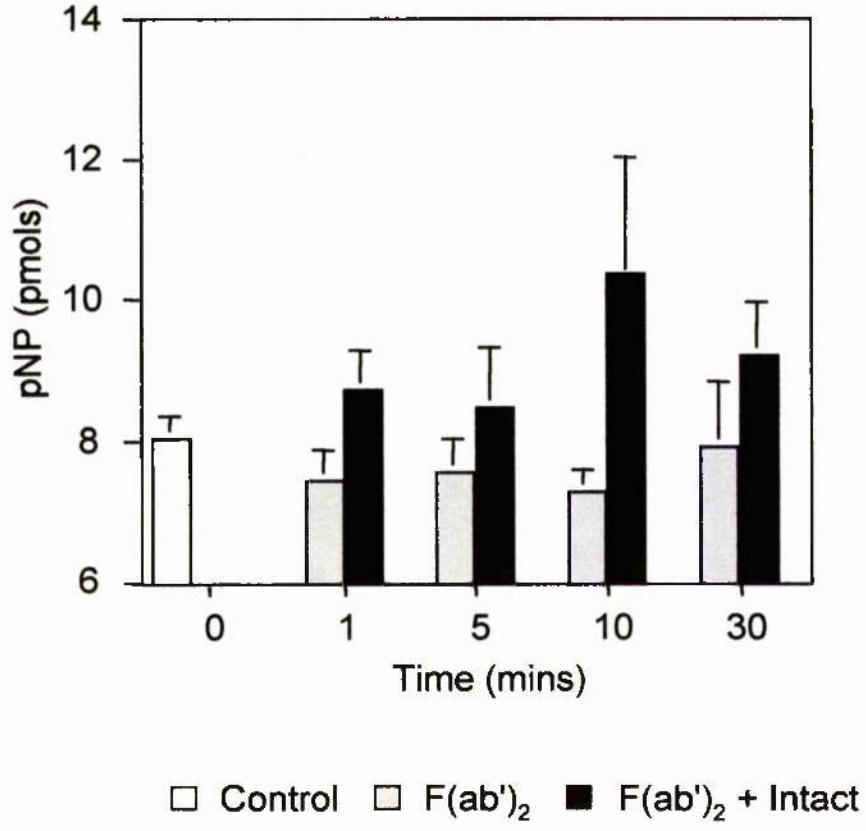

14
12
10
8
6
pNP (pmols)
0 1 5 10 30
Time (mins)
Control
F(ab')2
F(ab')2 + Intact

Figure 3.14 Effect of BCR and FcγRIIb co-ligation on PTEN 3'-inositol phosphatase activity.

Purified splenic B cells from Balb/c mice (1.5×10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control.

Anti-PTEN immune precipitates was assayed for inositol phosphatase activity by the hydrolysis of $[^3H]$-$InsP_4$ and the subsequent production of $[^3H]$-$InsP_3$, as described in Materials and Methods. Data are expressed as the mean ± standard deviation from triplicate measurements of a single experiment and representative of at least two other independent experiments. **Basal control level 208 ± 5 .**

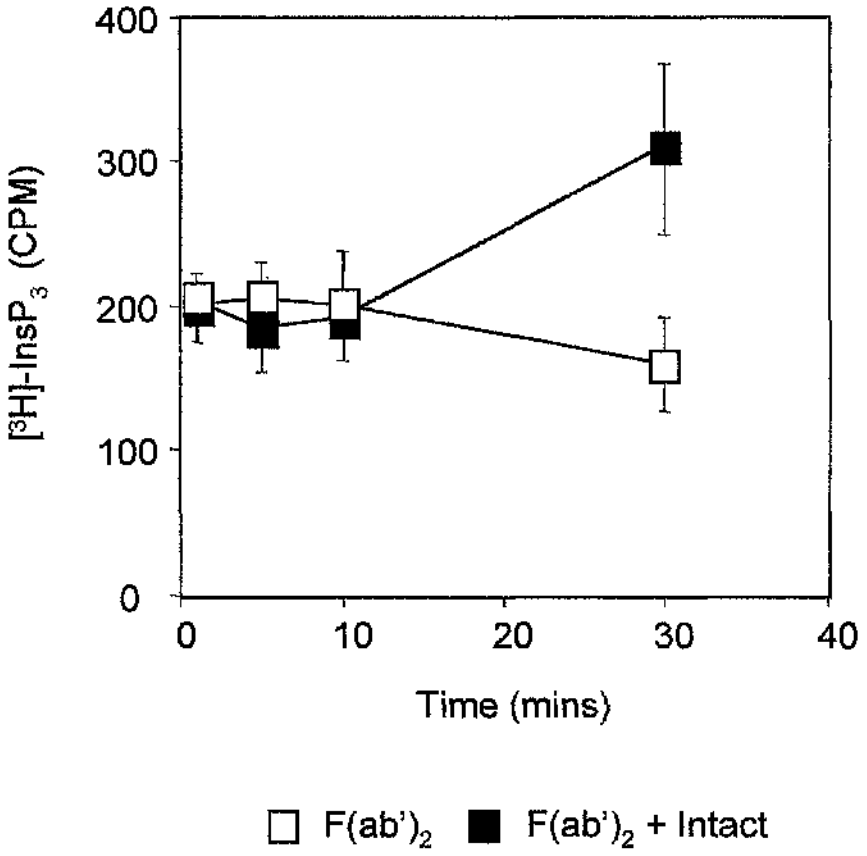

400
300
200
100
0
[3H]-InsP3 (CPM)
0
10
20
30
40
Time (mins)
F(ab')2
F(ab')2 + Intact

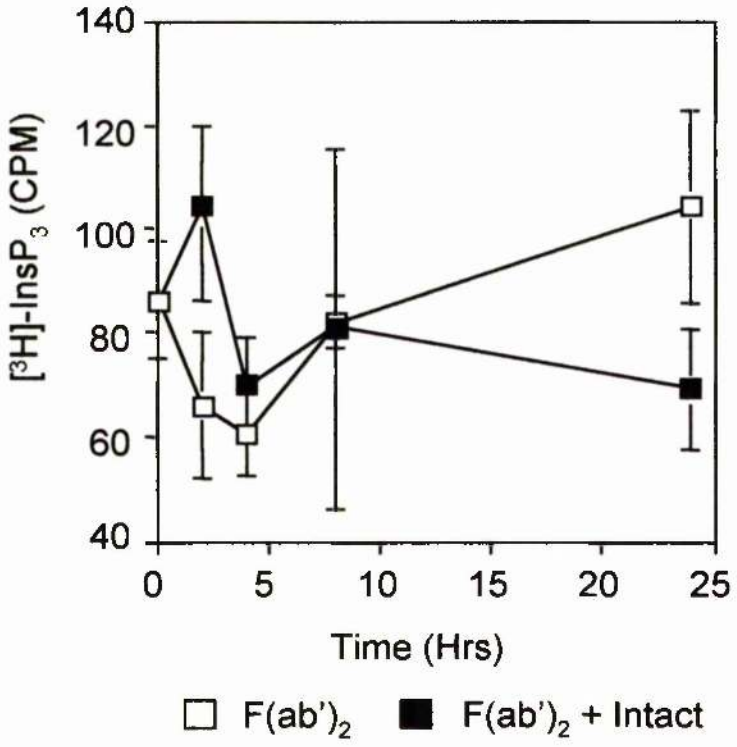
Figure 3.15 Effect of BCR and FcγRIIb co-ligation on long-term PTEN 3'-inositol phosphatase activity.

Purified splenic B cells from Balb/c mice (1.5×10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control.

(A) Anti-PTEN immune precipitates was assayed for inositol phosphatase activity by the hydrolysis of $[^3H]$-$InsP_4$ and the subsequent production of $[^3H]$-$InsP_3$, as described in Materials and Methods. Data are expressed as the mean ± standard deviation from triplicate measurements of a single experiment and representative of at least one other independent experiment.

(B) Anti-PTEN immune precipitates were subjected to Western blot analysis with the monoclonal anti-phosphotyrosine antibody, 4G10.

A


140
120
100
80
60
40
[3H]-InsP3 (CPM)
0
5
10
15
20
25
Time (Hrs)
F(ab')2
F(ab')2 + Intact

B

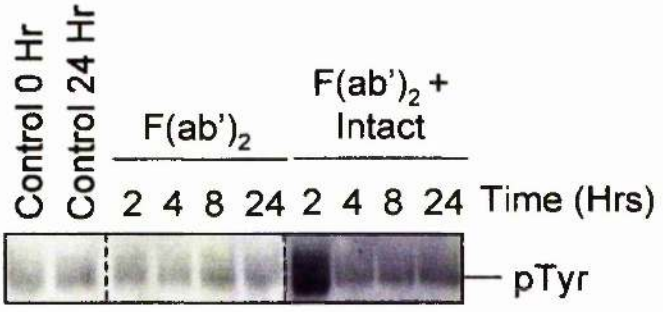

Control 0 Hr
Control 24 Hr
F(ab')2
F(ab')2 + Intact
2 4 8 24 2 4 8 24 Time (Hrs)
pTyr

Figure 3.16 Effects of FcγRIIb and BCR co-ligation on JNK/SAPK activity

Purified splenic B cells from Balb/c mice (3×10^7) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a basal level control, whilst cells stimulated for 30 minutes in the presence of PMA (100 ng/ml) and ionomycin (1 μg/ml) were included as a positive control. Data are from a single experiment, representative of at least one other independent experiment. This study was done in collaboration with a fellow post-graduate student in the laboratory, Derek Blair.

JNK activity was assayed for by the phosphorylation of a c-Jun fusion protein, as described in Materials and Methods. c-Jun fusion protein pull-downs were subjected to Western blot analysis with an anti-phospho-c-Jun antibody, stripped and reprobed with anti-c-Jun antibody. The numbers beneath the blots represent the relative band densities as assessed by densitometry, compared to the band densities obtained for the basal control and arbitrarily set as 1.

A

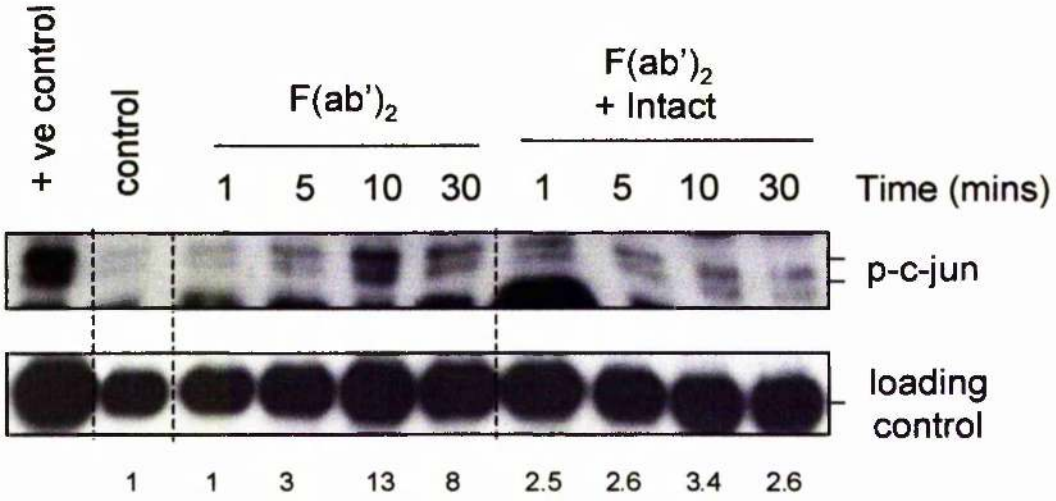

+ ve control
control
F(ab')2
F(ab')2 + Intact
1 5 10 30 1 5 10 30
Time (mins)
p-c-jun
loading control
1 1 3 13 8 2.5 2.6 3.4 2.6

Figure 3.17 FcγRIIb ligation modulates BCR-mediated phosphorylation of Rb and p53.

Purified splenic B cells from Balb/c mice (1 x 10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Data are from a single experiment, representative of at least one other independent experiment. This study was done in collaboration with a fellow post-graduate student in the laboratory, Derek Blair.

(A) Whole cell lysates (50 μg/lane) were subjected to Western blot analysis with a an anti-Cdk4 antibody.

(B) Whole cell lysates (50 μg/lane) were subjected to Western blot analysis with a anti-phospho-Rb antibody.

(C) Anti-p53 immune precipitates were subjected to Western blot analysis with the monoclonal anti-phosphoserine-15 antibody, stripped and reprobed with anti-p53 antibody.

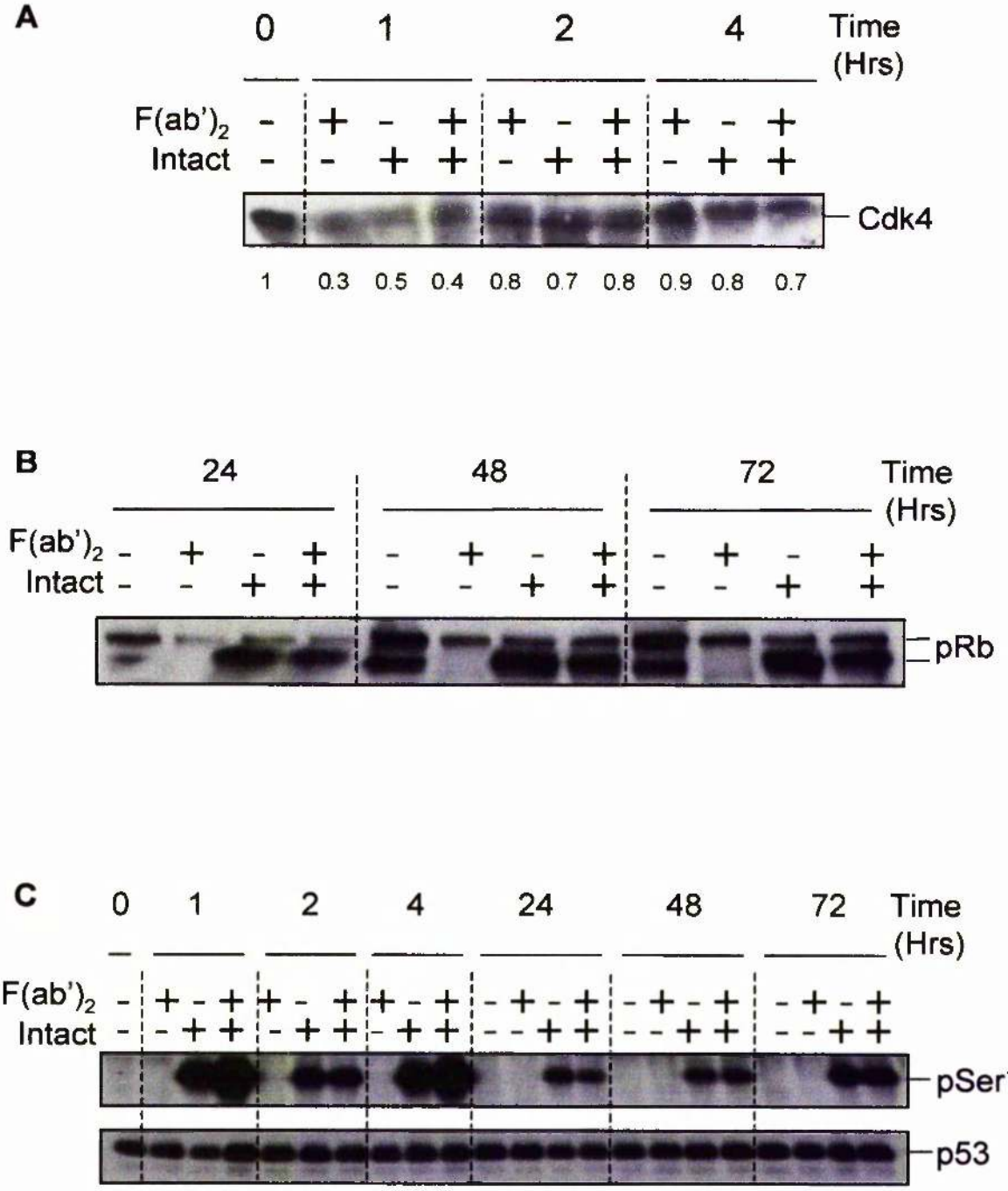

A
0
1
2
4
Time (Hrs)
F(ab')2
Intact
Cdk4
1 0.3 0.5 0.4 0.8 0.7 0.8 0.9 0.8 0.7
B
24
48
72
Time (Hrs)
F(ab')2
Intact
pRb
C
0
1
2
4
24
48
72
Time (Hrs)
F(ab')2
Intact
pSer15
p53

Figure 3.18 Effects of FcγRIIb and BCR co-ligation on NF-κB binding.

Purified splenic B cells from Balb/c mice (3 x 10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Nuclear protein extracts were assayed by EMSA for binding to a ^{32}P-labelled oligonucleotide probe that represents a NF-κB binding site of the iNOS promoter. Arrows denote binding complexes. Data are from a single experiment, representative of at least two other independent experiments.

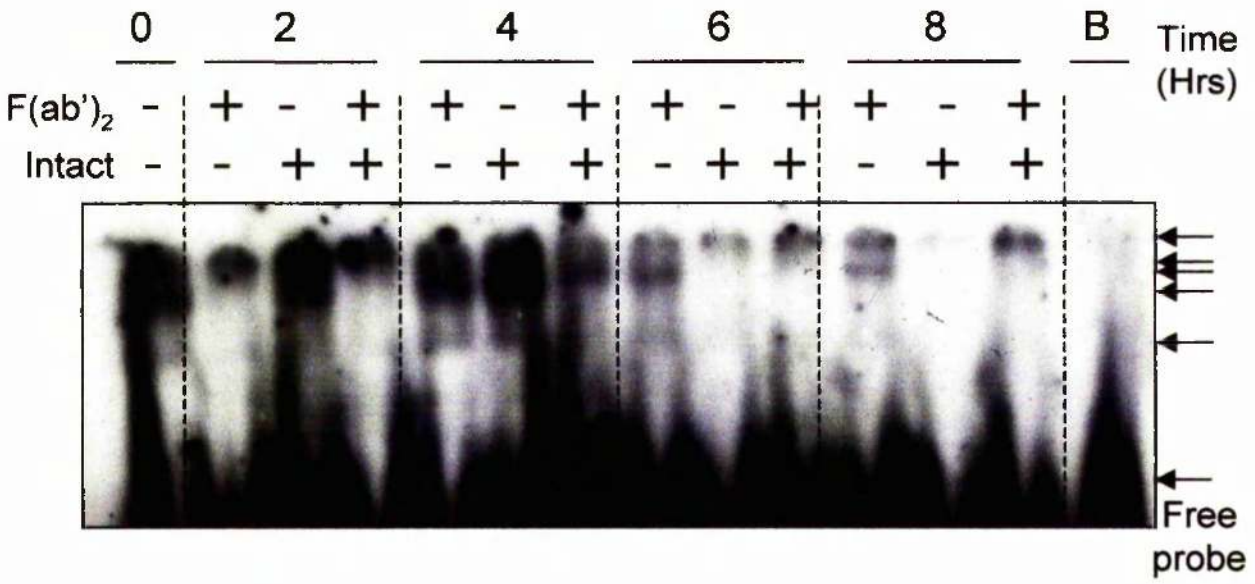

0
2
4
6
8
B
Time (Hrs)
F(ab')2 - + - + + - + + - + + - + +
Intact - - + + - + + - + + - + +
Free probe

Figure 3.19 FcγRIIb-mediated mechanisms of B cell inhibition result in a decrease in mitochondrial membrane potential.

Purified splenic B cells from Balb/c mice (1 x 10^6 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM alone, or in combination as indicated for 72 hours. Cells stimulated in the presence of medium alone were included as a control. The mitochondrial membrane potential was determined by $DiOC_6(3)$ staining and FACS analysis, as described in the Materials and Methods. Data represents the $DiOC_6(3)^{LOW}$ cell population, as determined on a logarithmic FL-1 axis, and expressed as a percentage of the total cells analysed of a single experiment and representative of at least two other independent experiments.

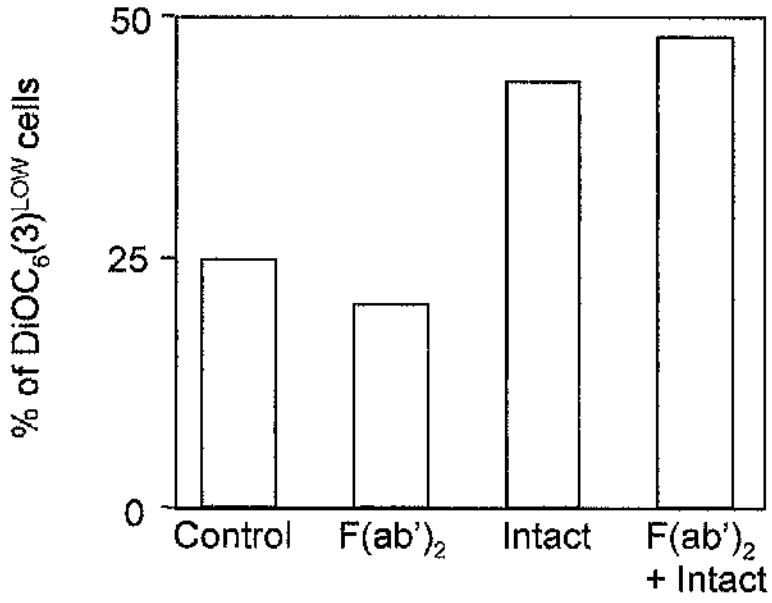

50
25
0
% of DiOC6(3)LOW cells
Control
F(ab')2
Intact
F(ab')2
+ Intact

Figure 3.20 Effect of BCR and FcγRIIb co-ligation on the mRNA expression levels of FcγRIIb1 and FcγRIIb2 in splenic B cells.

Purified splenic B cells from Balb/c mice (5 x 10^6 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM alone, or in combination for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Total RNA was isolated and subjected to reverse transcription in the presence of 100 U of Superscript RT II. FcγRIIb1 (upper panel) and FcγRIIb2 (lower panel) isoform transcripts were assayed for by real-time PCR (TaqMan). Levels are expressed as a percentage relative to HPRT mRNA level ± standard deviation from triplicate measurements of a single experiment.

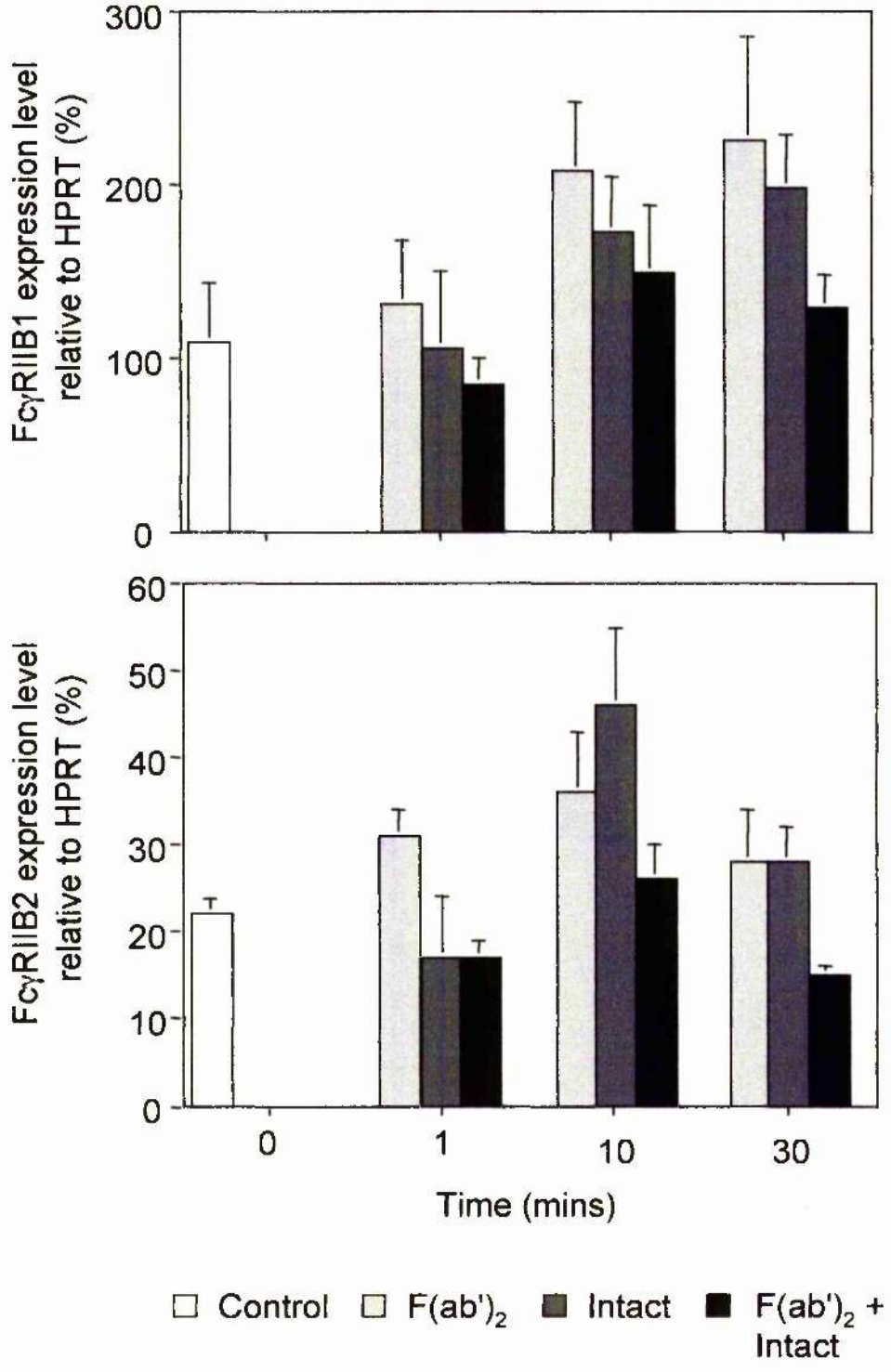

FcγRIIB1 expression level relative to HPRT (%)
300
200
100
0
FcγRIIB2 expression level relative to HPRT (%)
60
50
40
30
20
10
0
0
1
10
30
Time (mins)
Control
F(ab')2
Intact
F(ab')2 + Intact

Figure 3.21 Effect of BCR and FcγRIIb co-ligation on the ErkMAPKinase pathway

Co-ligation of the BCR with FcγRIIb results in the phosphorylation of the FcγRIIb cytoplasmic ITIM motif by the Src-family PTK, Lyn **(1)**. Following recruitment to the phosphorylated ITIM the SH2-domain containing inositol 5'-phosphatase, SHIP, prevents the induction of the BCR-coupled Ras/MAPKinase cascade via interactions with the adaptor molecule Shc and the RasGAP binding protein, p62Dok **(2)**. In addition to the activation of SHIP, FcγRIIb appears to uncouple the BCR from the Ras/MAPKinase pathway through the rapid activation and recruitment of the dual-specific nuclear MAPK phosphatase, Pac-1 **(3)** and by promoting the BCR recruitment of Lyn-SHP-1 protein tyrosine phosphatase complexes **(4)**.

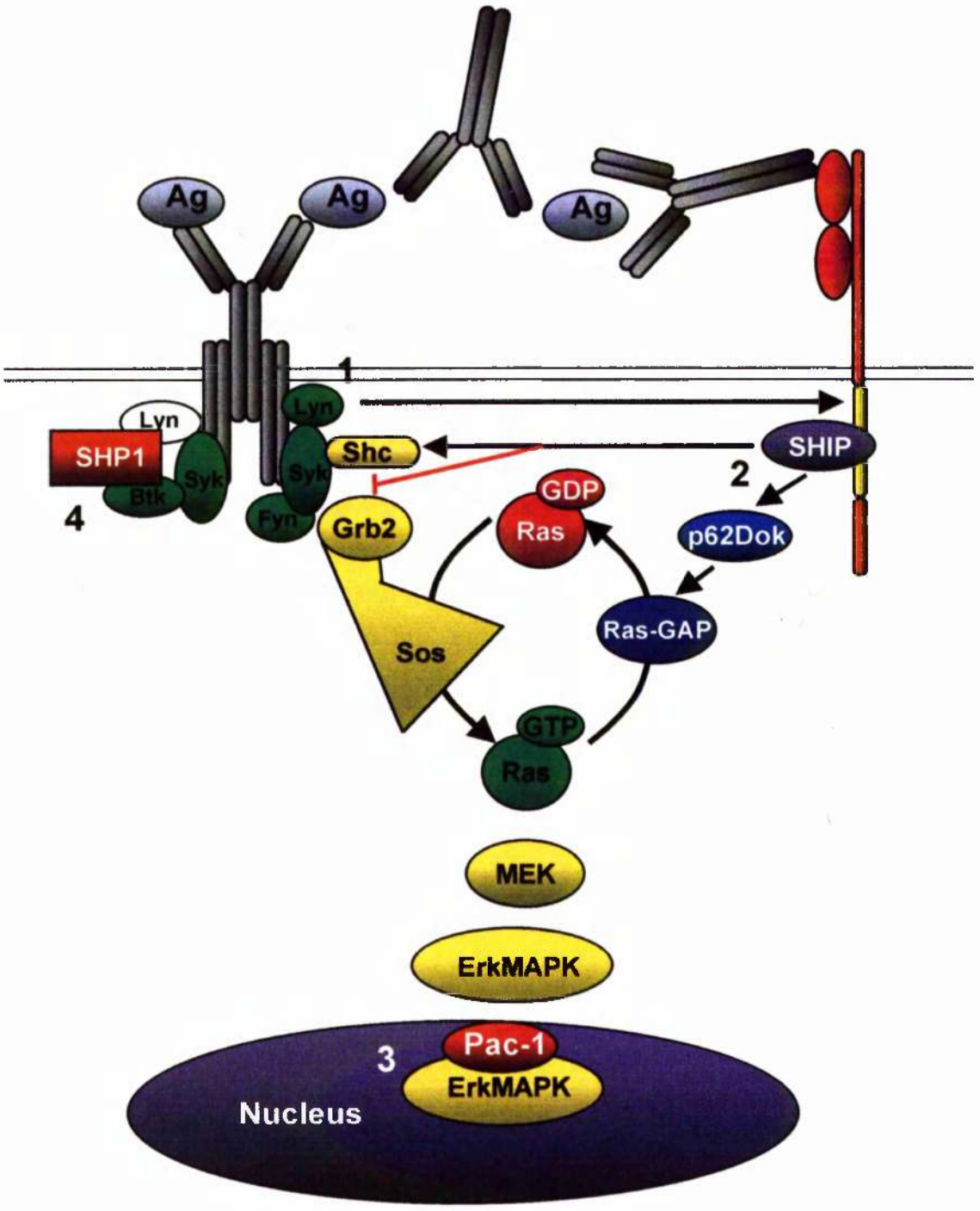

Ag
Ag
Ag
1
Lyn
Lyn
SHP1
Syk
Syk
Shc
SHIP
2
Btk
4
Fyn
Grb2
GDP
Ras
p62Dok
Ras-GAP
Sos
GTP
Ras
MEK
ErkMAPK
Pac-1
3
ErkMAPK
Nucleus

Figure 3.22 Effect of BCR and FcγRIIb co-ligation on the Akt survival pathway

BCR ligation is known to induce the activation of the pro-survival factor Akt and promote B cells survival. Co-ligation of the BCR with FcγRIIb results in the termination of this signal, through the conversion of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 by the inositol 5'-phosphatase, SHIP. However, PtdIns(3,4)P_2 is also known to activate Akt, thus FcγRIIb may inhibit BCR-activated Akt by the recruitment of the inositol 3'-phosphatase, PTEN. Through the conversion of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, PTEN could impair BCR mediated activation of Akt by counteracting the activities of PI-3-Kinase.

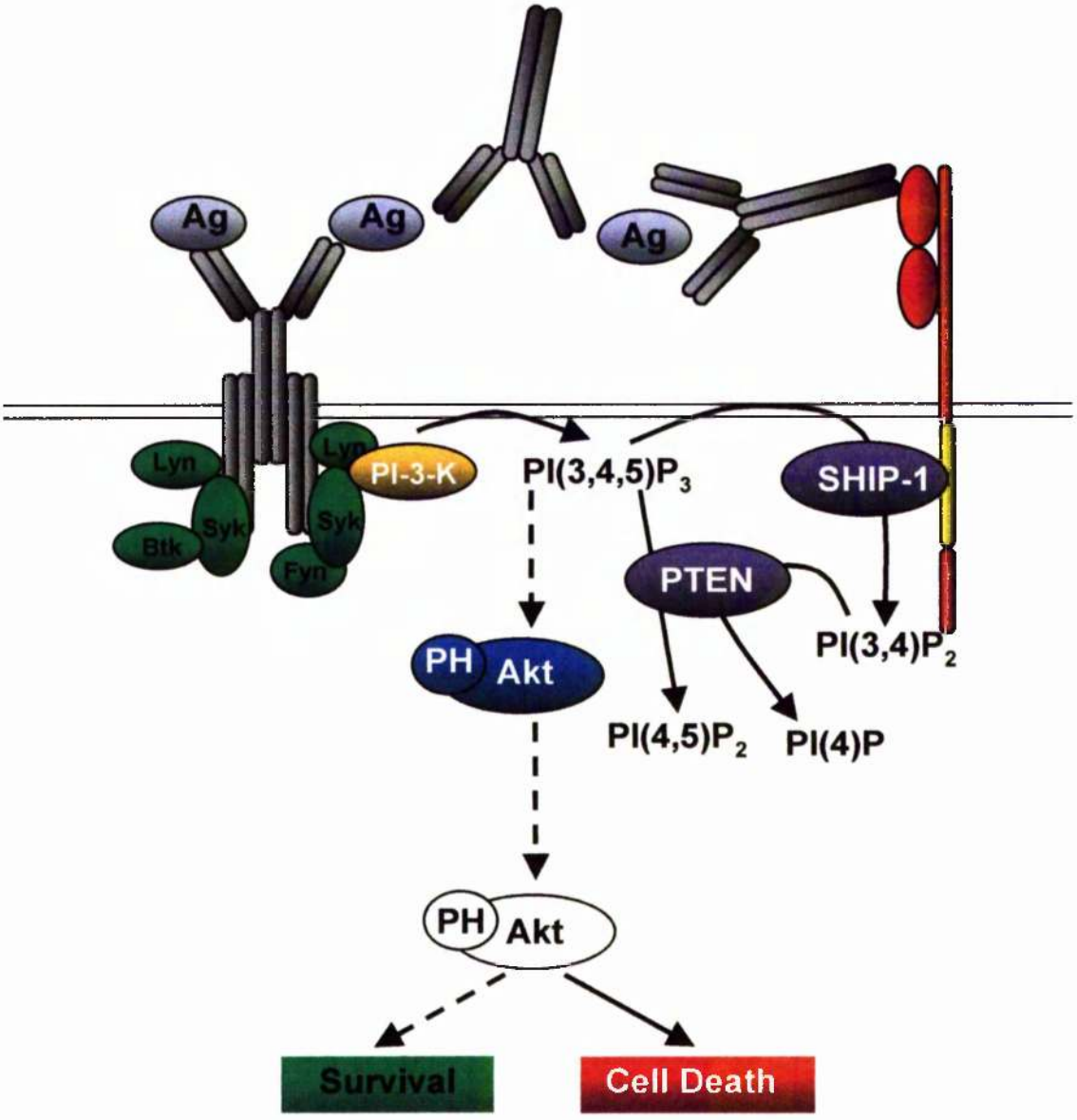
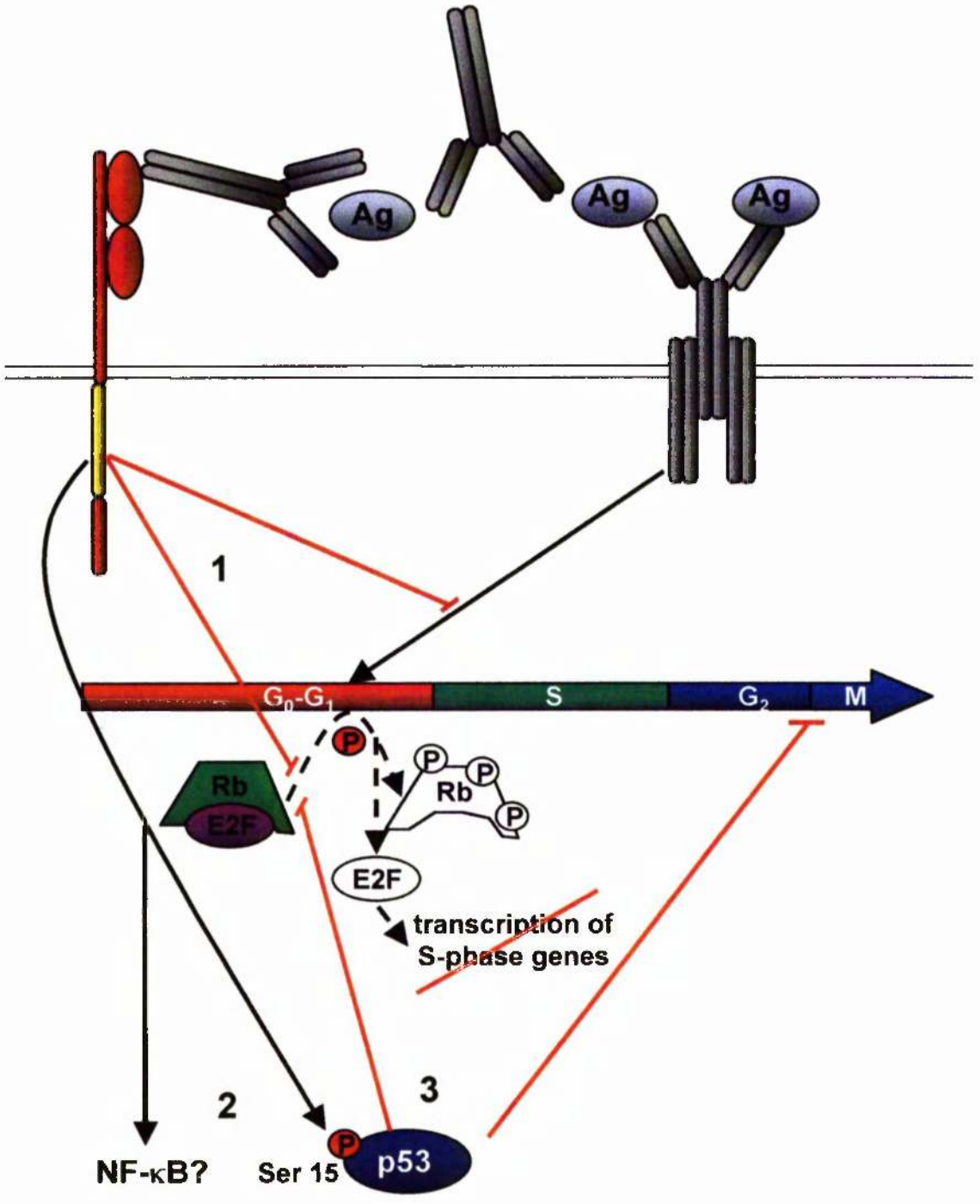

Ag
Ag
Ag
Lyn
Lyn
PI-3-K
Syk
Syk
Btk
Fyn
PI(3,4,5)P3
SHIP-1
PTEN
PI(3,4)P2
PH
Akt
PI(4,5)P2
PI(4)P
PH
Akt
Survival
Cell Death

Figure 3.23 Effect of BCR and FcγRIIb co-ligation on the regulation of the cell cycle.

FcγRIIb-mediated growth-arrest leads to the modulation of cell cycle regulatory proteins. BCR-mediated G_1-S phase progression results in an increase in Rb phosphorylation. However, FcγRIIb co-ligation results in the partial suppression of BCR-induced Rb phosphorylation, potentially preventing the release of E2F and the transcription of S phase genes **(1)**. FcγRIIb ligation also disrupts BCR-mediated cell-cycle progression by the modulation of NF-κB activation and the rapid phosphorylation of p53 on serine 15 **(2)**. The activation of p53 can lead to cell cycle arrest at the G_1-S and G_2-M transition points **(3)** and DNA repair or apoptosis.


Ag
Ag
Ag
1
G_0-G_1
S
G_2
M
P
Rb
E2F
P
P
P
Rb
E2F
transcription of
S-phase genes
2
3
NF-κB?
Ser 15
P
p53

Chapter 4 - FcγRIIb and Murine Autoimmune Disease Models

4.1 Autoimmunity and autoimmune disease

As with any balanced system, inappropriate activation and a loss of homeostasis can result in detrimental effects. In the case of the immune response, deregulation may result in a variety of chronic inflammatory processes, and in some situations, may cause serious disease. Autoimmunity is the inappropriate response of the immune system to self-antigens, which can lead to autoimmune disease. Autoimmune disease-mediated tissue damage can be caused by a variety of mechanisms involving autoimmune T cells or the production of autoantibodies (antibodies against self-antigens) (reviewed by Ada *et al.*, 1997).

Tissue damage may result from a number of aberrant responses including direct attack on cells bearing the self-antigen, from the formation of immune complexes or from local inflammation. For example, in autoimmune haemolytic anaemia, autoantibody recognition of self-antigens on the surface of red bloods cells, mediates their destruction. The autoantibody-coated cells are cleared by Fc receptor expressing phagocytic cells in the spleen or by complement receptor bearing macrophages. The binding of certain rare autoantibodies, with the ability to fix complement efficiently, can also cause haemolysis via the formation of a complement membrane attack complex. Systemic lupus erythematosus arises from the production of autoantibodies to common cellular constituents, such as dsDNA and ribonuclear proteins (section **5.1.1**). Due to the large quantities of self-antigen available, large numbers of immune complexes are deposited in small blood vessels in the renal glomerulus, joints and other organs. This tissue-deposition leads to the release of inflammatory mediators, infiltration of inflammatory cells and complement fixation. The consequent tissue damage results in the release of more nuclear-protein complexes from the damaged cells. The increase in self-antigen concentrations results in the further production of immune complexes and a cyclical

exacerbation of the disease. Finally, inflammatory injury in Goodpasture's syndrome is caused by the production of autoantibodies to the basement membrane collagen of the renal glomeruli. Antibody binding results in complement activation and the influx of neutrophils causing inflammatory injury to the glomerulus and a rapidly fatal disease if untreated.

4.1.1 The role of B cells in the loss of tolerance and production of autoantibodies.

In order to determine how an autoimmune response is induced, an understanding of (i) how tolerance is maintained normally in the immune system and (ii) why responses occur in autoimmune disease, is required. Tolerance is the failure of the immune system to respond to a specific antigen and is required to prevent inflammatory responses to the many innocuous substances that are encountered on a daily basis. When the specific antigen is borne by self-tissues, then tolerance is described as self-tolerance. Tolerance to self-antigens is important in preventing the mounting of an immune response against the tissues of the body.

The most important mechanisms of tolerance to self involve clonal deletion and inactivation (anergy) of lymphocytes by exposure to the antigen during the maturation stages of lymphocyte development (see section **1.2.1**). Clonal deletion of B cells that express antigen-receptors to ubiquitous self-antigens occurs in the bone marrow and secondary lymphoid tissue follicles. Deletion is dependent on BCR signal strength or a lack of co-stimulatory T cell help in the form of CD40 and IL-4. Thus, immature B cells with low affinity or redundant antigen specificites undergo receptor editing or apoptosis in the bone marrow whilst a lack of co-stimulatory T cell-derived cytokines will also promote apoptosis. These processes prevent the maturation of those B cells that would potentially recognise self-structures present during the maturation stages, resulting in the generation of autoantibodies (reviewed by Cushley and Harnett, 1993).

Despite the stringent selective processes of clonal deletion and receptor editing in the maturation stages, B cells expressing antigen-receptors for self are still present in the periphery. This is presumably because not all self-antigens can be sufficiently presented during the selection processes as many self-molecules are available only in the periphery. Therefore mechanisms exist to ensure that B cells reactive to these self molecules do not cause autoimmune disease. B cell recognition of self-antigen in the T cell zone of peripheral lymphoid organs, results in the arrest of their migration. Inactivation of B cells in the periphery is also achieved through the induction of B cell anergy by the downregulation of sIgM and/or partial inhibition of sIgM-mediated intracellular signalling pathways. Monomeric soluble antigens can induce this mechanism when presented in the absence of co-stimulatory signals from T cells in the periphery. A subsequent lack of self-antigen specific $CD4^+$ T cell help, results in apoptosis of the trapped B cells.

Autoreactive B cells may also be forced to undergo apoptosis by activated autoreactive T cells that express Fas ligand (Fas-L) (reviewed by Sigel, *et al.*, 2000). Although negative selection in the thymus usually eliminates such T cells, when an autoreactive T cell is activated, it is able to kill autoreactive B cells through the binding of Fas-L to Fas (CD95) on the B cell surface. In the absence of normal co-stimulatory signals the autoreactive B cells show enhanced sensitivity to apoptosis following Fas-L/Fas interactions. Indeed, humans and mice that are deficient in Fas, or Fas-ligand, develop severe autoimmune disease associated with the overproduction of lymphocytes (Ada, *et al.*, 1997; Sigel, *et al.*, 2000). B cells bearing antigen receptors for self-antigens, developed as a result of somatic hypermutation, may also undergo Fas-dependent apoptosis within a few hours if they encounter soluble antigen in the absence of T cell co-stimulatory signals (Alt, *et al.*, 1997). Indeed, if there is no co-stimulatory help at the time of antigen encounter, the antigen is likely to be ignored by the immune system.

In addition, immune-complexes consisting of antigen and specific antibody can provide a feedback mechanism for the inhibition of B cells through co-

engagement of the low affinity receptor for IgG, FcγRIIb. In a normal immune response co-ligation of FcγRIIb acts to uncouple antigen-receptor signalling and thus, acts to suppress B cell activation and the production of antibodies. Thus self-antigen immune complexes should act to inhibit autoreactive B cell activation via the BCR through the co-ligation of FcγRIIb.

The mechanisms leading to the breakdown in immune tolerance and self-recognition are many and varied. These can include genetic susceptibility factors which can predispose an individual to autoimmunity, such as the association of specific MHC class II alleles with insulin-dependent diabetes mellitus and rheumatoid arthritis. An autoimmune response may also be directed at a cross-reactive microbial antigen which mimics a self-antigen. For example, the initiation of Lyme arthritis is thought to be due to infection with the spirochete *Borrelia burgdorferi* which shares an antigenic determinant in its outer surface protein with the leukocyte function associated antigen 1 (LFA-1) (Regner and Lambert, 2001). Similarly, aberrant presentation of self-antigens may also result in activation of otherwise "silent" autoreactive lymphocytes. Thus, disease exacerbation of systemic lupus erythematosus occurs when tissue damage results in the release of cellular components not normally in immune circulation, thus promoting an immune response (section **5.1.1**). Autoantibodies may also arise due to random mutations in the antigen-receptor genes of B cells during a normal immune response. The new self-reactive B cell clones may be selected for by mutant self-reactive T cells concomitantly binding to self-epitopes. The produced autoantibodies may then bind to surface receptors and stimulate or block normal cellular responses.

The propensity of B-1 cells to produce low-affinity autoreactive antibodies implicates a role for these cells in autoimmune disease. (reviewed by Hayakawa and Hardy, 2000). The restricted diversity of B-1 cell antigen-receptors helps provide a population of antibodies of degenerate specificity that are able to bind to common bacterial antigens and mimic self-structures. Indeed, B-1 cell populations that produce autoantibodies are expanded in autoimmune disease models such as NZB and motheaten, SHP-1 deficient, mice and elevated levels

of B-1a cells have been found in a proportion of patients with rheumatoid arthritis and systemic lupus erythematosus (Porakishvili *et al.*, 2001). A final model for the breakdown of immune tolerance proposes that suppressive regulatory T cells, responsible for inhibiting self-reactive T or B cells, are somehow deleted thus permitting activation by self-antigens (Maloy and Powrie, 2001). However, the cellular mechanisms responsible for this model are, as yet, unclear.

4.1.2 Immune-complex formation and autoimmunity

Immune complexes are formed whenever there is an antibody response to antigen. Normally these complexes are cleared efficiently by Fc and complement receptor bearing cells of the phagocytic and red blood cell system, such that they cause little tissue damage. However, this system can fail in three circumstances (reviewed by Ada *et al.*, 1997). If high levels of antigen are introduced into the system, this can lead to the formation of large amounts of immune complexes that can overwhelm the normal clearance response. This is exemplified in the case of serum protein injections, which can result in a hypersensitivity reaction known as serum sickness. The immune-complex-mediated reaction is initiated by the development of antibodies to the injected foreign protein. The antigen-antibody complexes are deposited in small blood vessels and subsequently bind and activate Fc and complement receptor bearing effector cells. Fever and symptoms of nephritis and arthritis are induced. However, these effects are transient and resolve when the foreign protein is cleared. Similarly, chronic bacterial infection, where there is a continual strong antibody response, results in the deposition of immune complexes in the small blood vessels of organs such as the kidney and the skin. Tissue injury occurs through the activation of complement and the recruitment of inflammatory cells. Finally, the immune system may suffer from an inherent inability to clear immune complexes, as seen in diseases such as systemic lupus erythematosus. This immune complex driven autoimmune disease, will be discussed in more detail in later sections and **Chapter 5**.

4.1.3 The role of FcγRs in autoimmune disease

The cellular responses to, and the effective clearance of, immune complexes not only depends on the immunoglobulin subtype(s) involved but also on the repertoire of immunoglobulin-binding receptors expressed. Studies in mice deficient in either Fc or complement receptors have revealed distinct functions for these two classes of molecules in the immune response to antigen (reviewed by Ravetch and Clynes, 1998; Heyman, 2000). Results have indicated that immune complex binding by complement is essential for innate immunity against microbial pathogens, whereas FcγRs have emerged as the essential component for coupling antigen specific IgG antibodies to effector cells to trigger inflammatory responses.

Murine models of inflammatory disease have supported the concept that a balance between stimulatory and inhibitory FcγRs, is a determinant of the susceptibility to, and severity of disease (Clynes *et al.,* 1999; Heyman, 2000, Dijstelbloem *et al.,* 2001). This is because the initiation and propagation of an inflammatory response results, in part, from the interaction of immune complexes and antibodies with ITAM containing IgG Fc receptors, such as FcγRIIa. In general, activation and inhibitory FcγRs are co-expressed on the same cell. Their co-expression provides a physiologically important mechanism for setting the threshold for activating stimuli, because the IgG ligand will co-engage both receptors. The ratio of expression of these two opposing signalling systems will directly influence the signalling pathways engaged and determine the cellular response of the expressing cell.

Support for the role of FcγRs in immune complex-mediated disease comes from mice with targeted gene deletion of the γ-chain, such that they do not express the activating FcγRs, FcγRI and FcγRIIIa (Clynes *et al.,* 1998). Deletion of these FcγRs protects the mice from spontaneous or induced antibody-mediated autoimmune disease without altering the production of autoantibodies. This absence of disease induction is thought to be due to a lack of FcγR-mediated phagocytosis resulting in the subsequent lack of initiation of type II and type III

hypersensitivity antibody-mediated reactions. These include Arthus' reactions, autoimmune glomerulonephritis and experimental antigen-induced arthritis (Sylvestre and Ravetch, 1994; Clynes and Ravetch, 1995, Ravetch and Clynes, 1998; Clynes *et al.,* 2000).

In contrast, disruption of the inhibitory FcγR, FcγRIIb, by gene targeting has a pro-inflammatory effect. FcγRIIb deficient mice have augmented humoral (type II and type III) hypersensitivity responses and anaphylactic (type I) responses (Takai *et al.,* 1996). In studies of inflammatory disease, these mice display high levels of antigen specific serum IgG in response to thymus-dependent and thymus-independent antigens, with an increased incidence of autoimmune disease (Takai *et al.,* 1996). Furthermore, studies have demonstrated that in specific strains, FcγRIIb -/- mice are able to develop an autoimmune syndrome resulting in the production of autoantibodies, accelerated antibody-induced glomerulonephritis and immune complex mediated alveolitis (Suzuki *et al.,* 1998; Clynes *et al.,* 1999; Nakamura *et al.,* 2000).

Relevant to this, studies of the FcγRIIb promoter region in strains of autoimmune-prone mice, have highlighted that deletions in this area are associated with reduced surface expression and function of FcγRIIb on macrophages and B cells (Luan *et al.,* 1996; Pritchard *et al.,* 2000). Indeed, studies in disease-prone mice have shown a correlation between FcγRIIb promoter polymorphisms and the subsequent down-regulation of surface expression with the promotion of autoimmune disease (Jiang, 2000). The same group also found that antigen stimulation of germinal-centre B cells resulted in reduced surface expression of FcγRIIb and a concomitant up-regulation of IgG antibody responses (Jiang, 1999). Bone marrow transfer experiments have also suggested that the autoimmune phenotype displayed by certain strains of FcγRIIb -/- mice, is dependent upon FcγRIIb deficiency in B cells but not in macrophages (Bolland and Ravetch, 2000). This suggests that the absence of FcγRIIb mediated inhibitory signals in B cells leads to a spontaneous induction

of autoimmune disease in these mice. Indeed, FcγRIIb may prevent autoimmune disease by immune complex-mediated induction of B cell tolerance.

Overall, these data appear to support a role for FcγRIIb in maintaining immune tolerance and preventing autoimmune disease. Moreover, they imply that FcγRIIb may function *in vivo* to suppress autoimmunity by regulating both B cell and effector cell activation. Hence, lesions in the expression or signalling of FcγRIIb receptors may contribute to the development of B cell-mediated autoimmune disease. Preliminary studies into the modulation of FcγR signalling represent an exciting potential therapeutic strategy into the treatment of human autoimmune disease for which no effective treatment currently exists (see **Chapter 5**). Strategies based on the downregulation of activating FcγRs and the upregulation of inhibitory FcγRs, like FcγRIIb, may represent a realistic approach to ameliorating immune complex driven autoimmune disease.

Therapeutic mechanisms involving the modulation of FcγRIIb expression on B cells must also account for the two alternatively spliced isoforms, FcγRIIb1 and FcγRIIb2. Both isoforms possess an inhibitory ITIM motif in their cytoplasmic domains and are able to mediate negative feedback regulation of ITAM containing receptors. However, whilst FcγRIIb2 is capable of mediating rapid IgG endocytosis, a sequence insertion in the cytoplasmic tail of FcγRIIb1 disrupts the cytoskeletal attachment domain responsible for modulating receptor internalisation. Therefore, differences in the relative expression levels of the two isoforms of FcγRIIb may influence the regulation of B cell tolerance by immune-complexes and an individuals overall risk of developing autoimmune disease.

With a wealth of data supporting a potential role for FcγRIIb in the prevention of autoimmune disease, we decided to investigate whether abnormal FcγRIIb signalling or isoform expression in B cells contributes to the generation of disease in established autoimmune diseases models. In particular, we investigated its regulatory role in the collagen-induced arthritis (CIA) and the systemic lupus erythematosus (SLE, MRL-*lpr/lpr*) murine models.

4.1.4 Collagen-induced arthritis (CIA)

Experimental models, which imitate human rheumatoid arthritis (RA) (section **5.1.2**), have been characterised as a means to understanding disease pathophysiology and identifying new strategies for treatment. The two animal models that have been studied in the greatest depth are the adjuvant arthritis (AA) model in rats and the collagen-induced arthritis (CIA) model in mice. The AA model relies on the induction of an immune response against a bacterial antigen whereas in CIA an autoimmune response is raised against a major component of articular cartilage. However, as CIA has histopathological features in common with rheumatoid arthritis (Durie, *et al.,* 1994; Staines and Wooley, 1994) it is considered the preferred model of investigation and has been widely used for the evaluation of potential anti-rheumatic agents.

CIA is a chronic inflammatory polyarthritis, characterised by hyperplasia of the synovium, mononuclear infiltration of the synovial tissue, osteolysis and eventual destruction of joint cartilage (Stuart, *et al.,* 1988). The development of the disease is highly influenced by MHC genes (Wooley, *et al.,* 1981) with additional roles implicated for non-MHC genes (Watson and Townes, 1985). CIA can be induced in susceptible strains of mice which express the major histocompatibility complex (MHC) class II $H\text{-}2^q$ or $H\text{-}2^r$ haplotype, by the intradermal injection of native type II collagen (CII) in complete Freund's adjuvant (CFA) (Trentham, *et al.*, 1977). The $H\text{-}2^q$ haplotype codes for a MHC class II molecule that binds the immunodominant CII peptide region, whilst the $H\text{-}2^r$ haplotype confers susceptibility to CIA when bovine or porcine CII is used.

The essential role of this susceptibility factor is exemplified by the DBA mouse strains, where DBA/1 ($H\text{-}2^q$) mice are susceptible to CIA whilst the DBA/2 ($H\text{-}2^d$) are not. CII antigen presentation leads to activation of CII-reactive CD4+ T cells and the generation of a T_H1-type immune response, which is crucial for the development of arthritis in this model (reviewed by Luross and Williams, 2001). In addition, a strong B cell response is activated in CIA, producing complement

fixing IgG autoantibodies directed towards CII-specific structures. B cells have been shown to be crucial for the development of CIA, as B cell deficient mice of a susceptible background do not develop disease (Svensson *et al.*, 1998). Importantly, mice with MHC class II haplotypes, normally associated with resistance to CIA (*H-2^b*), become susceptible to autoimmune arthritis when FcγRIIb is deleted (Yuasa *et al.*, 1999).

4.1.5 MRL-*lpr/lpr*

Several animal models have aided the investigation into the pathogenesis of systemic lupus erythematosus (SLE) (reviewed by Theofilopoulos and Dixon, 1985; Cohen and Eisenberg, 1991 and section **5.1.1**). Two well characterised models are the F_1 hybrid of New Zealand black (NZB) (*H-2^d*) and New Zealand white (NZW) (*H-2^z*) mice and the MRL mouse (reviewed by Vyse and Kotzin, 1998). Independently, NZB mice develop autoantibodies and haemolytic anaemia and NZW a mild, late onset of autoimmune nephritis. However, the (NZW x NZB)F_1 results in the development of lupus-like disease and a more severe autoimmune phenotype than that predicted by the additive effect of the parental phenotypes. A minimum of three genetic loci are required for full autoimmune glomerulonephritis in the (NZW x NZB)F_1, two from NZB and one from NZW mice. The heterozygosity is associated with enhanced production of pathogenic IgG autoantibodies to double-stranded (ds) DNA and chromatin.

MRL mice are a mixture of LG/J , AKR/J and C3H/Di strains with *H-2^k* backgrounds. The MRL mice most frequently studied also carry a single gene mutation that accelerates lupus-like disease, either homozygosity for the *lymphoproliferation (lpr)* mutation in the Fas or the *gld* mutation in the Fas ligand genes. Although the mechanisms by which mutations in *Fas* lead to enhanced autoimmunity are unknown, the consensus is that self-reactive T and B cells arise when they fail to undergo normal apoptosis.

However, no counterparts to the *lpr* and *gld* phenotypes exist in human SLE, and studies have not provided evidence of defects in the Fas or Fas-L homologous genes in human SLE patients. Indicating that there many factors involved in the initiation of lupus-like disease. Therefore, the (NZW x NZB)F_1

and MRL-*lpr/lpr* strains are primarily models for lupus-like glomerulonephritis associated with the production of IgG antibodies towards dsDNA. These autoantibodies mediate nephritis, probably as a result of *in situ* immune complex deposition in the glomerulus. However, genetic studies have demonstrated that these strains also produce autoantibodies against endogenous retroviral glycoproteins, which are implicated in the pathogenesis of murine nephritis (reviewed by Vyse and Kotzin, 1998).

In both human and murine SLE, one susceptibility allele for IgG hypergammaglobulinemia has been mapped to the interval linked to the FcγRII gene on chromosome 1 (Vyse and Kotzin, 1998). Whilst the dysfunction of FcγRIIb has not yet been determined in MRL-*lpr/lpr* mice, FcγRIIb-deficient mice produce high levels of anti-nuclear antibodies of a comparable titre to that found in sera from this well studied autoimmune model (Bolland and Ravetch, 2000). Furthermore, studies in (NZW x NZB)F_1 mice deficient in the γ-chain have demonstrated that the balance of activation and inhibitory FcγR expression on effector cells is important for immune-complex-mediate nephritis (Clynes *et al.*, 1998). Consistent with this, one study on MRL-*lpr/lpr* mice, has examined the effect of administering peptides that bind the constant region of IgG and thus inhibit the subsequent interactions with FcγRs (Marino *et al.*, 2000). These results demonstrated that whilst the peptides had little effect on the serum level of anti-DNA autoantibodies, they had a much more dramatic effect on reducing proteinuria and increasing survival in the mice. These results support the role of FcγRs in SLE pathogenesis whilst indicating a possible divergence in the roles of immune-complex binding receptors and their abilities to mediate autoimmune disease.

4.2 Aims and objectives of this study

The production of autoantibodies in the collagen-induced arthritis (CIA) model implies the involvement of aberrant B cell activation. FcγRIIb is known to play an important role in maintaining normal B cell responses to antigen and thus may be involved in the pathogenesis of CIA. In addition, systemic lupus

erythematosus (SLE) is a classic immune-complex mediated disease that is characterised by the production of autoantibodies against ubiquitous self-antigens. FcγRIIb is the only Fcγ receptor present on B cells that can interact with IgG containing immune-complexes. Therefore, the primary objectives of this study were to investigate whether FcγRIIb-mediated negative regulation of B cell activation becomes defective during onset and progression of disease in CIA and MRL-*lpr/lpr* models. The relative expression levels of the FcγRIIb isoforms on B cells may represent a physiologically important mechanism for setting the threshold of BCR-mediated activation. Thus, the ratio of expression of FcγRIIb1 and FcγRIIb2 may directly influence the modulation of B cell responses by immune-complexes. It was therefore also planned to determine whether B cells from CIA and MRL-*lpr/lpr* mice develop aberrant FcγRIIb isoform mRNA expression during the disease status transition and progression.

Finally, previous studies from this laboratory had shown that an anti-inflammatory molecule, ES-62 could "anergise" B cells. It was therefore planned to investigate whether FcγRIIb mediates, at least in part, the therapeutic effects of this inflammatory molecule, ES-62, in the CIA model.

4.3 Results

4.3.1 The role of FcγRIIb-mediated B cell inhibition in the progression of collagen-induced arthritis (d0-d47).

Genetic studies have suggested a potential role for FcγRIIb in suppressing the induction of collagen-induced disease (CIA) in certain murine strains (Yuasa *et al.*, 1999; Bolland and Ravetch, 2000). To investigate whether abnormal FcγRIIb signalling or isoform expression contributes to the generation of disease in an established murine arthritis model, the integrity of FcγRIIb signalling in *ex vivo* B cell responses during onset and progression of CIA was tested. Susceptible male DBA/1 mice ($H\text{-}2^q$) were injected intradermally with type II collagen (CII) in CFA and a gradual onset of arthritis was observed when challenged intraperitoneally with collagen 21 days later (see **Figure 2.1**). Mice were monitored for a further 5 weeks for signs of arthritis; disease incidence rates, mean clinical score, and paw swelling. Littermates, which had been injected and subsequently challenged with CFA alone, did not develop arthritis. Naïve or CFA alone DBA/1 mice were taken as baseline controls.

4.3.1.1 FcγRIIb co-ligation results in the inhibition of BCR–mediated ErkMAPK phosphorylation in splenic B cells from both CFA control and CIA mice, *ex vivo* (d0-d47).

In order to investigate putative signalling lesions following FcγRIIb ligation on B cells from CFA/CII, CFA control and naïve DBA/1 mice, the effect of FcγRIIb ligation on BCR-signalling was determined. Ligation of the BCR on splenic B cells is known to induce the phosphorylation and activation of the Ras/ErkMAPK pathway (reviewed by Campbell, 1999), which is inhibited by the co-ligation of FcγRIIb (reviewed by Cambier, 1995). Previous DNA synthesis studies in primary B cells by our laboratory had indicated that 48 hours was the optimal timepoint to observe maximal BCR-mediated proliferative and FcγRIIb-mediated inhibitory effects (**Chapter 3**). Thus, the negative regulation of BCR-mediated ErkMAPK signalling by FcγRIIb was investigated in CFA/CII, CFA control and naïve DBA/1 mice. Anti-ErkMAPK immunoprecipitates were prepared from

whole cell lysates of splenic B cells treated with intact or $F(ab')_2$ fragments of anti-mouse IgM/IgG antibody for 48 hours. Western blot analysis was carried out with an antibody specific to the dually phosphorylated (active) form of p44/p42 ErkMAPK, as an indication of ErkMAPK activation.

BCR stimulation of splenic B cells from CFA/CII, CFA control and naïve DBA/1 mice, results in the phosphorylation of ErkMAPK and this is sustained at 48 h (**Figure 4.1**). Interestingly, ErkMAPK phosphorylation, particularly that of p44 Erk, is enhanced in the CFA/CII mice following BCR stimulation. This increased level of ErkMAPK activation, however, could possibly also reflect the collagen-specific co-stimulatory effect of bystander inflammatory cells *in vivo*. However, although co-ligation of FcγRIIb either alone or in combination with mitogenic signalling via the BCR, resulted in reduced levels of ErkMAPK phosphorylation, compared to BCR stimulation a reduction in ErkMAPK activation back to basal control levels is not demonstrated. Indeed, BCR-coupled p42 ErkMAPK is only partially inactivated (~40% inhibition) following intact antibody stimulation of CFA/CII B cells. Furthermore, whilst BCR and FcγRIIb co-ligation abrogated signalling by an average of 67% in CFA control B cells, only an average of 43% inhibition of the BCR-mediated ErkMAPK phosphorylation levels by FcγRIIb, was achieved in CFA/CII B cells. Interestingly, in contrast to the situation in naïve mice, stimulation with intact anti-Ig alone induced a substantial amount of Erk activation at 48 hours, particularly in B cells derived from CFA-treated mice.

Thus, ErkMAPK phosphorylation is differentially inhibited by FcγRIIb in CFA control and CFA/CII mice, but with both types of mice exhibiting preferential inhibition of the p44 form of ErkMAPK. In agreement with published studies on the induction of inflammatory and autoimmune disease in FcγRIIb deficient mice (Takai *et al.*, 1996; Nakamura, A. *et al.*, 2000) these results indicate that FcγRIIb inhibition of BCR-activated ErkMAPK appears to be dysregulated in CFA/CII mice throughout the progression of disease.

4.3.1.2 FcγRIIb co-ligation inhibits the BCR–mediated proliferative response of splenic B cells from both CFA control and CIA mice, *ex vivo* (d0-d47).

CIA is dependent on the dysregulation of both humoral and cell-mediated responses. To determine whether the putative dysregulation of FcγRIIb functionality on B cells observed above reflected uncoupling of negative feedback inhibition of B cell responses in CIA mice through disease onset and progression, the proliferative response of splenic B cells derived from CFA/CII, CFA control and naïve DBA/1 mice on days 0, 7, 23, 34 and 47, was investigated.

Antigenic stimulation of splenic B cells with $F(ab')_2$ fragments of anti-mouse Ig resulted in increased levels of B cell proliferation in naïve, CFA control and CFA/CII primed DBA/1 mice (**Figure 4.2**).The levels of DNA synthesis observed were similar in both CFA primed groups of mice (but greater than those of B cells from naïve DBA/1 mice) throughout the disease onset and progression, until day 47, when particularly enhanced DNA synthesis levels were obtained (**Figure 4.2**). With the exception of day 47, stimulation of FcγRIIb by means of intact antibody alone resulted in a lack of DNA synthesis in all three groups. The increased levels of DNA synthesis on day 47 following coligation of BCR and FcγRIIb were presumably as a result of an increase in the percentage of activated cells and this might perhaps reflect signals generated by CFA-induced inflammation resulting in T cell-derived signals overcoming FcγRIIb-mediated anergy and/or apoptosis.

Co-ligation of the BCR and FcγRIIb in the presence of mitogenic BCR signalling, resulted in similar percentage levels of inhibition of BCR-mediated DNA synthesis in all mice. However, FcγRIIb inhibition of BCR-induced DNA synthesis was slightly reduced in the CFA/CII mice on day 23, 2 days post-collagen (CII) challenge (30% compared to 54% in the CFA control mice). In our laboratory, previous DNA synthesis studies in primary B cells have indicated that FcγRIIb-mediated suppression of BCR-induced DNA synthesis, following co-ligation of the receptors, is approximately 50% (c.f. typical example in **Figure 3.1**). Thus, it was possible that, in agreement with the ErkMAPK results, the

reduction in FcγRIIb-mediated suppression in the CFA/CII mice on day 23 indicated that FcγRIIb-mediated inhibition of B cell proliferation may be dysregulated at the initial onset of acute inflammatory disease.

In light of studies suggesting that FcγRIIb deficiency is a susceptibility factor for inflammatory and autoimmune disease in mice, the finding that FcγRIIb appeared to be functional throughout the remaining disease progression was somewhat surprising. This result implies that a deregulation of the BCR-mediated B cell response by FcγRIIb at the time of antigen re-challenge, could be sufficient to initiate an inflammatory and subsequent autoimmune response.

4.3.1.3 FcγRIIb ligation results in growth-arrest and/or apoptosis of splenic B cells from both CFA control and CIA mice, *ex vivo* (d0-d47).

The above experiment confirmed that BCR-FcγRIIb co-ligation in splenic B cells was unable to induce DNA synthesis in CFA/CII, CFA control and naïve DBA/1 mice and that co-ligation in the presence of mitogenic stimulation via the BCR resulted in the inhibition of BCR-mediated proliferation. However, DNA synthesis studies give no indication as to whether such FcγRIIb-mediated inhibition of DNA synthesis is due to apoptosis, anergy or growth arrest. This is of importance since recent studies have indicated that FcγRIIb ligation can lead to distinct cellular responses of growth arrest and/or apoptosis in B cell lines, dependent on the mechanism of receptor aggregation (Ashman *et al.*, 1996; Ono *et al.*, 1997). Thus, co-ligation of BCR and FcγRIIb with intact anti-Ig antibodies is known to result in lower levels of DNA synthesis than those of control unstimulated B cells reflecting induction of a pro-apoptotic signal. In contrast, co-ligation of FcγRIIb with the BCR during mitogenic signalling via the BCR results in a survival signal and the induction of growth-arrest (Pearse *et al.*, 1999). This finding has important implications for the selection and activation of B cells by immunocomplexes in the development of both normal and autoimmune responses. The mechanisms reflect the differential requirements of the immune system to both dispose of autoreactive naïve cells whilst only suppressing antibody production by activated cells during an ongoing infection. As such a B cell may require re-activation if the immune

system re-encounters antigen. Therefore, to confirm the results obtained in the DNA synthesis experiment, suggesting that FcγRIIb mediated negative feedback regulation is dysregulated in BCR-FcγRIIb co-ligated B cells at disease onset, the levels of FcγRIIb-induced apoptosis were measured.

The levels of apoptosis were determined by measuring the population of cells that expressed sub-diploid DNA, as measured by FACS analysis using propidium iodide (PI) staining. **Figure 4.3** shows that post-sacrifice 70% of splenic B cells from CFA/CII, CFA control and naïve DBA/1 mice are in the G_0/G_1 phase of the cell cycle and the majority of which, if left unstimulated for 48 hours, undergo apoptosis (sub-diploid population) (**Figures 4.3** and **4.4**). Stimulation of the BCR with $F(ab')_2$ fragments of anti-mouse Ig for 48 hours, resulted in increased cell numbers entering into the proliferative S and G_2/M stages in all three groups of mice, whilst, FcγRIIb-co-ligation resulted in enhanced levels of growth-arrest and apoptosis. Importantly, the percentage of cells committed to growth arrest or apoptosis following FcγRIIb co-ligation was shown to be similar in CFA/CII and CFA control mice on day 23. Indeed, levels of apoptosis following co-ligation of BCR-FcγRIIb in CFA/CII B cells were elevated compared to CFA control B cells. Thus, these results confirm the ability of B cells from CIA/CII and CFA control mice to readily undergo apoptosis or growth arrest following FcγRIIb-co-ligation throughout disease onset and progression. Interestingly, the responses of B cells derived from CFA/CII and CFA control mice resulted in enhanced proliferative or apoptotic effects with respect to naïve B cells. Indicating, perhaps, that the exposure of B cells to CFA results in an increase of sensitivity of B cells to both stimulation and inhibition.

To further examine the growth-arrest effects induced by FcγRIIb ligation, the DNA content of the living population of B cells was analysed in naïve (**Figure 4.5**) CFA control and CFA/CII primed DBA/1 mice (**Figure 4.6**). These figures clearly demonstrate that there is no appreciable difference in the percentage of B cells in the distinct stages of the cell cycle, when comparing CFA/CII and CFA control mice. Moreover, that FcγRIIb-mediated inhibition of cell cycle progression appears to be functioning in these cells. Indeed, the percentage of

viable cells in the G_0/G_1 phase of the cell cycle increases from 50%, when stimulated via the BCR, to 75% when FcγRIIb is co-ligated. When the two receptors are co-ligated in the presence of mitogenic signalling via the BCR the levels of growth-arrest are intermediate to these two values, suggesting an integration of signals from the positive and negative regulatory receptors. The discrepancy between the DNA synthesis and cell cycle data, may reflect the differential numbers of rounds of division at day 23 by cells within the population. Such that a small sub-group of cells, for example, collagen-specific B cells, may be responsible for the proliferative response observed in the DNA synthesis assay. Thus, analysis of cell division numbers following FcγRIIb co-ligation may help to explain the contradictory data. However, attempts to ascertain the number of rounds of cell division using the cell-permeable dye, CFSE, were prevented by a lack of dye reduction in actively proliferating cells.

4.3.2 The role of FcγRIIb-mediated B cell inhibition in the secondary immune response to collagen and the onset of collagen-induced arthritis, *ex vivo* (d21-d25).

The regulation of B cell clonal expansion following re-exposure to antigen is important for the production of a rapid and augmented secondary immune response. Whilst contradictory, the results obtained above implicated a potential dysregulation of FcγRIIb-mediated negative feedback regulation corresponding to the onset of a secondary immune response to collagen. To further investigate this putative deregulation of FcγRIIb, the effect of FcγRIIb-ligation on the collagen specific B cell response in CFA/CII and CFA control mice was investigated, during the days immediately pre- and post-collagen challenge (d21-d25).

4.3.2.1 FcγRIIb co-ligation modulates BCR–mediated ErkMAPK phosphorylation in splenic B cells from CFA control and CIA mice, *ex vivo* (d21-d25).

To determine whether the negative regulation of BCR-mediated ErkMAPK signalling by FcγRIIb was functional in CFA/CII and CFA control mice during the period of collagen re-challenge, the phosphorylation status of ErkMAPK

following FcγRIIb ligation or co-ligation with the BCR was investigated. Western blot analysis of anti-ErkMAPK immunoprecipitates from *ex vivo* stimulated B cells demonstrated that stimulation of the BCR with $F(ab')_2$ fragments of anti-IgM or collagen resulted in enhanced phosphorylation of ErkMAPK in CFA/CII mice (**Figure 4.7**). Similar to the stimulation with $F(ab')_2$ fragments, exposure to collagen stimulated ErkMAPK with preferential activation of the p42 form of ErkMAPK. Similar to the ErkMAPK results previously shown in **Figure 4.1**, FcγRIIb co-ligation with the BCR on CFA/CII B cells resulted in a small reduction of p44 ErkMAPK phosphorylation levels. Interestingly, stimulation with collagen or $F(ab')_2$ fragments of anti-Ig antibodies demonstrated differential ErkMAPK phosphorylation profiles during the period of investigation. Collagen stimulation promoted a strong ErkMAPK response in d21 cells, correlating with the time of collagen challenge, which returned to basal levels over time. In contrast, $F(ab')_2$ stimulation promoted a maximal ErkMAPK response on day 23 which remained above basal levels for the remainder of the time period. Importantly, FcγRIIb-mediated negative feedback regulation of ErkMAPK appeared to be functional on days 23-25, but inoperative on days 21-22. These data suggest that deregulation of FcγRIIb-mediated abrogation of ErkMAPK signalling may occur at the time of collagen challenge and could promote aberrant B cell responses.

4.3.2.2 FcγRIIb co-ligation inhibits the BCR–mediated proliferative secondary response of splenic B cells, *ex vivo* (d21-d25).

Similar to the results obtained in **4.3.1.2**, stimulation of the BCR with $F(ab')_2$ fragments of anti-mouse IgM antibody resulted in proliferation of splenic B cells from both CFA/CII and CFA mice (**Figure 4.8**). Moreover, this proliferative response could be inhibited in both groups of mice following FcγRIIb-co-ligation with the intact form of the antibody. Interestingly, the B cells appeared to be rather anergic to BCR-induced stimulation in the days corresponding to the onset of acute disease in the CFA/CII mice. In particular, the B cells appeared to be unresponsive to stimulation or inhibition on days 24 and 25. However, since the unresponsiveness is also demonstrated, albeit to a lesser degree by the CFA mice, it suggests an inherent problem with the experimental procedure,

as opposed to a suppressed immunological response. Importantly, no differences in the levels of FcγRIIb–mediated inhibition of BCR-induced proliferation were seen between the CFA/CII and CFA control mice, even on day 23 as previously observed in **4.3.1.2.**

Interestingly, although a low level collagen-specific B cell response was expected in the CFA control mice, B cells from both CFA/CII and CFA mice proliferated poorly when cultured in the presence of collagen (**Figure 4.9**). However, in the pre-exposed CFA/CII mice the poor proliferative response may be reflective of the low percentage of collagen-specific B cells in the purified splenic population. Thus the *in vitro* environment may also lack the co-stimulatory signals that would be necessary for a full immunological response to collagen *in vivo*. Whilst the lack of a collagen-induced response makes the determination of whether FcγRIIb is actively inhibiting the collagen-specific B cell population difficult, the DNA synthesis results following collagen-FcγRIIb co-stimulation suggest that FcγRIIb-mediated inhibition could be dysfunctional on days 21-23. However, the low percentage of inhibition is similar in both groups of mice, suggesting a lack of collagen-specificity and perhaps reflects the breaking of naïve B cell unresponsiveness by inflammatory stimuli discussed above.

4.3.2.3 FcγRIIb ligation induces growth-arrest and/or apoptosis of splenic B cells from CFA control and CIA mice, *ex vivo* (d21-d25).

The FcγRIIb-mediated growth arrest observed in **4.3.2.2** was confirmed by DNA content analysis. However, whilst data showed that FcγRIIb- co-ligation with the BCR results in the accumulation of cells in the G_0/G_1 phase of the cell cycle, (**Figure 4.10**) enhanced G_0/G_1 levels were also demonstrated by BCR and collagen stimulated cells on days 24 and 25. This result is supported following re-examination of the viable population of cells in **Figure 4.11**. These results show a correlation between the poor proliferative response following antigen receptor ligation or collagen stimulation in **4.3.2.2**, with a reduction in the number of cells entering S phase. Furthermore, these studies show no

differences in the commitment to growth-arrest or apoptosis between the two groups of mice following FcγRIIb- co-ligation with the BCR.

4.3.3 The role of FcγRIIb-mediated inhibition of peripheral lymphoid tissue mononuclear cells in the progression of collagen-induced arthritis, *ex vivo* (d8-d34).

The peripheral lymphoid organs are organised tissues where lymphocytes engage in important interactions with non-lymphoid cells for the initiation of an adaptive immune response. Although there are structural and anatomical differences, induction of immune responses in lymph nodes and the spleen occur by essentially similar mechanisms. Phagocytic and antigen-presenting cells bearing antigens are carried to the draining lymph nodes by the lymphatic system. Trapped in the nodes, naive B cells are localised into follicles and undergo intense proliferation after encountering the displayed specific antigen and helper T cells. Once antigen-specific B cells have undergone proliferation and differentiation they leave the lymph nodes and enter into circulation as effector cells. Whilst the organisation of the spleen is similar to that of the lymph node, antigen enters the spleen from the blood rather than from the lymph. The spleen is a larger lymphoid organ that also engages in the destruction of damaged red blood cells and thus the majority of the structure is dedicated to disposal of these cells.

In the spleen and lymph nodes, the distinct populations of cells are carefully regulated. A balance is required between the continuous supply of cells from the primary lymphoid tissues and their proliferation, terminal differentiation and death, in response to antigen. One method by which this achieved is through the expression of negative regulatory receptors, such as FcγRIIb, by B cells and effector cells. Co-ligation of this receptor with the B cell antigen receptor or with other ITAM-containing receptors can lead to inhibition of cellular responses. Thus, lesions in the expression or signalling of FcγRIIb may contribute to dysregulation of lymphocyte and other non-lymphoid cell responses and the development of inflammatory autoimmune disease. Indeed, published studies appear to support a role for FcγRIIb *in vivo* to suppress autoimmunity by

regulating both B cell and non-lymphoid cell activation (Suzuki *et al.*, 1998; Clynes *et al.*, 1999; Pearse *et al.*, 1999; Heyman, 2000; Nakamura, A. *et al.*, 2000).

In contrast, previous results in this study, from DNA synthesis and cell-cycle analysis, seem to indicate that FcγRIIb-mediated regulation of splenic B cells in CIA mice is intact and functional. In order to investigate the role of FcγRIIb-mediated inhibition of both non- and lymphoid cells in the peripheral lymphoid organs, throughout CIA disease onset and progression, the *ex vivo* cellular responses of whole spleen and nodal populations to FcγRIIb ligation were investigated. This method allows us to rule out the possible selective nature of the B cell purification process relating to distinct B cell sub-populations in the previous studies and to determine whether co-stimulatory signals from accessory cells are necessary for full FcγRIIb-mediated inhibition of a collagen-induced immunological response *ex vivo*. This method also permits an investigation of the effect of FcγIIb induced growth-arrest or apoptosis by ligation or co-ligation of the receptor in two distinct sites of antigen encounter where antigen regulation of B cells is of primary importance for their proliferation and differentiation.

Spleens and lymph nodes were obtained post-sacrifice from CFA/CII and CFA control mice at stages of pre-disease and during disease onset and progression. The following study first examines the effect of FcγRIIb ligation on the antibody-mediated proliferative response of splenic populations before looking at the nodal population responses.

4.3.3.1 FcγRIIb co-ligation inhibits the BCR–mediated proliferative response of splenic mononuclear cells from CFA control and CIA mice, *ex vivo* (d8-d34).

Using DNA synthesis assays as an indication of proliferation, **Figure 4.12** shows that stimulation of splenic cells with $F(ab')_2$ fragments of anti-IgM results in enhanced DNA synthesis in both groups of mice. This proliferative response

could be inhibited in both groups of mice following FcγRIIb-co-ligation with the intact form of the antibody. Similar to the results shown in (**Figures 4.8 and 4.9**), the splenic cells were less responsive to BCR-induced stimulation as the disease progressed. However, in contrast to that observed with purified B cells, these results indicate that intact antibody alone actually stimulates low levels of cell proliferation, possibly through the co-activation of effector cells bearing ITAM-containing Fc receptors.

4.3.3.2 FcγRIIb co-ligation modulates the collagen-induced proliferative response of splenic mononuclear cells from CFA control and CIA mice, *ex vivo* (d8-d34).

Similar to the results obtained in **Figure 4.9**, stimulation with collagen produced very little proliferative response thus confirming the low percentage of collagen-specific cells in the splenic population (**Figure 4.13**). Due to the lack of a collagen-induced response it is difficult to determine whether the intact antibody and thus, FcγRIIb, is actively inhibiting proliferation of the collagen-specific cell population. Indeed, due to the ability of intact antibody alone to slightly enhance proliferation of the splenic population, co-stimulation with collagen results in an enhancement of the collagen initiated response.

4.3.3.3 FcγRIIb ligation induces growth-arrest and/or apoptosis in splenic mononuclear cells from CFA control and CIA mice, *ex vivo* (d8-d34).

To confirm the induction of FcγRIIb-mediated growth-arrest of the splenic cells, DNA content was analysed. **Figures 4.14** and **4.17** show that BCR stimulation of the splenic population from naïve DBA/1 mice results in increased levels of cells entering the proliferative S and G_2/M phases of the cycle. In contrast, BCR-FcγRIIb co-ligation results in the accumulation of cells in the G_0/G_1 phase of the cell cycle and blocks entry into the S phase. Ligation of FcγRIIb did not appear to modulate the cell cycle of the naïve cells co-stimulated with collagen. FcγRIIb co-ligation was shown to inhibit cell cycle progression and induce growth-arrest and/or apoptosis in both BCR (**Figures 4.15** and **4.18**) and

collagen (**Figures 4.16** and **4.19**) stimulated splenic cells from both CFA control and CIA mice. Interestingly, in BCR stimulated cells FcγRIIb co-ligation enhanced both growth-arrest and apoptosis levels whilst in collagen stimulated cells, FcγRIIb co-stimulation appeared to modulate apoptosis levels only. The differential response to FcγRIIb co-ligation in the two stimulations may be due to the fact $F(ab')_2$ anti-Ig stimulation will provide a positive survival signal to all the cells, whereas collagen stimulation will promote survival in only a small percentage of the cells.

4.3.3.4 Effect of FcγRIIb co-ligation on the mitochondrial membrane potential of splenic mononuclear cells from CFA control and CIA mice, *ex vivo* (d8-d34).

In many cells, one of the consequences of the commitment to apoptosis is the disruption of mitochondrial function and depolarisation of the mitochondrial membrane potential ($\Delta\psi_m$). This effect can be investigated in cells by the incorporation of the cationic lipophilic dye, $DiOC_6(3)$, where uptake of the dye is directly proportional to the $\Delta\psi_m$. To investigate whether FcγRIIb-induced apoptosis in splenic mononuclear cells correlated with a reduction in $\Delta\psi_m$, BCR, FcγRIIb and collagen stimulated cells from CFA/CII and CFA control mice were loaded with $DiOC_6(3)$ and subjected to FACS analysis. **Figures 4.20 and 4.21** clearly demonstrate that stimulation of the BCR results in a stabilisation of the mitochondria and a high $\Delta\psi_m$ in splenic mononuclear cells from all groups of mice. In contrast, co-ligation of FcγRIIb on the splenic cells with intact anti-IgM/IgG antibodies induced a decrease in $\Delta\psi_m$. Indeed, this decrease was more profound in cells stimulated with intact alone, whereas co-ligation with the BCR (**Figures 4.21** and **4.22**) in the presence of a mitogenic stimulus via the BCR produced an intermediate $\Delta\psi_m$. In agreement with the DNA synthesis and cell cycle analysis results, collagen stimulation resulted in minimal stabilisation of the mitochondrial membrane potential which was partially modulated following FcγRIIb ligation (**Figure 4.22**). Thus, the FcγRIIb-mediated mitochondrial depolarisation in splenic mononuclear cells correlates with the commitment and induction of apoptosis. Importantly, in agreement with the DNA synthesis and

content analysis data, no differences between CFA/CII and CFA control mice were observed.

4.3.3.5 FcγRIIb co-ligation inhibits the BCR–mediated proliferative response of mononuclear cells from lymph nodes of CFA control and CIA mice, *ex vivo* (d8-d34).

Inguinal and popliteal draining lymph nodes from CFA/II and CFA control mice were obtained at stages of pre-disease and during CIA onset and progression (days 8, 23 and 34 after the primary immunisation). The overall proliferative response of the cells to stimulation with $F(ab')_2$ fragments of anti-mouse Ig was reduced in comparison to that of the splenocyte population (**Figure 4.23**). This difference can presumably be explained by the low percentage of B cells in the lymph node compared to in the spleen (nodes, 20%; spleen 45%, results not shown). During disease progression, the response to proliferative and inhibitory stimuli was also reduced in the nodal population. Despite the reduced proliferative response, FcγRIIb-ligation resulted in the inhibition of the BCR-mediated proliferative response by nodal mononuclear cells from both CFA control and CIA mice.

4.3.3.6 FcγRIIb co-ligation modulates the collagen-induced proliferative response of mononuclear cells from lymph nodes of CFA control and CIA mice, *ex vivo* (d8-d34).

Similar to the results obtained for the splenic population, the collagen-induced proliferative response of the nodal population was minimal (**Figure 4.24**) and stimulation with intact antibody alone resulted in enhanced DNA synthesis. Co-stimulation of the nodal populations from both groups of mice, with collagen and intact antibody, did not inhibit the collagen-induced proliferative response. This lack of inhibition is presumably not due to deregulation of FcγRIIb-mediated inhibitory effects, but is simply a reflection of the stimulatory effect of intact antibody alone. Furthermore, the co-stimulation also resulted in enhanced DNA synthesis in the CFA mice on day 8.

4.3.3.7 FcγRIIb ligation induces growth-arrest and/or apoptosis in mononuclear cells from lymph nodes of CFA control and CIA mice, *ex vivo* (d8-d34).

FcγRIIb-mediated growth-arrest and/or apoptosis of nodal B cells was confirmed by DNA content analysis of the cells after 48 hours stimulation, *ex vivo*. **Figure 4.25** demonstrates that pre-disease onset, FcγRIIb co-stimulation prevents the BCR-mediated S phase transition of nodal mononuclear cells and promotes G_0/G_1 arrest (**Figures 4.26**) and the commitment to apoptosis. However, at later stages of disease progression, FcγRIIb co-ligation does not modulate the BCR-mediated cell cycle progression. This is accounted for by the minimal $S/G_2/M$ phase progression observed following BCR stimulation, when compared to control basal levels. The discrepancy of the results with the DNA synthesis data, may, once again, reflect differential numbers of rounds of division by the population of cells.

4.3.3.8 Effects of FcγRIIb co-ligation on the mitochondrial membrane potential of mononuclear cells from lymph nodes of CFA control and CIA mice, *ex vivo* (d8-d34).

In agreement with the DNA synthesis and content data, **Figure 4.27** demonstrates that BCR stimulation results in minimal stabilisation of $\Delta\psi_m$ in the nodal mononuclear cells. Interestingly, whilst co-ligation of FcγRIIb induces a substantial decrease in $\Delta\psi_m$ in nodal mononuclear cells from CFA/CII mice, the FcγRIIb induced decrease in $\Delta\psi_m$ of nodal mononuclear cells from CFA control mice is less pronounced as BCR-stimulation does not appear to protect the cells. This differential mitochondrial response to FcγRIIb ligation between the two groups perhaps indicates that nodal cells from CIA mice are more responsive to apoptotic signals than mice primed with CFA alone.

4.3.3.9 FcγRIIb1 and FcγRIIb2 mRNA expression levels in splenic B cells from CFA control and CIA mice (d21-d25).

As discussed in section **3.2.16**, FcγRIIb is expressed on B cells as two differentially-spliced isoforms, FcγRIIb1 and FcγRIIb2. These isoforms appear to

be identical except for an amino acid insert in the cytoplasmic domain of FcγRIIb1, which abolishes the endocytic capacity of the receptor. Importantly, B cell expression levels of the two isoforms are thought to be modulated in response to activation by crosslinking of the BCR or culture with cytokines (Gergely, *et al.*, 1994). Thus, the relative expression levels of the different isoforms may be involved in the regulation of B cell response to antigen as downregulation of the endocytosis-competent isoform would prevent the interaction between antigen-specific B and T cells and hence suppress T cell-mediated help. To quantify the relative expression levels of the two FcγRIIb isoforms RT-PCR was performed. Total RNA was extracted from purified, unstimulated splenic B cells from CFA/CII and CFA control mice during the days immediately pre- and post-collagen challenge. The RNA was reversed transcribed and the relative expression levels of the isoform transcripts were determined by quantitative TaqMan analysis.

The levels of FcγRIIb1 mRNA detected were higher than that of FcγRIIb2 in both groups of mice throughout the period of study (**Figure 4.28**). Interestingly, Gergely *et al.*, 1994 have shown that in human B cells the expression levels of FcγRIIb2 greatly exceed that of FcγRIIb1 but activation by antigen-receptor stimulation induces alternative splicing of FcγRIIb1 and simultaneous suppression of FcγRIIb2 mRNA. However, in both groups of mice the FcγRIIb1 message levels remain relatively constant through the period of disease onset, whilst FcγRIIb2 is enhanced in CFA/CII mice following the second collagen challenge. These preliminary results suggest that FcγRIIb1 may be constantly expressed by murine B cells and predominantly mediate the negative regulation of B cell activation, whilst FcγRIIb2 expression is modulated depending on the availability of antigen and possibly influences the presentation of antigen by B cells. However, since these results are preliminary, the fluctuations observed may represent variation inherent within the assay. Thus, only through repeat experiments and the examination of surface expression of the two isoforms could this hypothesis be confirmed.

4.3.4 Modulation of the immune response by a filarial nematode excretory-secretory product (ES-62).

Filarial nematodes are a large group of arthropod-transmitted vertebrate parasites that mediate lifelong infection. Moreover, individual parasites are capable of surviving in the host for in excess of five years. They are known to cause a range of debilitating diseases known collectively as filariasis, which includes lymphatic filariasis and onchocerciasis (WHO, 1987). Clinical manifestations of the disease can include blindness, swollen limbs, genitals and severe damage to the kidneys and lymphatic system, much of this pathology resulting as a consequence of the host immune response. The general success of the parasites, however, reflects the fact that the majority of infected individuals exhibit much less severe symptoms as a consequence of their ability to modulate or suppress the host immune response. Indeed, filarial infection has been demonstrated to modulate both parasite-specific and general immune cell mediated responses. (Nutman *et al.,* 1987; Ottesen, 1984; Kwa and Mak, 1984; Haque and Capron, 1986; King and Nutman, 1991; Maizels and Lawrence, 1991).

Despite numerous investigative studies the mechanisms of modulation are, as yet, undetermined. However, molecules released by the adult nematode during infection have been implicated as a causative factor for lymphocyte hyporesponsiveness (Weiss, 1978; Soboslay, *et al.,* 1991). Work by this laboratory has previously shown that ES-62, a homologue of phosphorylcholine (PC)-containing glycoproteins secreted by human parasites, is the active inhibitory molecule secreted by the rodent filarial parasite *Acanthocheilonema viteae.* Infection by this parasite leads to the targeting and subversion of the responses of a number of cells of the immune system including T and B cells dendritic cells and macrophages. For example, exposure to ES-62 not only renders B and T cells anergic to subsequent stimulation via the antigen receptors but also polarises immune responses to a T_H2 phenotype by inhibiting pro-inflammatory cytokine production and driving differentiation of DCs to a DC2, T_H2-promoting phenotype (reviewed by Harnett and Harnett, 1999).

Extensive studies in lymphocytes have shown that ES-62 mediates its anti-proliferative effects by uncoupling the antigen receptors from key downstream proliferative signalling pathways. In particular, by selectively downregulating certain PKC isoforms and by desensitising protein tyrosine kinase-dependant coupling of the antigen receptors to PI-3-kinase and the Ras/ErkMAPKinase signalling cascades (**Figure 4.29**). Thus, this uncoupling results in a novel mechanism by which ES-62 can inhibit antigen receptor-mediated proliferative responses and subvert the normal immune response of the host.

Similarities between the inhibitory signalling mechanisms of ES-62 and FcγRIIb on B cells suggested that they could share similar effector pathways. Specifically, ES-62 has been shown to desensitise the BCR-mediated activation of ErkMAPK by the recruitment of the tyrosine phosphatase SHP-1 and the MAPKinase phosphatase, Pac-1 (Deehan *et al.*, 2001) (**Figure 4.30**). Moreover, the ability of ES-62 to initiate a polarised T_H2 response may represent a novel therapeutic strategy for the treatment of inflammatory-type autoimmune diseases, since the generation of a T_H1-type immune response by collagen-specific $CD4^+$ T cells, is crucial for the development of arthritis in the CIA model. Thus, the ability of ES-62 to suppress B cell activation and antibody production was of interest when investigating the role of FcγRIIb in the regulation of CIA.

4.3.4.1 Effect of *in vivo* exposure to ES-62 on the FcγRIIb inhibition of BCR-mediated proliferative responses of nodal mononuclear and splenic B cells, *ex vivo*.

To determine whether ES-62 subverts the negative regulation of B cells by FcγRIIb, the antibody-mediated proliferative responses of nodal mononuclear and splenic B cells, pre-exposed to ES-62 *in vivo*, were investigated. Through the use of osmotic pumps to deliver ES-62, the constant release of the parasite product during infection is mimicked efficiently *in vivo*, whilst minimising animal handling and stress which may influence the overall immune response (Goodridge *et al.*, 2001). **Figure 4.31** demonstrates that proliferation of mononuclear cells from the draining lymph nodes and B cells from the spleens of ES-62 treated mice, in response to anti-mouse Ig stimulation, is suppressed in comparison to the control mice treated with PBS. Furthermore, this suppression is shown to be dose-dependent. Importantly, FcγRIIb mediated inhibition of B cell proliferation is shown to be functional at all concentrations of ES-62 treatment, resulting in enhanced suppression of the BCR-mediated proliferative response.

4.3.4.2 Collagen-induced Arthritis/ES-62 prophylactic model

The generation of a T_H1-type immune response by collagen-specific CD4+ T cells, is thought to be essential for the development of arthritis in the CIA model. The ability of ES-62 to bias the immune system in favour of an anti-inflammatory T_H2 response has prompted the investigation into the use of ES-62 as a possible therapeutic candidate for the treatment of arthritis. A prophylactic model for the prevention of disease onset by ES-62 is shown in **Figure 4.32**. Results of clinical measurements and histological assessments taken by Dr. Bernard Leung (Department of Immunology, University of Glasgow) are shown in **Figure 4.33**. The results clearly show that prophylactic use of ES-62, either in limited or multiple dosage, results in a reduction of the mean articular index, disease incidence rate and characteristic paw thickening throughout the onset and progression of CIA in DBA/1 mice. *In vitro* analysis also demonstrated that ES-62 modulates the anti-collagen response of nodal

mononuclear cells by reducing the levels of pro-inflammatory mediators TNF-α, IFN-γ and IL-6 whilst promoting the production of the anti-inflammatory cytokine, IL-10 (d33; results not shown). Continual *in vivo* exposure to ES-62 also suppressed the production of anti-collagen IgG2a antibodies whilst enhancing the levels of collagen-specific IgG1 (d50). This modulation was also shown to last beyond the time period of treatment, until the termination of the study at day 50.

4.3.4.3 Effect of FcγRIIb co-ligation on the BCR and collagen-mediated proliferative response of splenic B cells from CFA/Collagen primed DBA/1 mice following prophylactic ES-62 treatment, *ex vivo.*

Previous results from the CIA model demonstrated that FcγRIIb-mediated negative regulation of B cell activation appeared to be intact throughout the onset and progression of arthritis. To determine whether the inhibitory role of FcγRIIb is independent of the suppressive effects of ES-62 on the induction of CIA, the antibody-mediated proliferative responses of splenic B cells from CFA/Collagen primed DBA/1 mice following prophylactic ES-62 treatment were investigated. **Figure 4.34** shows that on day 50 of the study, the proliferation of splenic B cells from ES-62 treated mice, in response to BCR and collagen stimulation, is suppressed in comparison to mice treated with PBS alone. In addition, this suppression is enhanced in mice treated with multiple doses of ES-62. Importantly, FcγRIIb mediated inhibition of BCR and collagen-specific proliferation is shown to be functional and independent of ES-62 treatment.

4.3.5 The role of FcγRIIb-mediated inhibition of B cells in a murine model of systemic lupus erythematosus.

Several studies have demonstrated that an essential component for the development of lupus-like disease in mice is the presence of autoreactive B cells. These are found at relatively higher numbers and are substantially activated compared with non-autoimmune mice (reviewed by Schlomchik *et al.*, 1994). These autoreactive B cells produce a variety of autoantibodies which are virtually diagnostic of lupus, such that agents that interfere with autoantibody production have been shown to attenuate disease (Tumlin, 1999). To

investigate whether abnormal FcγRIIb negative regulation or isoform expression in B cells contributes to the generation of disease in an established murine lupus model, the involvement of FcγRIIb on *ex vivo* B cell immunological responses of MRL/Mp-*lpr/lpr* mice was tested.

Initial results from a preliminary experiment using an in-house, in-bred mouse colony seemed to indicate that FcγRIIb might be dysregulated in this disease. **Figure 4.35** shows that splenic B cells from a female MRL-*lpr/lpr* mouse with chronic lupus-like disease, have enhanced levels of DNA synthesis when stimulated with $F(ab')_2$ fragments of anti-mouse IgM/IgG. In contrast, no stimulation of DNA synthesis is observed following co-ligation of the BCR with FcγRIIb with the intact form of the antibody alone. Importantly, BCR-FcγRIIb co-ligation, with a combination of both $F(ab')_2$ and intact anti-Ig together, does not result in a significant inhibition of BCR-induced DNA synthesis.

This result was in direct contrast to the induction of growth-arrest normally observed when co-crosslinking FcγRIIb and the BCR on splenic B cells from Balb/c or DBA/1 (Fas^+) mice. These initial observations suggested that Fas, or Fas related signalling pathways, could play a role in the regulation of FcγRIIb-mediated inhibition of splenic B cell activation. Thus, Fas may be required for the successful inhibition of BCR-mediated activation by antigen-antibody complexes.

To investigate the potential involvement of dysfunction of Fcγ–mediated regulation of B cell activation in murine lupus, the proliferative responses of splenic B cells from MRL/Mp-*lpr/lpr* mice was investigated throughout the onset and progression of disease. The SLE model strain used in these secondary studies (MRL/Mp-*lpr/Ola/Hsd,* H-2^k) was developed in the Jackson Laboratory, Bay Harbor, USA, by backcrossing the congenic mutant gene *lpr* (*lymphoproliferation*) by 5-cycles of cross-intercross matings from substrain MRL/1 to substrain MRL/n (now designated MRL/Mp-+/+). Male MRL/Mp-*lpr/lpr* mice were monitored for the physiological signs of disease onset from 13 weeks, in particular for the development of lymphadenopathy and diseased ear

lobes. Male MRL/Mp-*lpr/lpr* lupus model mice normally have a mean survival age of 22 weeks, compared to 20 weeks in the females. This difference is presumably due to the presence of oestrogen in female mice which is thought to slightly accelerate the disease onset. However, mortality in both genders usually arises from renal failure, due to glomerulonephritis.

4.3.5.1 Effect of FcγRIIb co-ligation on the BCR-mediated proliferative response of splenic B cells from MRL/MP-*lpr/lpr* mice, *ex vivo*.

DNA synthesis experiments demonstrated that FcγRIIb was capable of inducing B cell growth-arrest following co-ligation with the BCR in male MRL/MP-*lpr/lpr* mice up to 21-26 weeks of age (**Figure 4.36**). This inhibition is also shown to be overcome by co-culturing the cells with IL-4, a B cell stimulatory cytokine produced by T_H2 CD4+ T cells. Whilst FcγRIIb co-ligation is shown to inhibit BCR-mediated proliferation during weeks 14-21, the inhibitory effect is reduced as the disease progresses. Thus, by week 26, FcγRIIb co-ligation does not significantly inhibit BCR induced DNA synthesis. In agreement with previous results, no DNA synthesis is associated with intact antibody stimulation alone, throughout the period of study.

4.3.5.2 FcγRIIb ligation induces growth-arrest and/or apoptosis splenic B cells from MRL/MP-*lpr/lpr* mice, *ex vivo*.

DNA content analysis confirmed the induction of growth-arrest following BCR-FcγRIIb co-ligation in the early weeks of disease, whilst also demonstrating that commitment to apoptosis by FcγRIIb is Fas independent (**Figures 4.37** and **4.38**). In contrast to the DNA synthesis results, stimulation with intact antibody alone, or in combination with $F(ab')_2$ fragments, resulted in similar levels of growth-arrest and apoptosis in splenic B cells from mice aged 26 weeks. Furthermore, following stimulation with intact anti-Ig antibodies fewer cells accumulated in G_0/G_1 than in the control, with an increase in the sub-diploid population. These results may indicate that only a small population of cells were responsible for the enhanced levels of proliferation seen in the DNA synthesis assay.

4.3.5.3 Effects of BCR and FcγRIIb co-ligation on the mitochondrial membrane potential of splenic B cells from MRL/MP-*lpr/lpr* mice, *ex vivo*.

Cell death receptors such as Fas (CD95) initiate apoptotic pathways by directly recruiting pro-caspases, such as caspases 10 and 8 (see **1.2.4**). Activation of the caspase-cascade can lead to a loss of mitochondrial function and integrity, resulting in enhanced production of reactive oxygen species, calcium cycling and disruption of the inner mitochondrial potential. FcγRIIb ligation on splenic B cells has been shown in this study to result in a depolarisation of the mitochondrial membrane that is indicative of apoptosis. To investigate whether FcγRIIb-mediated mitochondrial disruption is Fas-dependent, the mitochondrial membrane potential of intact antibody stimulated B cells from MRL-*lpr/lpr* mice was assessed. **Figure 4.39** demonstrates that FcγRIIb stimulation of Fas deficient splenic B cells results in a loss of mitochondrial membrane potential, whilst stimulation via the BCR results in membrane potential stabilisation. Thus, the mechanism by which this is achieved is not dependent on the expression of Fas.

4.3.5.4 Effects of BCR ligation on the FcγRIIb1 and FcγRIIb2 mRNA expression levels in splenic B cells from MRL/MP-*lpr/lpr* mice, *ex vivo*.

To investigate whether the modulation of FcγRIIb isoform expression by splenic B cells is involved in the initiation of lupus-like disease in MRL/Mp-*lpr/lpr* mice, RT-PCR was performed to identify potential lesions in the expression of the isoforms at the mRNA. Throughout the progression of disease total RNA was extracted from splenic B cells of MRL/Mp-*lpr/lpr* mice, following BCR stimulation for 48 hours. The RNA was reversed transcribed, in the presence of Superscript RT™ II and the relative expression levels of the isoform transcripts were determined by quantitative TaqMan analysis. **Figure 4.40** shows preliminary results from these investigations. Interestingly, the data implies that during the progression of disease in MRL/Mp-lpr/lpr mice the relative expression levels of both FcγRIIb isoforms decreases. Thus, these results correlate with the dysregulation of FcγRIIb-mediated B cell inhibition found in the later stages of lupus-like disease in the MRL-*lpr/lpr* mice suggesting that FcγRIIb may be a

susceptibility factor, contributing to the genetic and environmental background that is required for the emergence of lupus-like disease in these mice.

4.4 Discussion

4.4.1 FcγRIIb and collagen-induced arthritis.

The role of inhibitory receptors and molecules in the regulation of lymphocyte homeostasis and tolerance to self-antigens has been demonstrated in numerous knockout and deletion studies. Studies that have investigated the involvement of FcRs in the onset of disease in CIA have highlighted their importance. In particular, mice deficient in the low affinity receptor for IgE (FcεRII), and FcγRIIa developed CIA with delayed onset and reduced severity compared with wild-type mice (Kleineau *et al.*, 1999). In contrast, mice deficient in the inhibitory receptor for IgG, FcγRIIb, have augmented immune responses, high levels of antigen specific serum IgG and an increased incidence of autoimmune disease (Takai *et al.*, 1996). Furthermore, mice with genotypes normally associated with resistance to collagen-induced arthritis, become susceptible to autoimmune arthritis when FcγRIIb is deleted (Yuasa *et al.*, 1999).

In this study, we have demonstrated that in an established murine model of inflammatory arthritis, CIA, FcγRIIb-mediated negative regulation of splenic B cell proliferation is functional throughout disease onset and progression, however there was a suggestion that FcγRIIb-mediated negative regulation of the BCR-mediated response may be deregulated in CIA mice at the time of collagen re-challenge. Furthermore, BCR-mediated ErkMAPK phosphorylation in CIA mice did not appear to be uncoupled following BCR-FcγRIIb co-ligation. However, these results were not reflected in the percentage of cells undergoing growth-arrest or commitment to apoptosis by cell cycle analysis of DNA content, following FcγRIIb co-ligation. Indeed, similar levels of growth-arrest and/or apoptosis were shown in both CIA and control CFA mice. To clarify this discrepancy and to determine whether FcγRIIb deregulation in CIA mice was potentially limited to collagen-specific B cells, the regulatory role of FcγRIIb-

ligation on the collagen-specific B cell response, during the days immediately pre- and post-collagen challenge, was investigated. Similarly, BCR-mediated ErkMAPK phosphorylation did not appear to be uncoupled in CIA mice following FcγRIIb co-ligation. In contrast, DNA synthesis and content analysis demonstrated that FcγRIIb-mediated negative regulation was functional in both general and collagen-specific B cells. This suggest that ErkMAPK may be coupled to mechanisms that lead to both proliferation and growth arrest in CIA mice or that FcγRIIb mediated disruption of ErkMAPK is not required to induce growth-arrest in splenic B cells from CIA mice. It also implicates the requirement for further positive signals, in addition to ErkMAPK, to promote a proliferative response. Thus, examination of alternative signalling components, in addition to ErkMAPK phosphorylation, is required to understand the mechanisms leading to proliferation, growth-arrest and/or commitment to apoptosis in these cells.

These results are in direct contrast to studies in FcγRIIb deficient mice which implicate FcγRIIb as a susceptibility factor for the onset and progression of spontaneous autoimmune disease and collagen-induced arthritis (Takai *et al.,* 1996; Yuasa *et al.,* 1999; Bolland and Ravetch, 2000). Interestingly, the incidence of arthritic disease in FcγRIIb deficient mice was lower than in the established DBA/1 CIA model (42% versus 95%) (Yuasa *et al.,* 1999), confirming that multiple defects are required for the full initiation of disease. Similar to our studies, the proliferative responses of mononuclear lymphoid cells to antigenic stimulation in CIA and FcγRIIb deficient mice with collagen (CII) were limited. Indeed, demonstrable differences in the collagen-specific responses were only observed by Yuasa *et al.* following priming and stimulation with twice the amount of CII used in our study. Furthermore, the CII specific responses were identical in FcγRIIb deficient, wild type and DBA/1 CIA mice at the normal levels of CII immunisation required for CIA induction. These results therefore suggest that the level of circulating antigen-antibody complexes dictate whether FcγRIIb is important for mediating negative feedback regulation of antigenic stimulation.

However, in the present study, the collagen-specific proliferative response in the CFA/CII mice was very low and implied that the percentage of collagen-specific B cells in the purified splenic population was minimal. Poor collagen-specific responses may also be due to the lack of lymphoid organ structure *in vitro,* resulting in the absence of co-stimulatory signals necessary for a full immunological response to collagen *in vivo.*

Thus, to examine the requirement for bystander lymphoid cells in the collagen-specific proliferative response, the *ex vivo* cellular responses of whole spleen and nodal populations to FcγRIIb ligation were investigated. DNA synthesis and content analysis confirmed that FcγRIIb-mediated negative regulation was functional in mononuclear cells from spleen and nodal populations. This finding was also supported by a demonstrable disruption of the mitochondrial membrane potential following FcγRIIb-ligation, an early indication of commitment to apoptosis.

Interestingly, in our study discrepancies were found in FcγRIIb-mediated inhibition of ErkMAPK phosphorylation in CIA and CIA-control mice. In particular, phosphorylation of the p44 (Erk1) form of ErkMAPK was differentially inhibited by FcγRIIb in CFA control and CIA mice. Studies in p44 (Erk1) MAPKinase knockout mice (Pagès *et al.*, 1999) have demonstrated that whilst lymphocyte maturation is normal in Erk1 deficient mice, proliferation in response to antigenic stimulation is reduced. Thus, whilst p42 (Erk 2) MAPKinase can compensate for the loss of p44 (Erk1) in lymphocyte development, their findings indicate that there may be physiological distinctions between the two isoforms in the regulation of lymphocyte proliferation. This suggests that there may be a potential requirement for ErkMAPK activity, in particular Erk1, in the basal proliferation of splenic B cells from DBA/1 mice and that the removal of this signal, due to FcγRIIb co-ligation, may be sufficient to induce growth arrest and/or apoptosis. However, the finding that FcγRIIb-mediated growth arrest and commitment to apoptosis are accompanied by the activation of ErkMAPK in B cells from CIA mice is of interest (see above). These data suggest that rather than being essential for proliferation, ErkMAPK may represent a permissive

signal for B cell proliferation in these mice and that further positive signals are required for the full proliferative effect. Thus, these results may indicate the downregulation or inhibition of a further component of a BCR-mediated signalling pathway that modulates ErkMAPK derived proliferative signals.

A central role for phosphatases in the development of autoimmunity has been implicated in other murine models displaying deficiency in an inhibitory receptor, such as CD22 (O'Keefe *et al.,* 1996) or PD-1 (Nishimura *et al.,* 1999). B cells from CD22-deficient mice exhibit the cell surface phenotype and augmented intracellular calcium responses characteristic of hyper-responsive B cells, similar to that of SHP-1 deficient mice (Sato *et al.,* 1996). CD22 may control basal BCR signal transduction thresholds in resting B cells through the binding of SHP-1. Thus, in the absence of CD22 a lack of SHP-1 recruitment permits chronic tyrosine phosphorylation of the BCR in resting B cells and hyperactivation. Therefore, alterations in CD22 function or expression could contribute to autoimmunity or other disease in which B cell function is dysregulated. Indeed, reduced up-regulation of CD22 on activated B cells and aberrant expression of the CD22 allele, CD22a, is implicated in autoantibody production in NZW murine models of lupus-like disease (Mary *et al.*, 2000) providing support for CD22a as a possible candidate allele contributing to lupus susceptibility. Furthermore, genetic variations of human CD22 and the possible association with rheumatic diseases has been investigated in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Whilst no significant association was observed between any of the CD22 variations and RA, the data indicated that a number of genetic variants are present in CD22, and the authors suggest that CD22 could be considered as a candidate susceptibility gene for autoimmune diseases (Hatta *et al.*, 1999).

PD-1 is an ITIM-containing, transmembrane Ig Super-Family (IgSF) receptor (reviewed by Nishimura and Honjo, 2001). It is expressed on thymocytes and mature macrophages and lymphocytes following activation. Engagement of PD-1 with PD-1 ligand (PD-L) expressing antigen-presenting cells results in negative signalling. Similar to FcγRIIb, co-ligation of the BCR with the PD-1 cytoplasmic region *in vitro*, results in the recruitment of the tyrosine

phosphatase, SHP-2, and the inhibition of BCR mediated calcium influx and tyrosine phosphorylation of downstream signalling activation molecules, such as Syk, PI-3-Kinase, PLC-γ and Vav. The inhibitory effect also leads to the inhibition of the MAPKinase pathway, resulting in inhibition of B cell proliferation. Importantly, PD-1 deficiency causes lupus and arthritis like disease in a C57BL/6 mouse background (Nishimura *et al.*, 1999). Thus, similar to FcγRIIb, PD-1 could be involved in the onset and progression of autoimmune disease. Unfortunately, primary splenic B cells and mononuclear cells stimulated with LPS, $F(ab')_2$ fragments of anti-Ig antibodies or IL-4 failed to demonstrate upregulation of PD-1 expression, as determined by FACS analysis with a PD-1 specific monoclonal antibody. Thus, investigation of its potential role in autoimmune disease murine models proved impossible.

Examination of the relative message levels of the two isoforms of FcγRIIb during the period of collagen re-challenge indicated that differential isoform expression might play a role in the initiation of disease. Indeed, FcγRIIb1 mRNA levels remained relatively constant throughout the period of disease onset in both groups of mice, whilst FcγRIIb2 was enhanced in CFA/CII mice following the second collagen challenge. Alterations in FcγRIIb isoform expression may contribute to the disease initiation process by modulating antigen-presentation by autoreactive B cells. Thus in healthy strains of mice a balance of FcγRIIb isoform expression levels on B cells will prevent aberrant activation by immune-complexes and regulate IgG antibody responses through a negative feedback mechanism. In contrast, selective down-regulation of FcγRIIb1 in B cells might promote T cell mediated help and hence permit inappropriate activation of B cells by immune-complexes. An upregulation of FcγRIIb2 may also promote antigen uptake and presentation by the B cells and thus could break normally tolerant B cells. Moreover, co-stimulatory T cell help will also overcome the negative feedback regulation mediated by FcγRIIb1. Thus in CIA, an upregulation of FcγRIIb2 on B cells may promote the presentation of self-antigen to autoreactive T cells and initiate an autoimmune response.

As a low affinity receptor, FcγRIIb acts most successfully in areas with high concentrations of immunocomplex such as the germinal centre. The down-

regulation of FcγRIIb1 expression on germinal centre B cells in autoimmune disease prone mice is, however, unlikely to be an effect of disease, since a downregulation in the expression of FcγRIIb1 was found in mice at an age before the onset of disease (Jiang *et al.*, 1999). These results are in agreement with our proposal that downregulation of FcγRIIb1 expression may promote disease initiation.

B cells studies from CIA mice carried out *in vitro* are usually naive cells derived from spleens primed with high doses of antigen *in vivo*. Activated B cells have Fc receptors with a higher affinity for passively administered IgG than naïve B cells. However, disruption of FcγRIIb has been shown not to appreciably modify the antigen-specific T cell response in non-permissive animals and thus is not likely to account for the susceptibility of these animals to CIA (Bolland and Ravetch, 2000). However, the fact that FcγRIIb is thought to prevent the activation of low-affinity autoreactive cells in the periphery and that auto-antibody production is enhanced in FcγRIIb knockout mice, may support a role for this receptor in some experimental disease systems. Thus, further investigation of FcγRIIb function in B-1 cells, which display a propensity to produce low-affinity autoreactive antibodies, may provide further insight into how dysregulation of B cell activation promotes the initiation and progression of disease. Hyper-antibody production may also be attributed to a decrease in FcγRIIb2 expression on macrophages, as reported in NOD mice (Luan *et al.*, 1996), the result being a reduction in the clearance of IgG immune complexes leading to the accumulation of immune complexes in the circulation.

The finding that deletion of the FcγRIIb gene renders mice susceptible to CIA does not confirm the role of the receptor in the initiation of disease. Indeed, the development of autoimmune disease in FcγRIIb deficient mice has since been shown to be strain dependent (Bolland and Ravetch, 2000), such that the mechanisms by which B cell FcγRIIb deficiency result in autoimmunity may be dependent on the C57/BL6 ($H\text{-}2^b$) or 129/SvJ ($H\text{-}2^b$) backgrounds. Thus, FcγRIIb-deficient Balb/c ($H\text{-}2^d$) mice, remain non-susceptible to the development of CIA. Interestingly, deletion of the FcγRIIb gene backcrossed

into the DBA/1 (*H-2*^{q}) mouse, the strain used in these studies, leads to a dramatically enhanced development of CIA and also contributes to increased susceptibility to other autoimmune diseases, such as gastritis (Kleinau *et al.*, 2000). The causes for strains differences are still unknown but one candidate is the bias towards a T_H1 response in susceptible animals (Afonso *et al.*, 1994).

These results suggest that in conjunction with certain HLA haplotypes, that selectively bind and present self-peptides to autoreactive T cells, FcγRIIb plays an important role in regulating antigen-presentation in the pathogenesis of CIA and that the ability of the FcγRIIb2 isoform to mediate antigen-endocytosis, may dictate the involvement of the receptor in the onset and progression of disease The initiation of CIA may therefore rely on both enhanced humoral responses to type II collagen and augmented proinflammatory mediator release (TNF-α) by effector cells, such as macrophages. Further investigation of these disease susceptibility genes will help to characterise the pathogenesis of autoimmune disease and provide new prophylactic and therapeutic approaches. One such approach currently being investigated is the use of an immunomodulatory molecule, ES-62, secreted by filarial nematodes.

4.4.2 Role of FcγRIIb in ES-62 treatment of collagen-induced arthritis.

The generation of collagen-specific antibodies by B cells and the promotion of a T_H1-type immune response by collagen-specific CD4+ T cells are crucial for the development of arthritis in the CIA model. Thus, the inhibition of lymphocyte activation and the initiation of a T_H2 immune response by ES-62 may represent a novel therapeutic treatment strategy. *In vivo* exposure of B cells to ES-62 in this animal model confirmed the ability of the parasite product to inhibit BCR-mediated DNA synthesis. In contrast, FcγRIIb-mediated inhibition of B cell proliferation was shown to be functional at all concentrations of ES-62 treatment, resulting in enhanced suppression of the BCR-mediated proliferative response.

Prophylactic or therapeutic use of ES-62 in the treatment of CIA clearly demonstrated a reduction in the mean articular index, disease incidence rate

and characteristic paw thickening throughout the onset and progression of CIA. Initial observations by our laboratory had suggested that FcγRIIb and ES-62 might mediate their suppressive effects by similar routes of inhibition or that ES-62 might mediate its effects through binding or upregulation of FcγRIIb. However, ES-62 pre-treatment did not affect the ability of FcγRIIb to inhibit BCR-mediated DNA synthesis nor did it result in a synergistic inhibition of B cell activation, implying that ES-62 did not mediate its effects directly via FcγRIIb signalling. It would be interesting to speculate, however, that, in addition to its direct anti-proliferative effects on B cells, ES-62 may inhibit antigenic stimulation of the BCR by upregulating the expression of FcγRIIb1. This may prove important in the perpetuation of disease, where expansion of autoreactive B cells requires T cell help. However, preliminary investigations of FcγRIIb isoform expression on B cells following pre-exposure to ES-62 were inconclusive and were unable to support this theory.

Interestingly, ES-62 was shown to skew the anti-collagen antibody response from IgG2a to IgG1 phenotype. This modulation of IgG subclass may play an important role in the regulation of the immune response to collagen as it dictates which FcγRs are ligated and therefore determines the effector mechanism elicited. For example, IgG2a will only ligate the low affinity FcγR, FcγRIIa, on inflammatory effector cells. A switch to IgG1 permits binding to all FcγR members . Thus, ES-62 may mediate its anti-inflammatory effects through the upregulation of IgG immune complex binding receptors and FcγR-bearing phagocytic cells whilst switching off the generation of inflammatory mediators by antigen-presenting cells and autoantibody production by B cells

4.4.3 Role of FcγRIIb in a murine model of systemic lupus erythematosus (SLE).

As discussed above, mice deficient in FcγRIIb have been shown to develop lupus-like disease spontaneously (Bolland and Ravetch, 2000; Nakamura *et al.*, 2000) whilst some autoimmune disease prone mouse strains have a promoter that is associated with reduced surface expression and function of this receptor

(Pritchard *et al.*, 2000). These results indicate that FcγRIIb may have a preventative influence on the initiation of autoimmunity. Importantly, lupus-like disease is accelerated in mice deficient in Fas or Fas-ligand. Since B cell Fas expression is considered to most important in preventing inappropriate T_H dependent expansion of autoreactive B cells in the peripheral lymphoid tissues this could suggest that FcγRIIb-mediated commitment to apoptosis may occur via a Fas dependent mechanism. However, the exact mechanisms of FcγRIIb-induced apoptosis have not been elucidated and a requirement for Fas has not been investigated.

Initial studies in a female MRL-*lpr/lpr* mouse, in which lupus-like disease was well established, indicated that FcγRIIb-mediated inhibition was indeed dysregulated in Fas deficient mice. These data suggested that FcγRIIb may be involved in the initiation of lupus-like disease and that Fas may play a role in FcγRIIb killing. Analysis of FcγRIIb-mediated negative regulation in male MRL-*lpr/lpr* mice before disease onset and during onset and progression demonstrated that prior to disease onset FcγRIIb-mediated negative regulation was functional. However, FcγRIIb inhibition of BCR-induced DNA synthesis was lost during disease progression. Interestingly, this loss of FcγRIIb-mediated negative regulation correlated with an increase of disease severity in the mice, thus the animals analysed at 26 weeks displayed chronic physiological signs of inflammatory disease. Analysis of apoptosis levels demonstrated that FcγRIIb-induced apoptosis was functional throughout the disease progression and contradicted the DNA synthesis data. However, the data also indicated that FcγRIIb-mediated growth arrest was functional. It is difficult to explain these obvious differences in DNA synthesis and content but these results suggest that FcγRIIb mediated growth-arrest is Fas-independent but that disease progression in these mice could induce this additional lesion. Furthermore, disruption of the mitochondrial membrane potential, suggests that apoptosis is Fas-independent. However, studies of Fas-mediated death have indicated that Fas can initiate apoptotic mechanisms that are independent of mitochondrial

disruption (reviewed by Krammer, 2000) and thus supports a Fas-independent mechanism for FcγRIIb induced cell death.

The strongest hypothesis for the initiation of lupus-like disease in these mice is that self-reactive T and B cells arise when they fail to undergo normal apoptosis therefore it is of interest that FcγRIIb-mediated apoptosis appears to be functional and this requires further investigation. Others, who suggest that B cell tolerance may be relatively independent of Fas, may support the theory that FcγRIIb mediated effects are perhaps more important for inducing self-tolerance at other stages of B cell development. Consistent with this, our preliminary data from MRL-*lpr/lpr* mice investigating the relative B cell expression levels of FcγRIIb isoforms following BCR-stimulation, suggest that FcγRIIb expression may be modulated in these mice. Disease progression was accompanied by a downregulation in mRNA levels of both isoforms of FcγRIIb. Suggesting that aberrant expression of FcγRIIb could promote perpetuation of lupus-like disease in these mice.

Discrepancies in the results obtained from the female and male MRL/*lpr/lpr* mice may be accounted for by differences in supply and disease onset. The females were supplied in house from an in-bred colony, whereas the males were bred commercially. The females displayed advanced physiological stages of disease by 18 weeks. In contrast, the males only demonstrated chronic physiological signs of disease by 26 weeks. It is therefore of interest that the male mice demonstrated aberration in FcγRIIb-mediated B cell inhibition at week 26, at a similar stage of disease progression as the females. Furthermore, there are marked age- and sex related variations in the isotype of anti-DNA antibodies seen in murine models of SLE. Importantly, there is an antibody isotype switch from IgM to IgG2a as the mice mature. This switch is seen earlier in females than males, coinciding with the onset of renal disease. This observation highlights the importance of antibody isotype in immune complex deposition and that FcγR-bearing antigen-presenting cells will only be able to bind the anti-DNA antibodies and promote an inflammatory response following the isotype switch.

In MRL-*lpr/lpr* no evidence for linkage of H-2 haplotype with nephritis is apparent, however, IgG autoantibody production was reduced by breeding $H\text{-}2^d$ onto B6-*lpr/lpr* mice suggesting that the disease is related to HLA and antigen presentation. However, the full expression of lupus-like disease is dependent on complex genetic contributions from other non-MHC genes. Indeed, a minimum of three distinct genetic loci is required for the development of disease in the F_1 cross of NZB/NZW. Spontaneous SLE models of NZB and (NZW x NZB) F_1 mice also demonstrate downregulation of FcγRIIb in germinal centre B cells associated with IgG hypergammaglobulinemia and IgG autoantibody production (Jiang, *et al.,* 1999) indicating a possible lack of negative feedback. Genetic linkage studies have associated Fas-L (*gld*) on chromosome 1 with FcγRIIb but Fas (*lpr*) is on chromosome 19 and not linked to FcγRIIb. In addition, nephritis in New Zealand hybrid mice (NBA2/*Sle*1) mice map to a region that is linked to FcγRIIb. Thus it would be of interest to repeat this study and investigate the role of FcγRIIb function and expression in these other murine models of lupus-like disease, such as female MRL/*gld/gld* mice, to confirm whether FcγRIIb does indeed play a role in the initiation of disease.

No counterparts to *lpr* or *gld* phenotypes exist in human SLE, indicating that there are multiple different factors involved in the initiation of lupus-like disease in humans and mice. However, a skewing of FcγRIIa allotype expression has been reported in SLE patients and linked with nephritis in African-Americans and Caucasians (Duits *et al.,* 1995). The differential expression results in the downregulation of the H131 allele of FcγRIIa, the only human FcγR that recognises IgG2a efficiently, implying that the skewing may lead to the downregulation of endocytic capability of phagocytes and insufficient clearance of immune complexes (Vyse and Kotzin, 1998).

Except for the complete deficiencies of classical pathway complement proteins the association of SLE with non-MHC genes or loci remains for the most work in progress. However, FcγRIIb-deficiency appears to be a susceptibility factor, contributing to the genetic and environmental background that is required for the emergence of disease.

Figure 4.1 Effect of FcγRIIb co-ligation on BCR–mediated ErkMAPK phosphorylation in splenic B cells from naïve, CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d0-d47).

Purified splenic B cells from naïve, CFA control or CFA/Collagen primed DBA/1 mice (1×10^7 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Whole cell lysates were immunoprecipitated with anti-ErkMAPK antibody (Erk23) and Western blotted for phospho-ErkMAPK. The numbers beneath the blots represent the relative band density as assessed by densitometry from a single experiment, compared to the band density obtained for the naïve control and arbitrarily set as 1. (–) represents bands that were too faint for accurate quantitation.

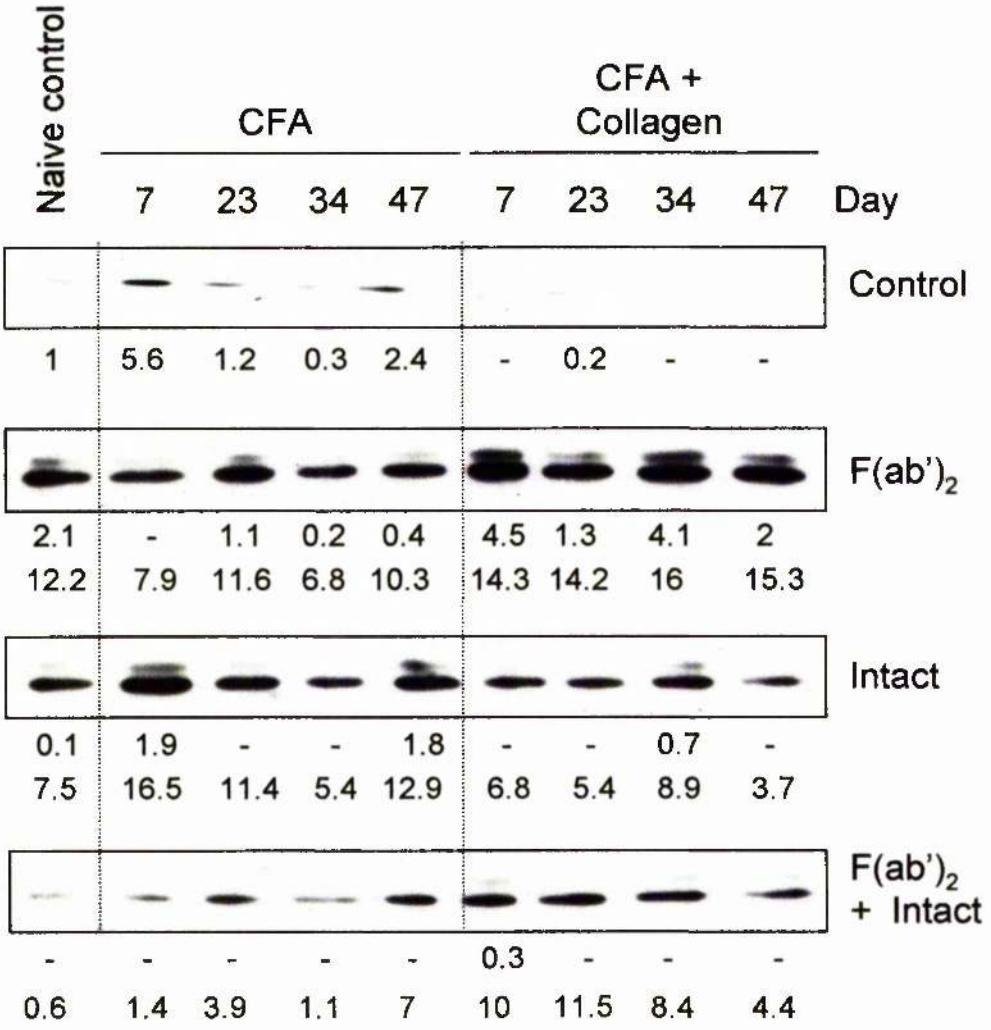

Naive control
CFA
CFA + Collagen
7 23 34 47 7 23 34 47 Day
Control
1 5.6 1.2 0.3 2.4 - 0.2 - -
F(ab')2
2.1 - 1.1 0.2 0.4 4.5 1.3 4.1 2
12.2 7.9 11.6 6.8 10.3 14.3 14.2 16 15.3
Intact
0.1 1.9 - - 1.8 - - 0.7 -
7.5 16.5 11.4 5.4 12.9 6.8 5.4 8.9 3.7
F(ab')2 + Intact
- - - - - 0.3 - - -
0.6 1.4 3.9 1.1 7 10 11.5 8.4 4.4

Figure 4.2 Effect of FcγRIIb co-ligation on the BCR–mediated proliferative response of splenic B cells from naïve, CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d0-d47).

Purified splenic B cells from naïve, CFA control or CFA/Collagen primed DBA/1 mice (2×10^5 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are expressed as the mean ± standard deviation from triplicate measurements of a single experiment.

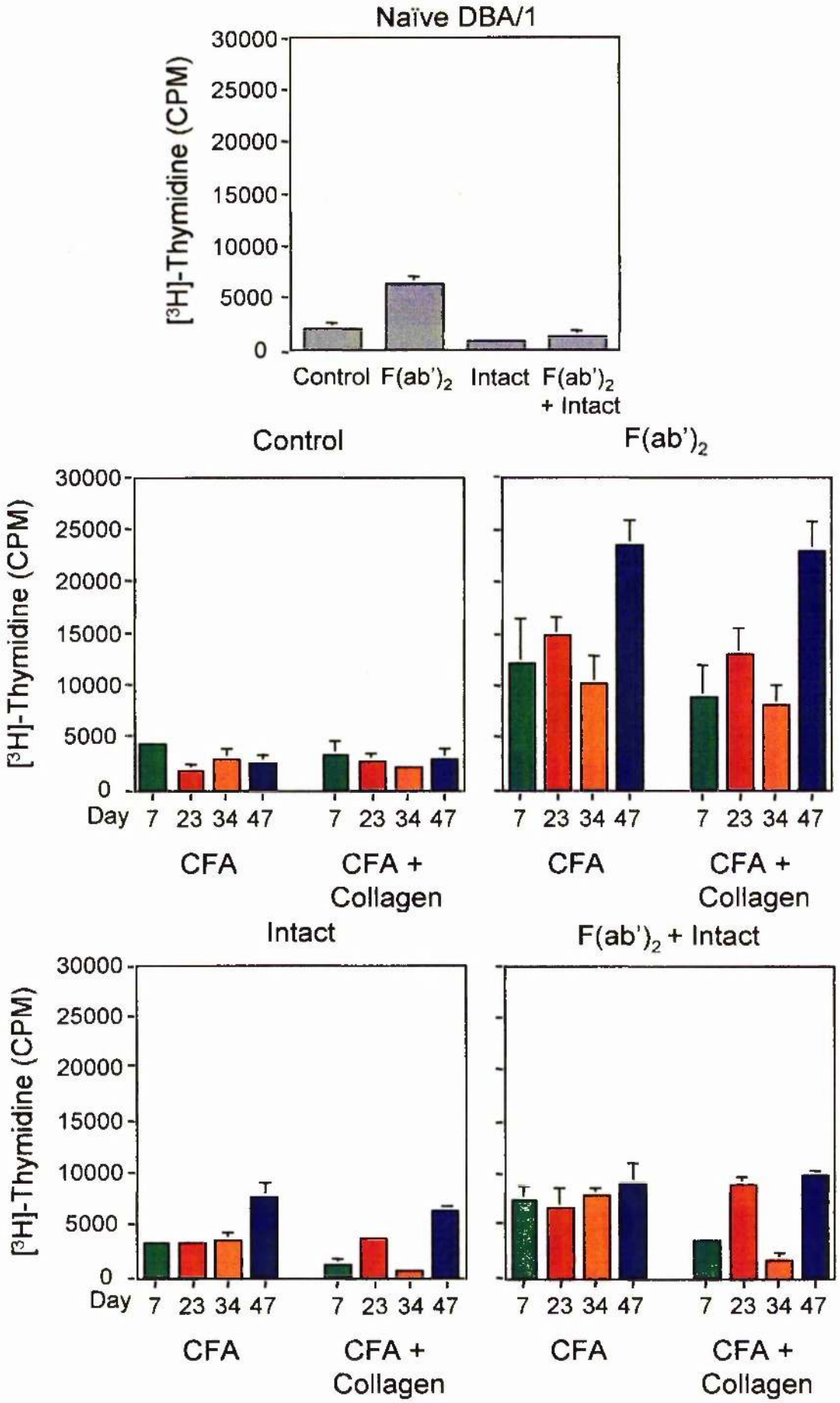

Naïve DBA/1
[3H]-Thymidine (CPM)
30000
25000
20000
15000
10000
5000
0
Control
F(ab')2
Intact
F(ab')2 + Intact
Control
F(ab')2
Day
7 23 34 47
CFA
CFA + Collagen
Intact
F(ab')2 + Intact

Figure 4.3 DNA content of splenic B cells from naïve, CFA control or CFA/Collagen primed DBA/1 mice post-sacrifice and following BCR and FcγRIIb stimulation of naïve splenic B cells, *ex vivo* (d0-d47).

Purified splenic B cells from naïve, CFA control and CFA/Collagen DBA/1 mice (1×10^6 cells) were subjected to DNA content analysis post-sacrifice. In addition, splenic B cells from naïve DBA/1 mice (1×10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total number of cells analysed from a single experiment.

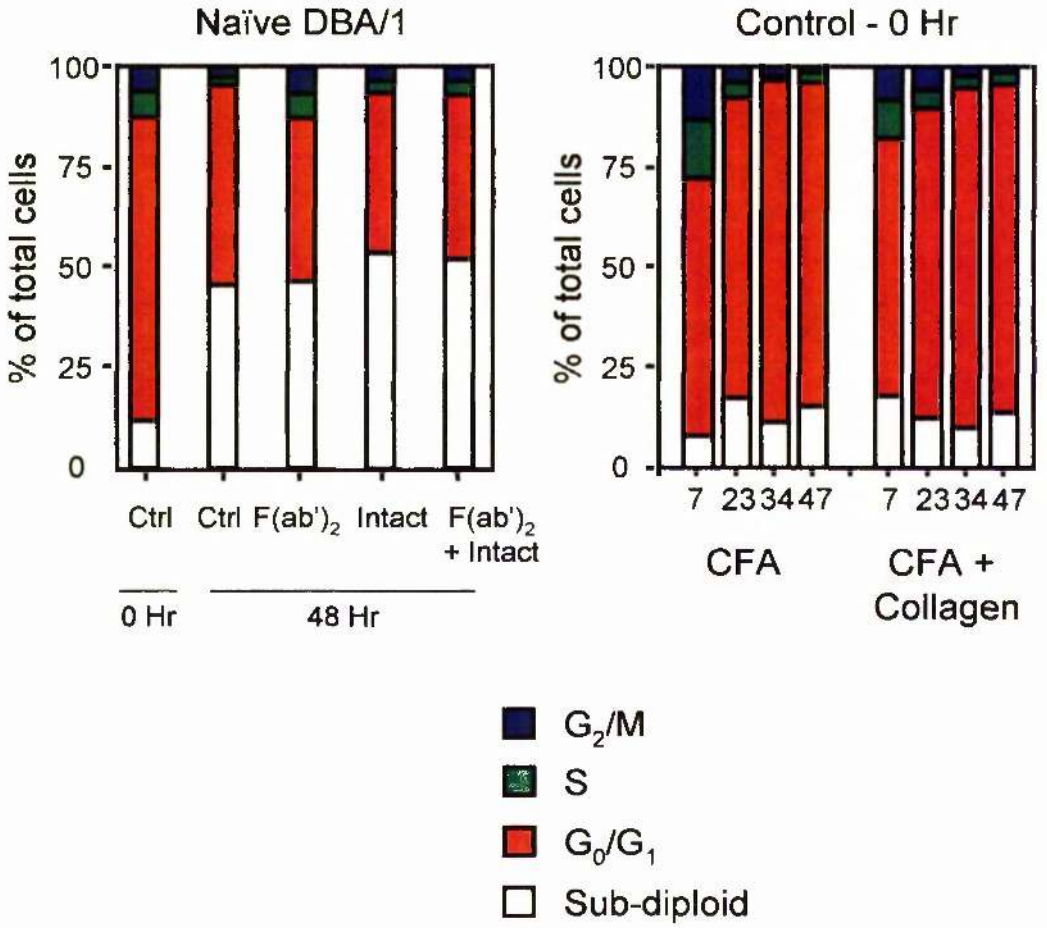

Naïve DBA/1
Control - 0 Hr
% of total cells
100
75
50
25
0
Ctrl
Ctrl
F(ab')2
Intact
F(ab')2 + Intact
0 Hr
48 Hr
7
23
34
47
7
23
34
47
CFA
CFA + Collagen
G2/M
S
G0/G1
Sub-diploid

Figure 4.4 Effects of BCR and FcγRIIb co-ligation on the DNA content of splenic B cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d0-d47).

Purified splenic B cells from CFA control or CFA/Collagen primed DBA/1 mice (1×10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total number of cells analysed from a single experiment.

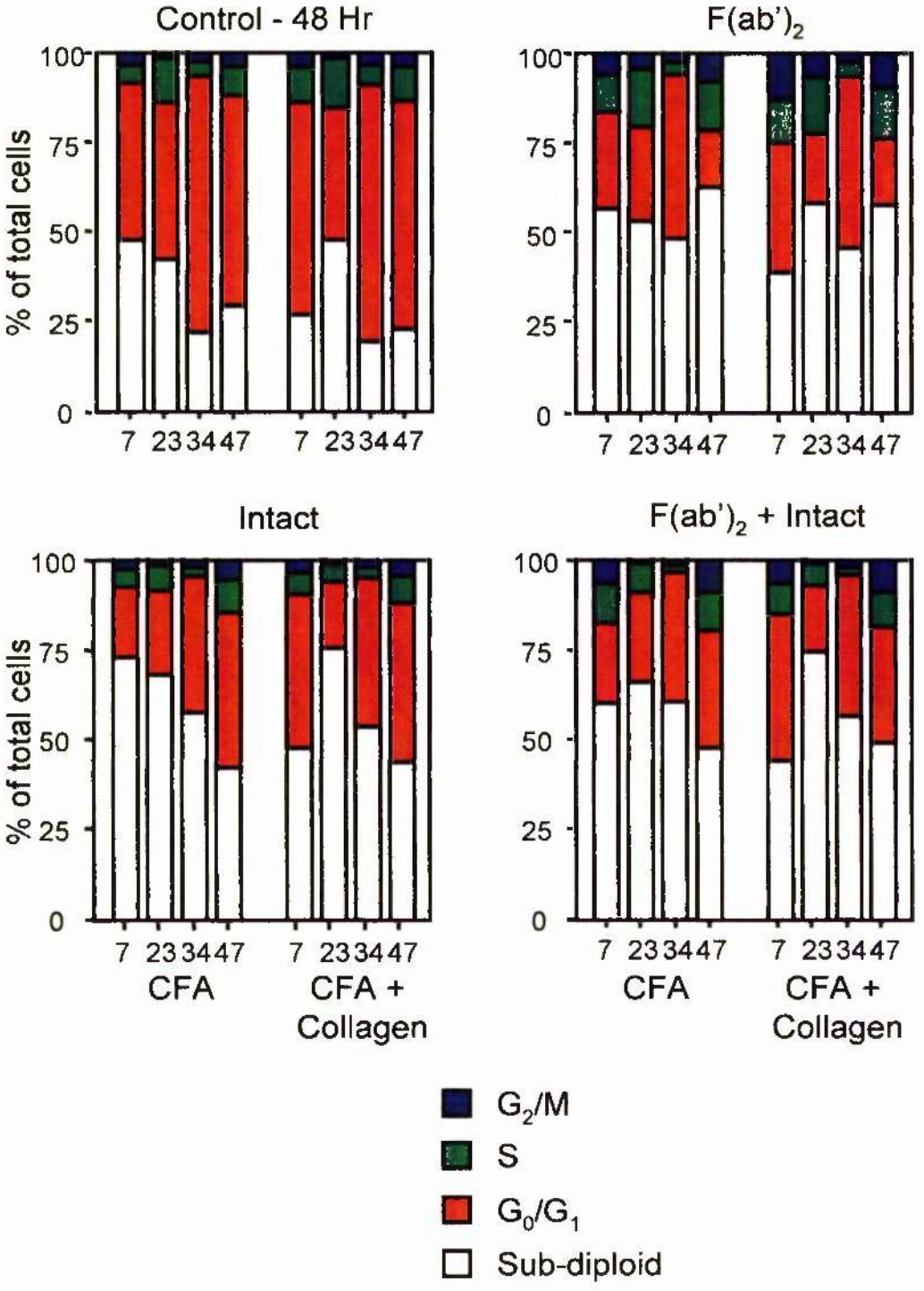

Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact
% of total cells
100
75
50
25
0
7 23 34 47 7 23 34 47
CFA
CFA + Collagen
G2/M
S
G0/G1
Sub-diploid

Figure 4.5 DNA content of the living population of splenic B cells from naïve, CFA control or CFA/Collagen primed DBA/1 mice post-sacrifice and following BCR and FcγRIIb stimulation of naïve splenic B cells, *ex vivo* (d0-d47).

Purified splenic B cells from naïve, CFA control and CFA/Collagen DBA/1 mice (1×10^6 cells) were subjected to DNA content analysis post-sacrifice. In addition, purified splenic B cells from naïve DBA/1 mice (1×10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total living population of cells analysed from a single experiment.

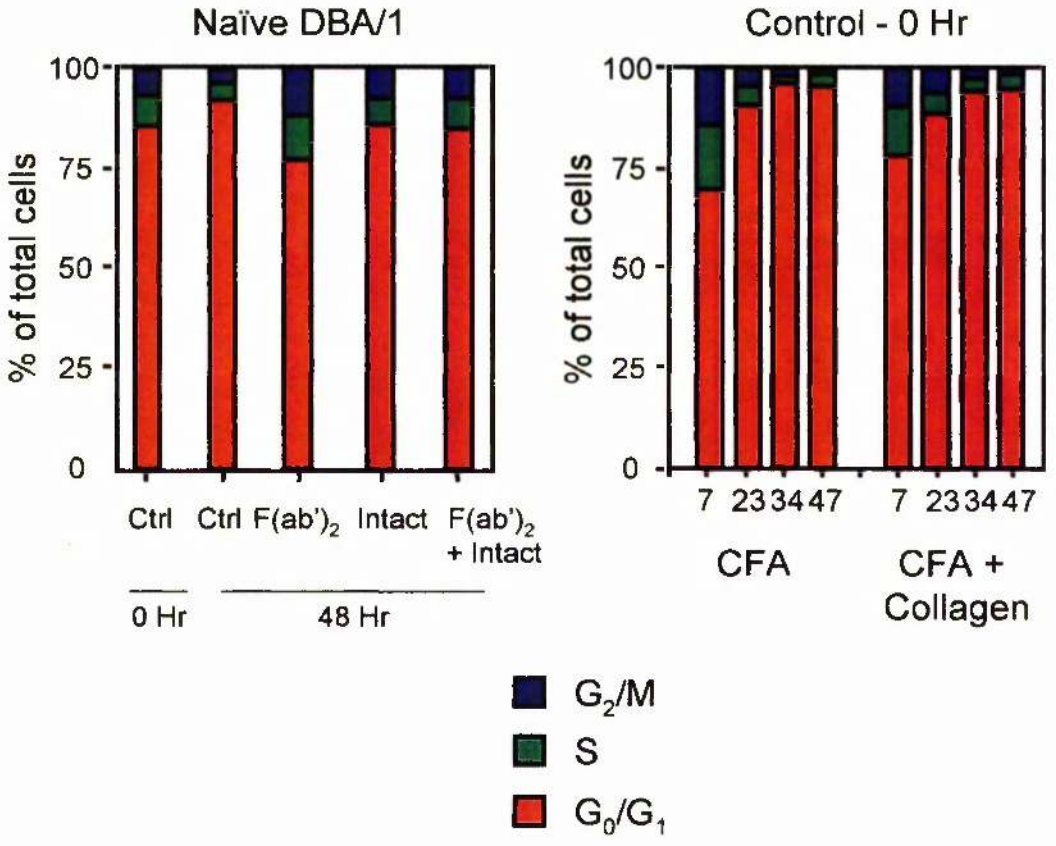

Naïve DBA/1
Control - 0 Hr
% of total cells
100
75
50
25
0
Ctrl
Ctrl
F(ab')2
Intact
F(ab')2 + Intact
0 Hr
48 Hr
7
23
34
47
CFA
CFA + Collagen
G2/M
S
G0/G1

Figure 4.6 Effects of BCR and FcγRIIb co-ligation on the DNA content of the living population of splenic B cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d0-d47).

Purified splenic B cells from CFA control or CFA/Collagen primed DBA/1 mice (1×10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total living population of cells analysed from a single experiment.

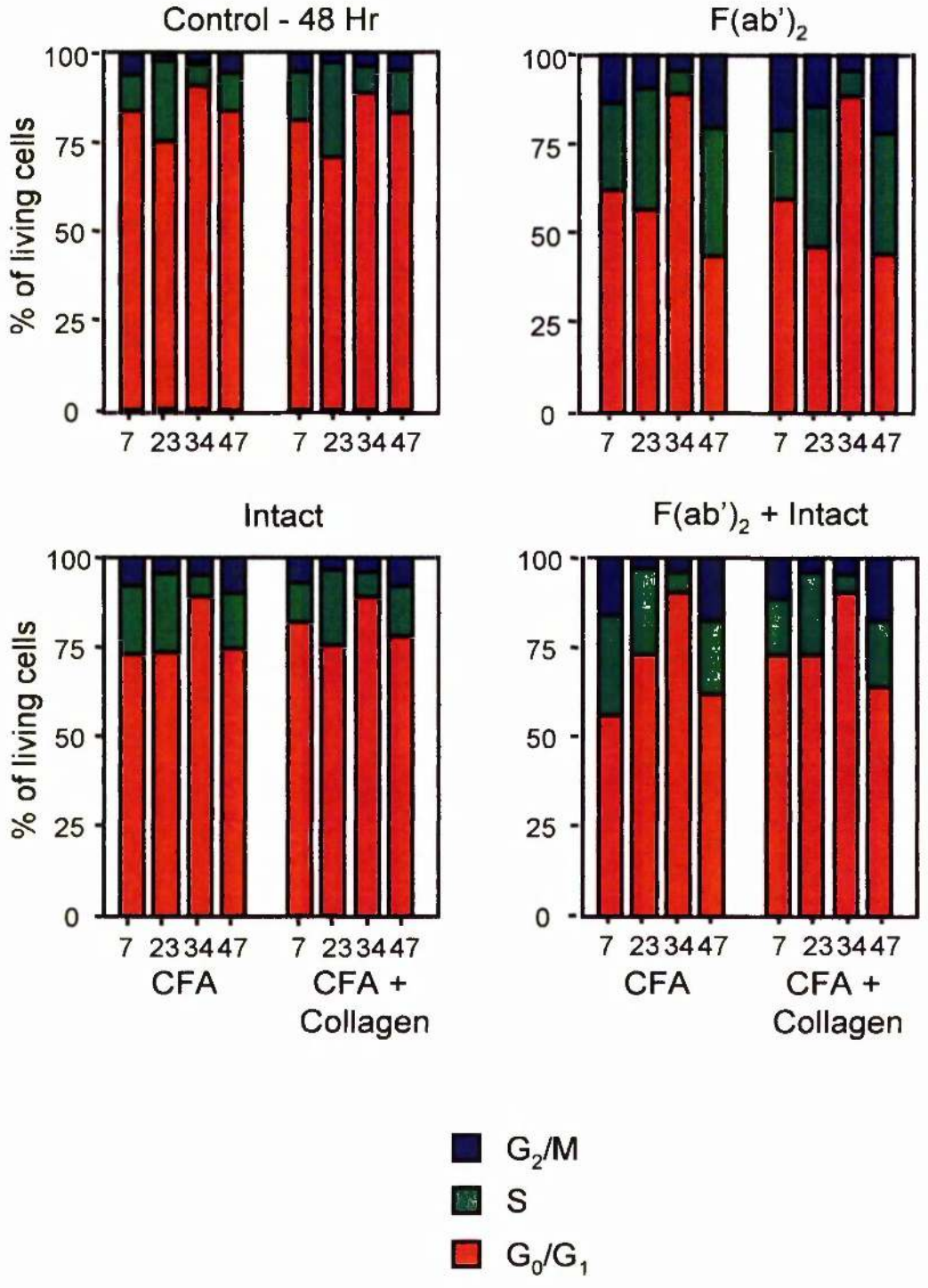

Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact
% of living cells
100
75
50
25
0
7 23 34 47 7 23 34 47
CFA
CFA + Collagen
G2/M
S
G0/G1

Figure 4.7 Effect of FcγRIIb co-ligation on BCR–mediated ErkMAPK phosphorylation in splenic B cells from CFA/Collagen primed DBA/1 mice, *ex vivo* (d21-d25).

Purified splenic B cells from CFA/Collagen primed DBA/1 mice (1.5×10^7 cells) were stimulated for 48 hours in the presence of 50 μg/ml collagen or 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Whole cell lysates were immunoprecipitated with anti-ErkMAPK antibody (Erk23) and Western blotted for phospho-ErkMAPK. The numbers beneath the blots represent the relative band density as assessed by densitometry from a single experiment, compared to the band density obtained for the individual control at 48 hours and arbitrarily set as 1. (–) represents bands that were too faint for accurate quantitation.

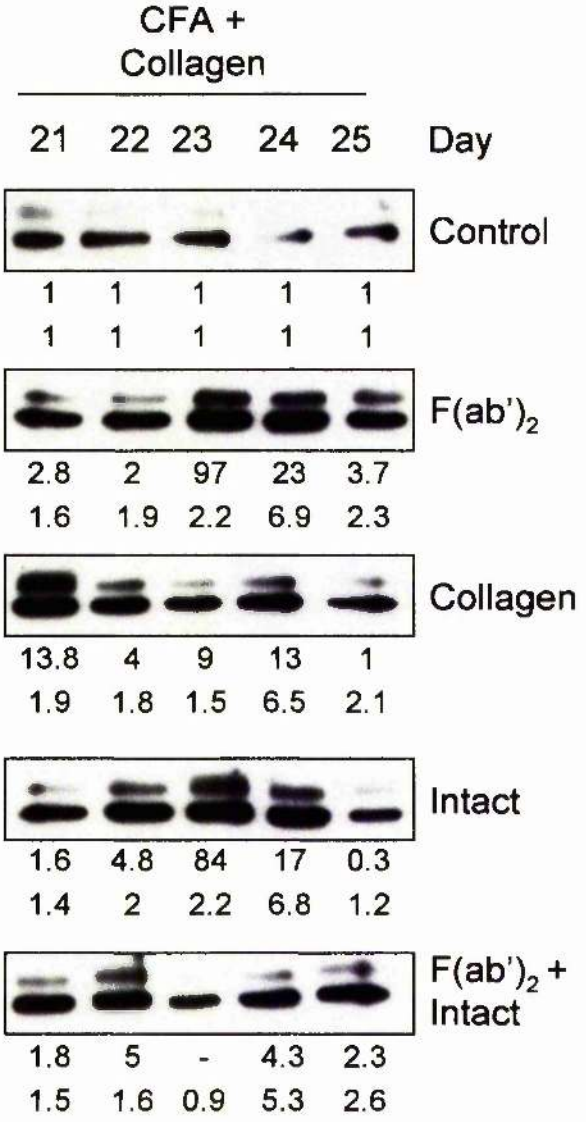

CFA +
Collagen
21 22 23 24 25 Day
Control
1 1 1 1 1
1 1 1 1 1
F(ab')2
2.8 2 97 23 3.7
1.6 1.9 2.2 6.9 2.3
Collagen
13.8 4 9 13 1
1.9 1.8 1.5 6.5 2.1
Intact
1.6 4.8 84 17 0.3
1.4 2 2.2 6.8 1.2
F(ab')2 +
Intact
1.8 5 - 4.3 2.3
1.5 1.6 0.9 5.3 2.6

Figure 4.8 Effect of FcγRIIb co-ligation on the BCR–mediated proliferative response of splenic B cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d21-d25).

Purified splenic B cells from CFA control or CFA/Collagen primed DBA/1 mice (2×10^5 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment.

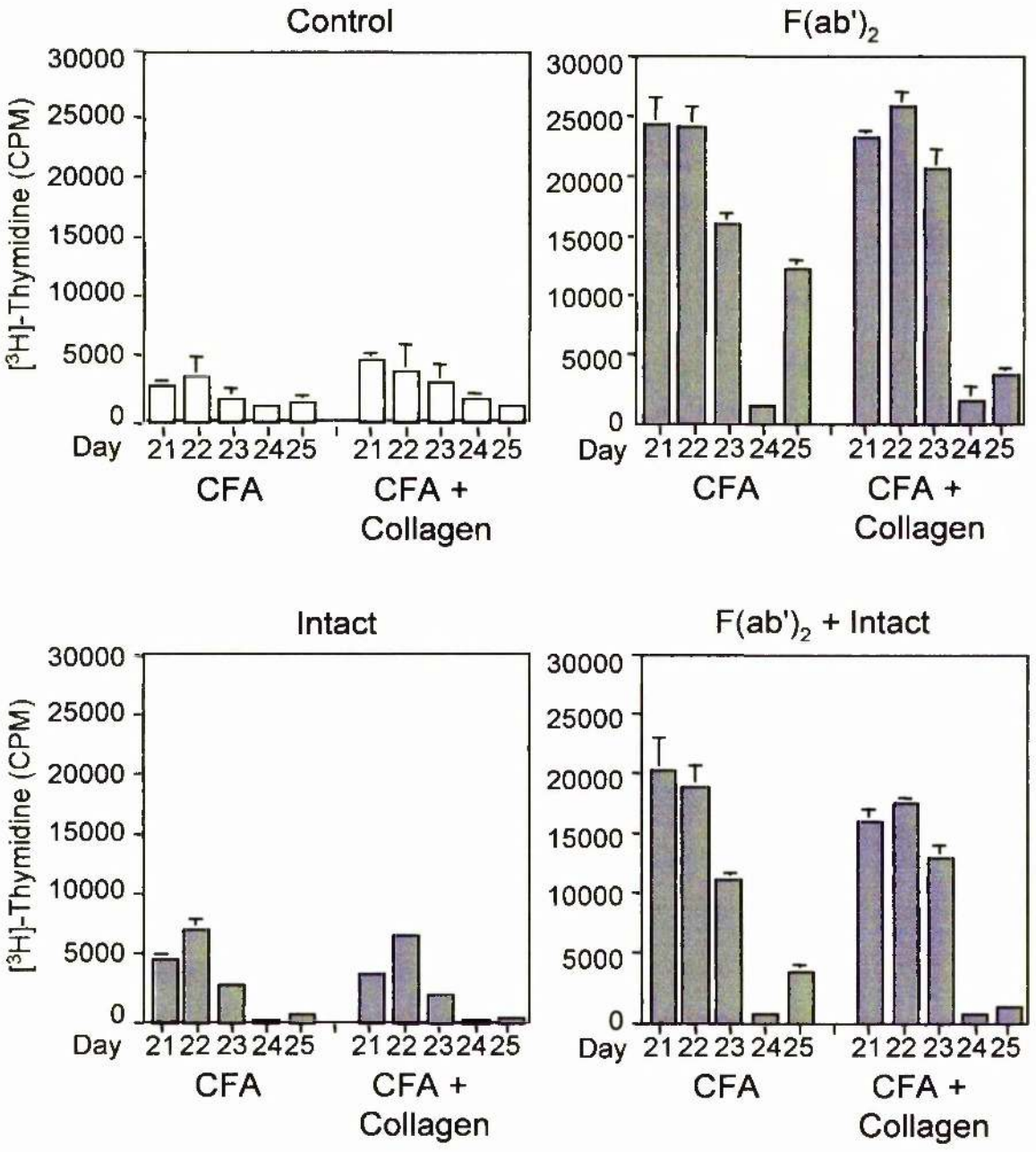

Control
F(ab')2
Intact
F(ab')2 + Intact
[3H]-Thymidine (CPM)
30000
25000
20000
15000
10000
5000
0
Day
21 22 23 24 25
CFA
CFA + Collagen

Figure 4.9 Effects of FcγRIIb ligation on the collagen-specific proliferative response of splenic B cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d21-d25).

Purified splenic B cells from CFA control or CFA/Collagen primed DBA/1 mice (2×10^5 cells) were stimulated for 48 hours in the presence of 50 μg/ml collagen or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment.

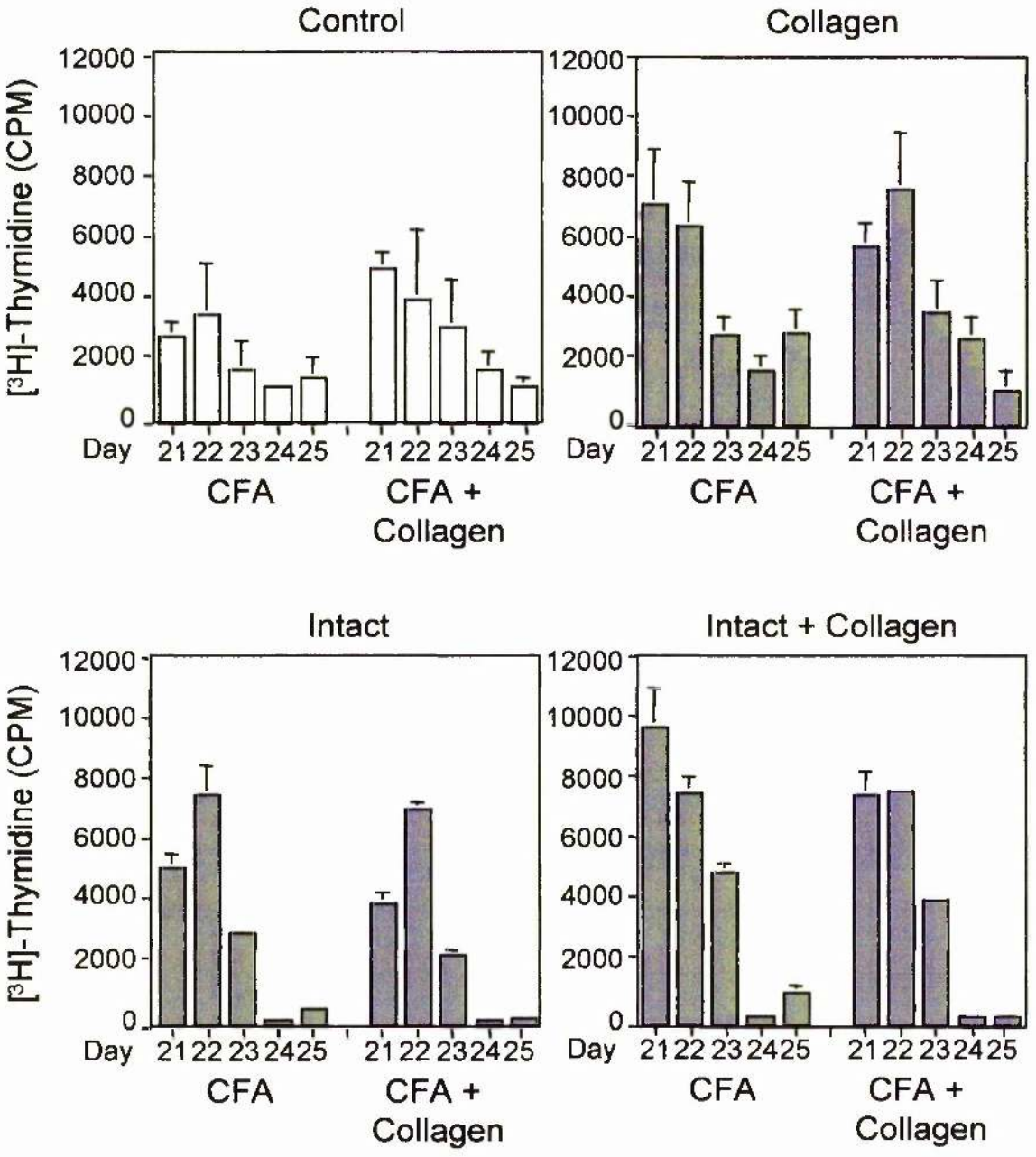

Control
Collagen
Intact
Intact + Collagen
[3H]-Thymidine (CPM)
12000
10000
8000
6000
4000
2000
0
Day
21 22 23 24 25
CFA
CFA + Collagen

Figure 4.10 DNA content of splenic B cells from CFA control and CFA/Collagen primed DBA/1 mice post-sacrifice or following BCR and FcγRIIb co-ligation, *ex vivo* (d21-d25).

Purified splenic B cells from CFA control and CFA/Collagen primed DBA/1 mice (1×10^6 cells) were subjected to DNA content analysis post-sacrifice or were stimulated for 48 hours in the presence of 50 μg/ml collagen or 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total number of cells analysed from a single experiment.

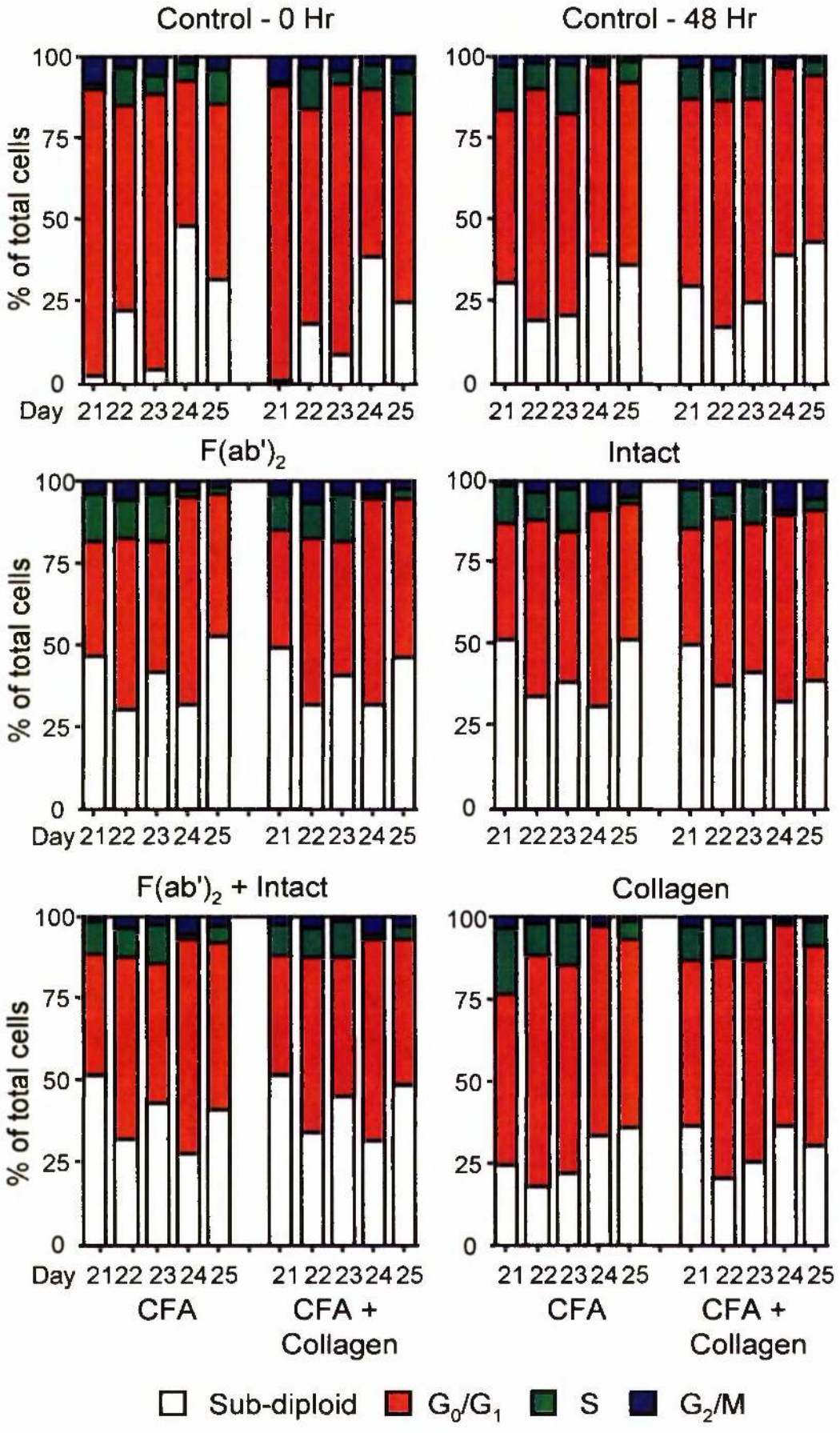

Control - 0 Hr
Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact
Collagen
% of total cells
100
75
50
25
0
Day 21 22 23 24 25 21 22 23 24 25
CFA
CFA + Collagen
Sub-diploid
G0/G1
S
G2/M

Figure 4.11 DNA content of the living population of splenic B cells from CFA control and CFA/Collagen primed DBA/1 mice post-sacrifice or following BCR and FcγRIIb co-ligation, *ex vivo* (d21-d25).

Purified splenic B cells from CFA control and CFA/Collagen primed DBA/1 mice (1×10^6 cells) were subjected to DNA content analysis post-sacrifice or were stimulated for 48 hours in the presence of 50 μg/ml collagen or 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total living population of cells analysed from a single experiment.

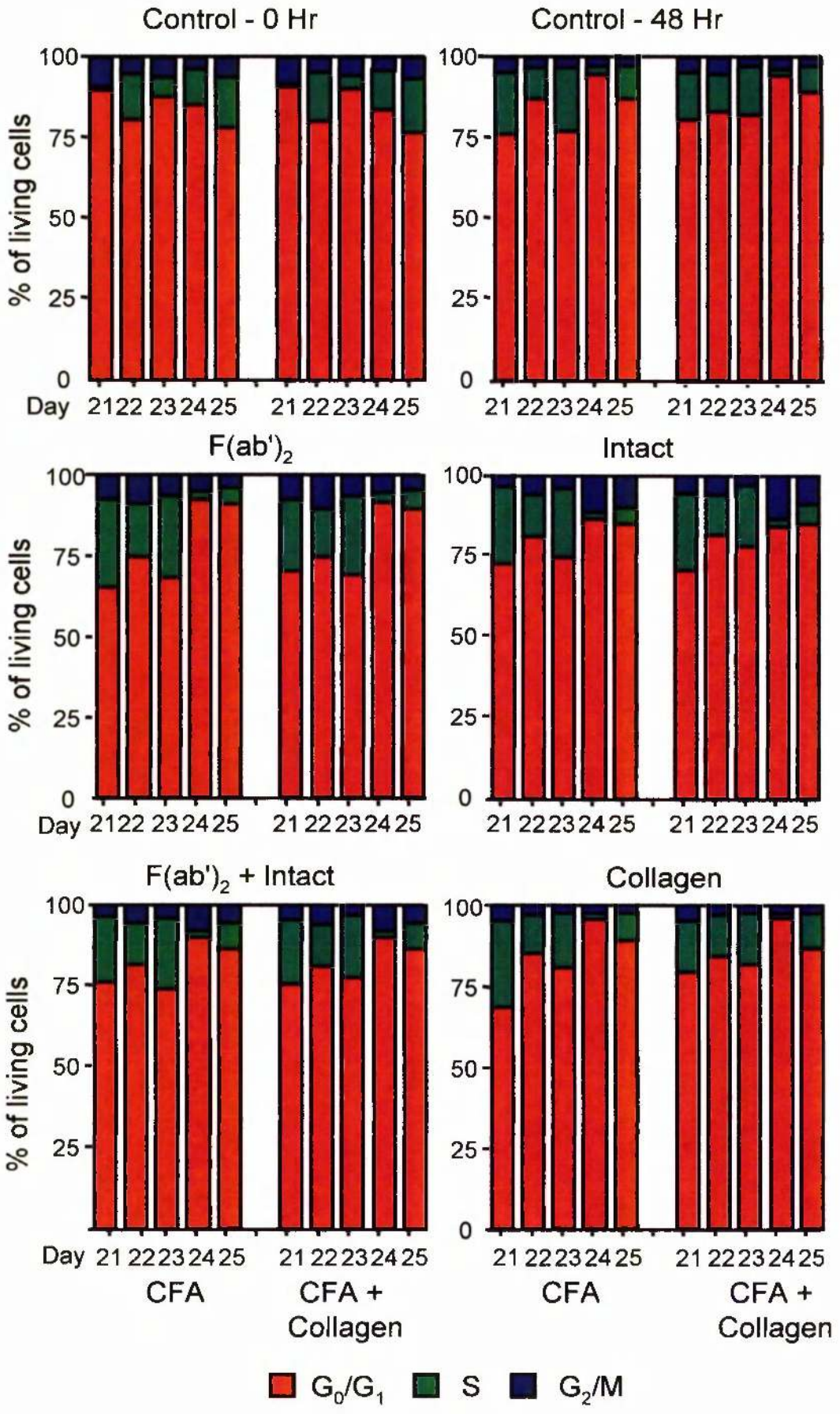

Control - 0 Hr
Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact
Collagen
% of living cells
100
75
50
25
0
Day 21 22 23 24 25 21 22 23 24 25
CFA
CFA + Collagen
G0/G1
S
G2/M

Figure 4.12 Effect of FcγRIIb co-ligation on the BCR–mediated proliferative response of splenic mononuclear cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d8-d34).

Splenic mononuclear cells from CFA control and CFA/Collagen primed DBA/1 mice (5×10^5 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements.

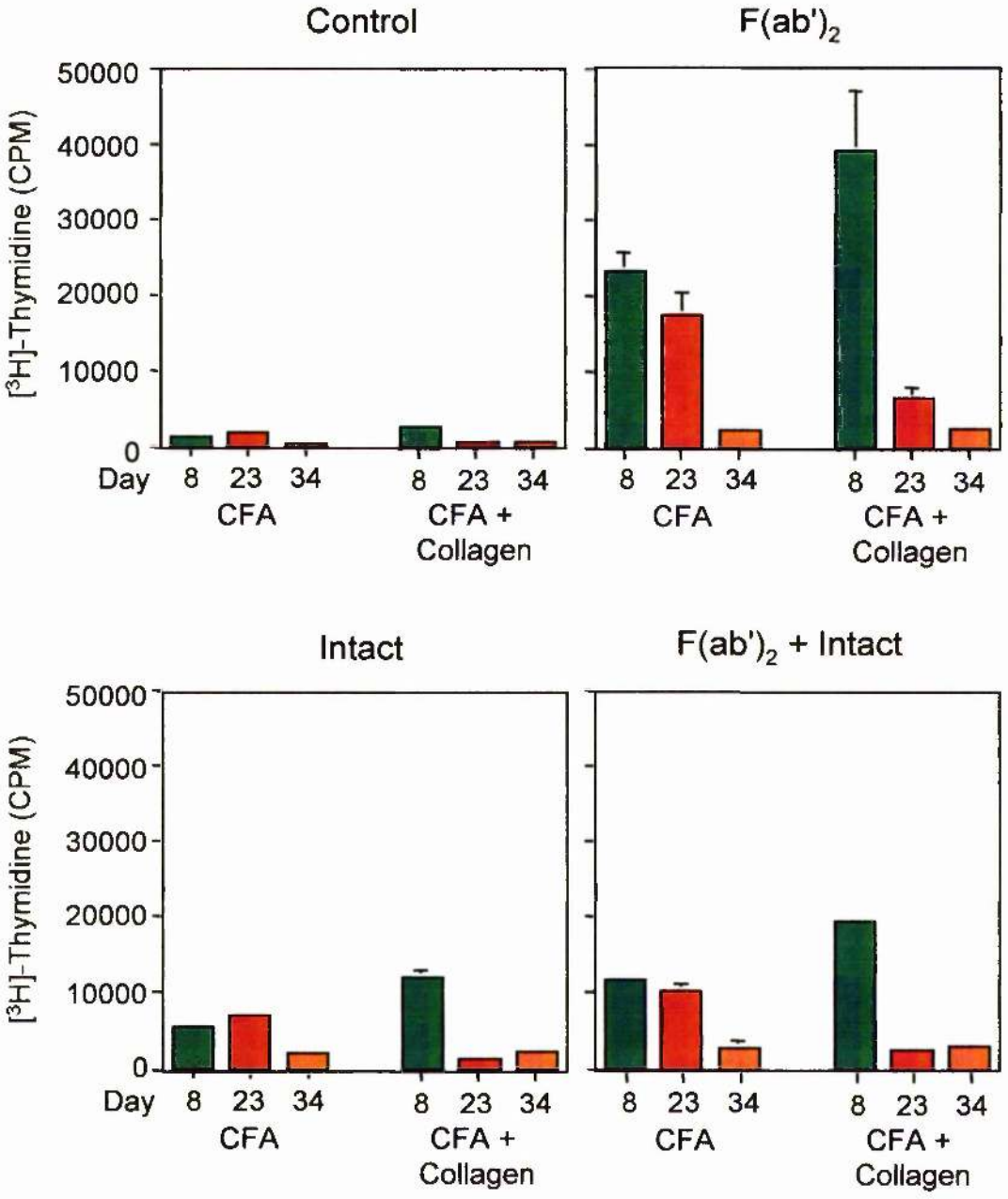

Control
F(ab')2
Intact
F(ab')2 + Intact
[3H]-Thymidine (CPM)
50000
40000
30000
20000
10000
0
Day
8
23
34
CFA
CFA + Collagen

Figure 4.13 Effect of FcγRIIb ligation on the collagen-specific proliferative response of splenic mononuclear cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d8-d34).

Splenic mononuclear cells from CFA control or CFA/Collagen primed DBA/1 mice (5×10^5 cells) were stimulated for 48 hours in the presence of 50 μg/ml collagen or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment.[^{3}H]-incorporation data from stimulation of splenic mononuclear cells from naïve DBA/1 mice: **Collagen** 3681 ± 394, **Intact + Collagen** 16181 ± 1731.

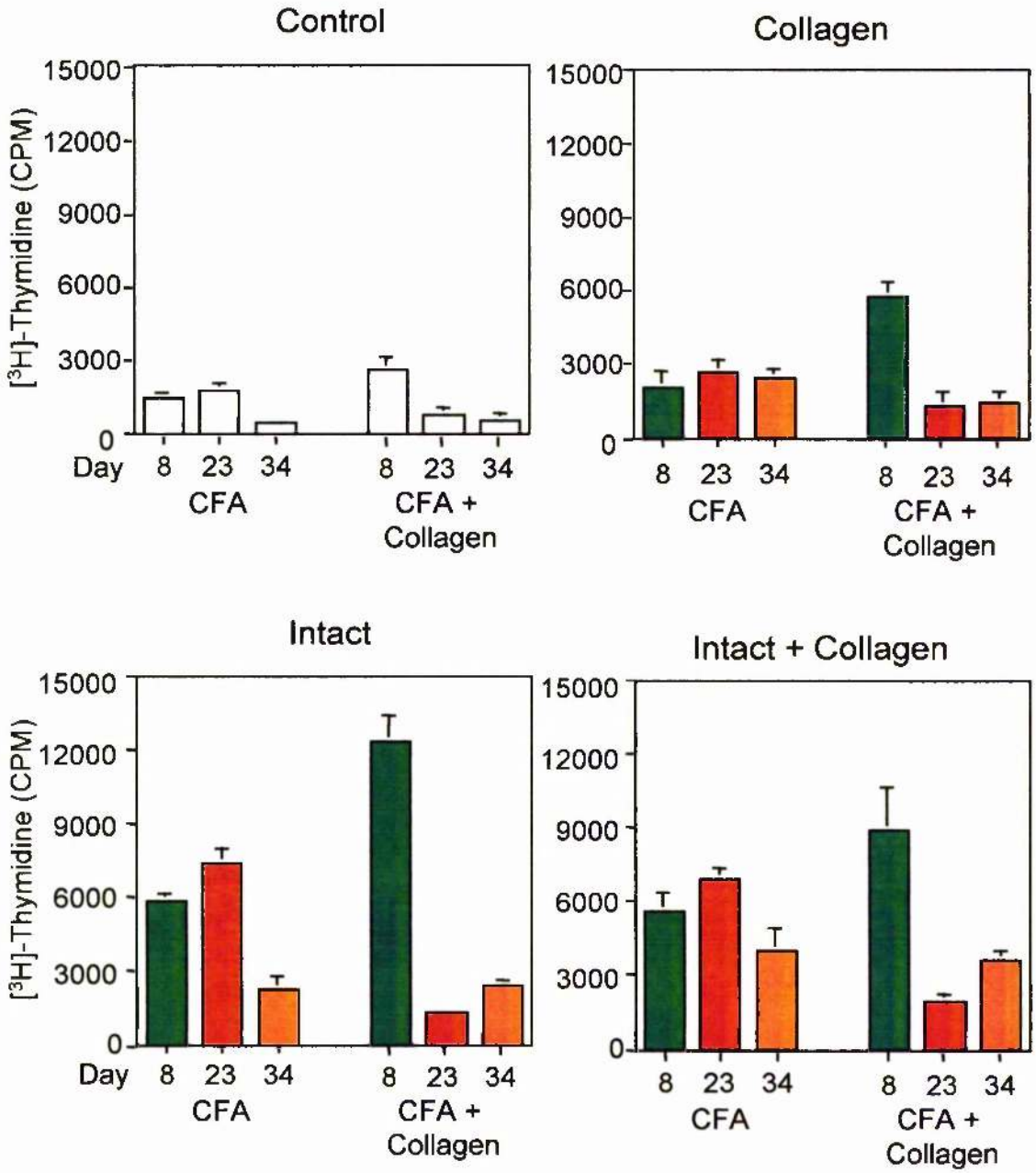

Control
Collagen
Intact
Intact + Collagen
[3H]-Thymidine (CPM)
15000
12000
9000
6000
3000
0
Day
8
23
34
CFA
CFA + Collagen

Figure 4.14 DNA content of splenic mononuclear cells from naïve, CFA control or CFA/Collagen primed DBA/1 mice post-sacrifice and following BCR, collagen or FcγRIIb stimulation of naïve splenic B cells, *ex vivo* (d8-d34).

Splenic mononuclear cells from naïve, CFA control and CFA/Collagen DBA/1 mice (1 x 10^6 cells) were subjected to DNA content analysis post-sacrifice. In addition, splenic mononuclear cells from naive DBA/1 mice (1 x 10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml collagen or 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total number of cells analysed from a single experiment.

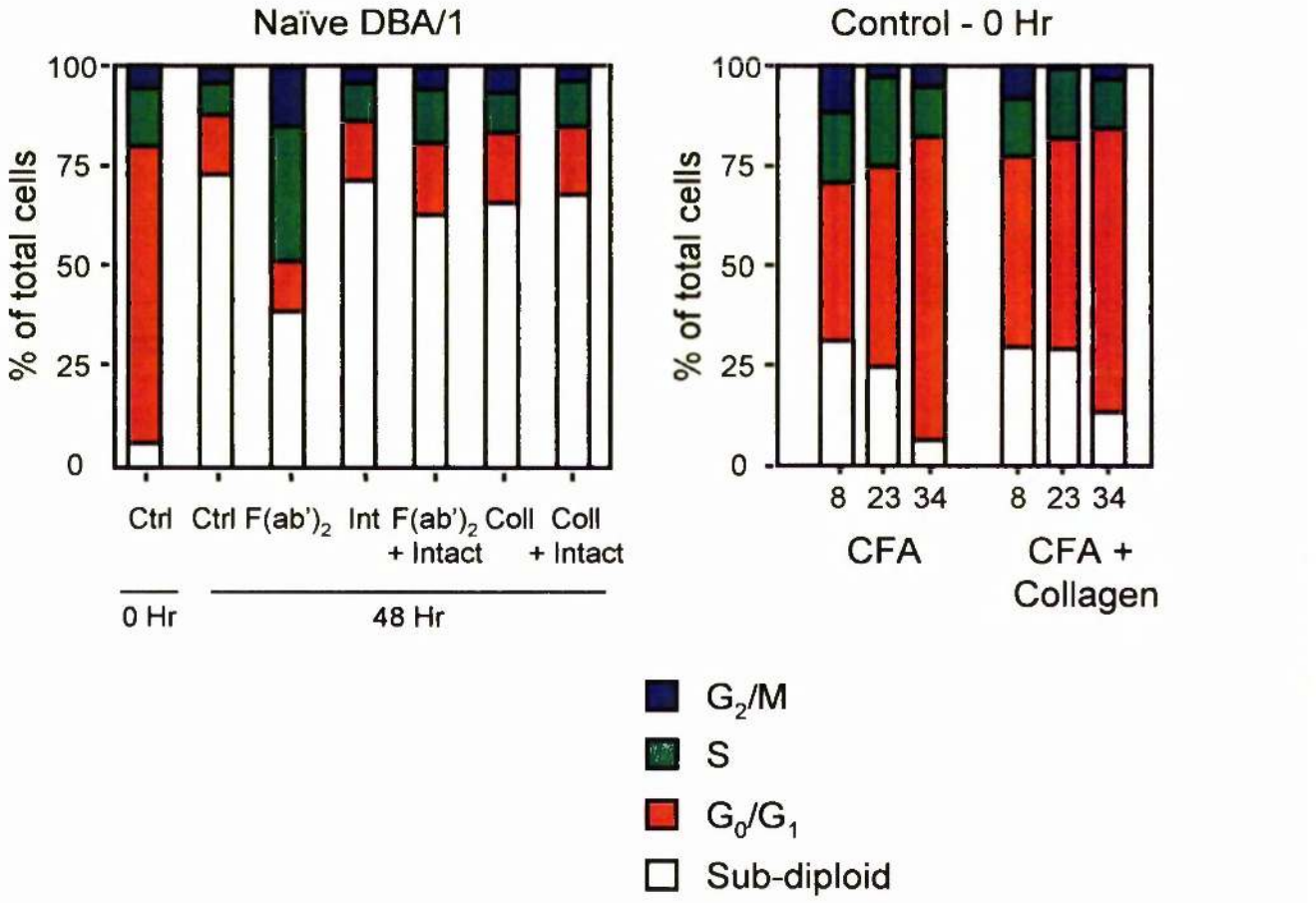

Naïve DBA/1
Control - 0 Hr
% of total cells
100
75
50
25
0
Ctrl
Ctrl
F(ab')2
Int
F(ab')2 + Intact
Coll
Coll + Intact
0 Hr
48 Hr
8
23
34
8
23
34
CFA
CFA + Collagen
G2/M
S
G0/G1
Sub-diploid

Figure 4.15 Effects of BCR and FcγRIIb co-ligation on the DNA content of splenic mononuclear cells from CFA control or CFA/Collagen primed DBA/1 mice, *ex vivo* (d8-d34).

Splenic mononuclear cells from CFA control and CFA/Collagen DBA/1 mice (1 x 10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total number of cells analysed from a single experiment.

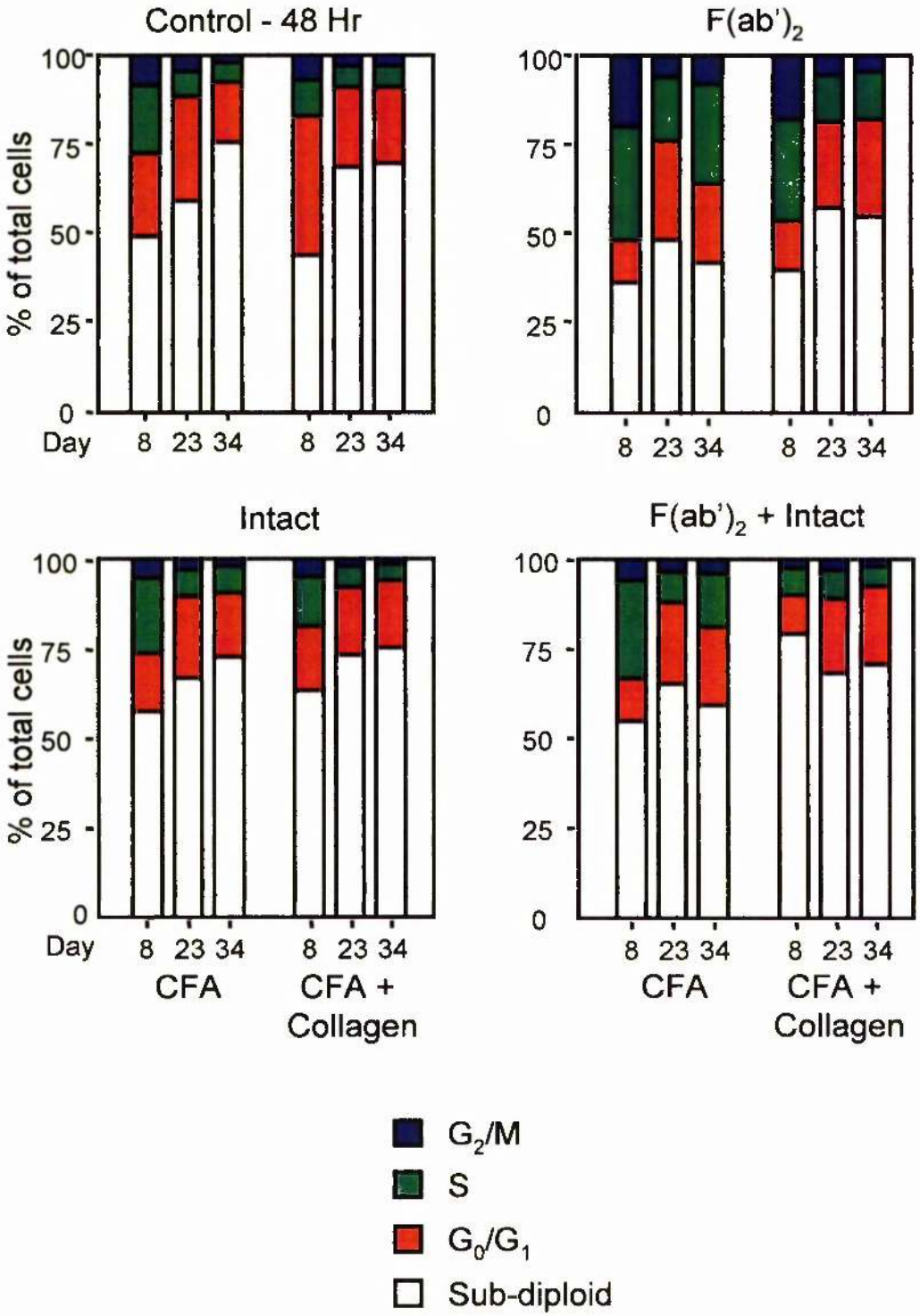

Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact
% of total cells
100
75
50
25
0
Day
8 23 34 8 23 34
CFA
CFA + Collagen
G2/M
S
G0/G1
Sub-diploid

Figure 4.16 Effect of FcγRIIb co-ligation on the DNA content of collagen-specific splenic mononuclear cells from CFA control and CIA mice, *ex vivo* (d8-d34).

Splenic mononuclear cells from CFA control or CFA/Collagen primed DBA/1 mice (1 x 10^6 cells) were stimulated for 48 hours in the presence 50 μg/ml collagen or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total number of cells analysed from a single experiment.

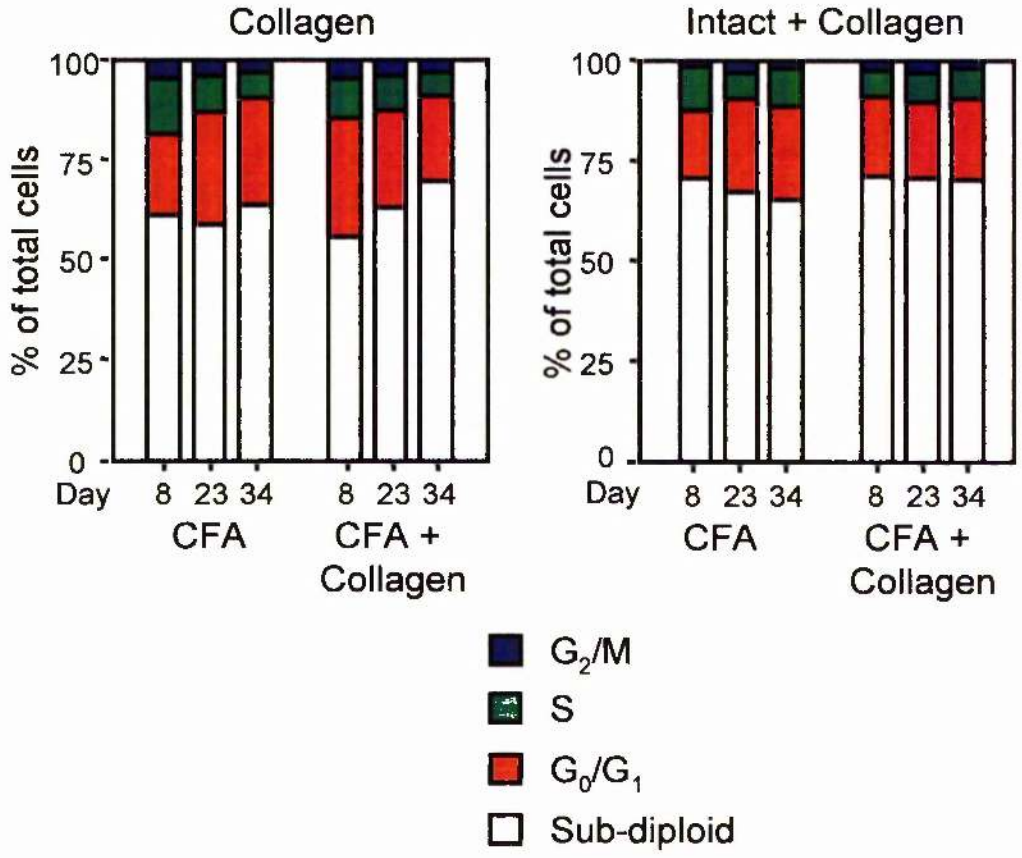

Collagen
Intact + Collagen
100
75
50
25
0
% of total cells
Day
8 23 34
8 23 34
CFA
CFA + Collagen
G_2/M
S
G_0/G_1
Sub-diploid

Figure 4.17 DNA content of the living population of splenic mononuclear cells from naïve, CFA control or CFA/Collagen primed DBA/1 mice post-sacrifice and following BCR, collagen or FcγRIIb stimulation of naïve splenic mononuclear cells, *ex vivo* (d8-d34).

Splenic mononuclear cells from naïve, CFA control and CFA/Collagen DBA/1 mice (1 x 10^6 cells) were subjected to DNA content analysis post-sacrifice. In addition, splenic mononuclear cells from naive DBA/1 mice (1 x 10^6 cells) were stimulated for 48 hours in the presence 50 μg/ml collagen or 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total living population of cells analysed from a single experiment.

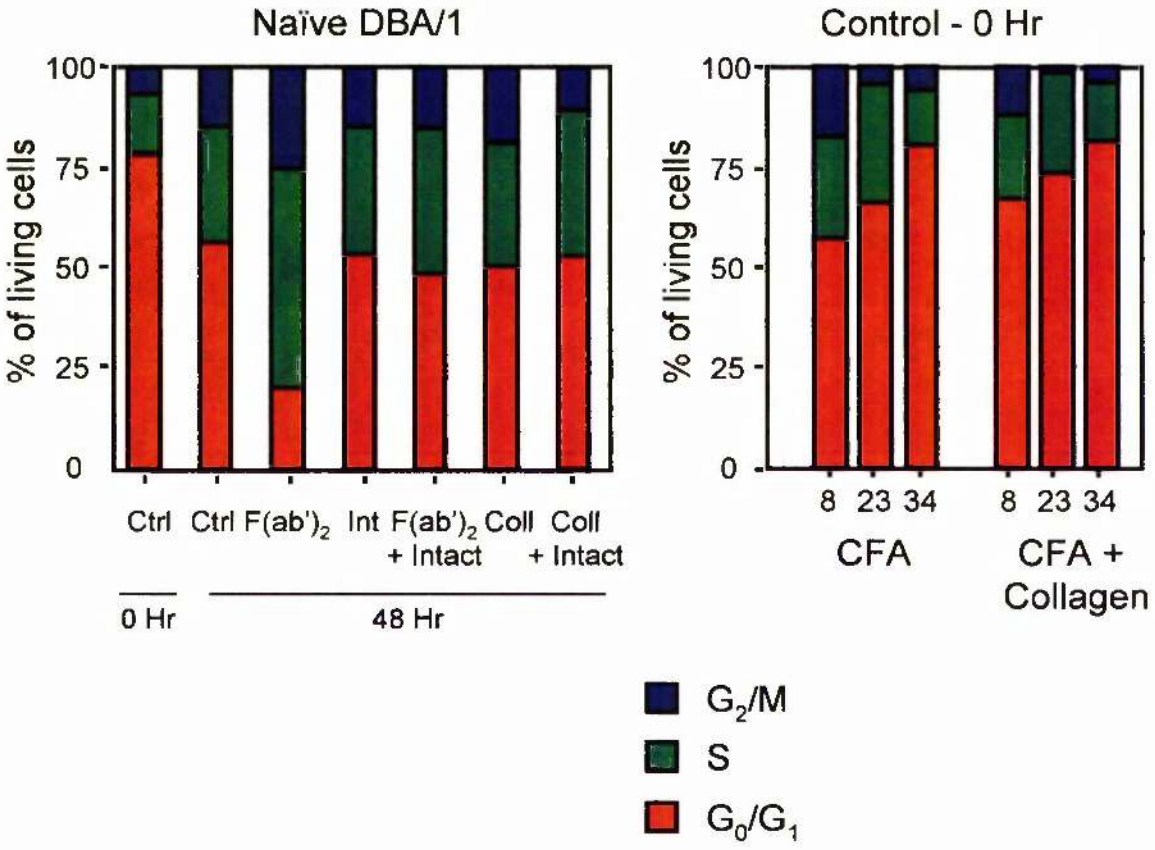

Naïve DBA/1
Control - 0 Hr
% of living cells
100
75
50
25
0
Ctrl
Ctrl
F(ab')2
Int
F(ab')2 + Intact
Coll
Coll + Intact
0 Hr
48 Hr
8
23
34
8
23
34
CFA
CFA + Collagen
G2/M
S
G0/G1

Figure 4.18 Effects of BCR and FcγRIIb co-ligation on the DNA content of the living population of splenic mononuclear cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d8-d34).

Splenic mononuclear cells from CFA control or CFA/Collagen primed DBA/1 mice (1 x 10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total living population of cells analysed from a single experiment.

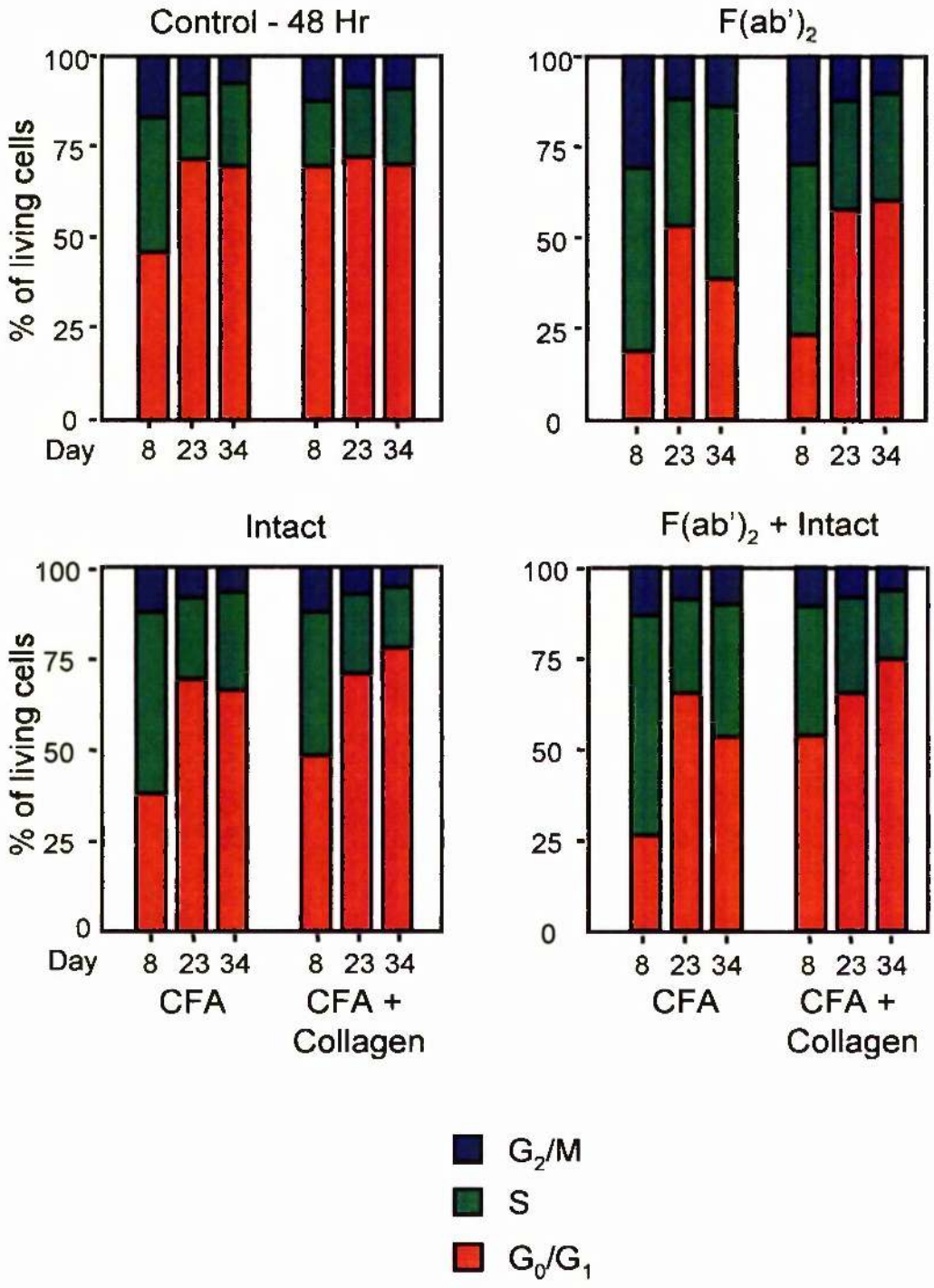

Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact
% of living cells
100
75
50
25
0
Day
8 23 34 8 23 34
CFA
CFA + Collagen
G2/M
S
G0/G1

Figure 4.19 Effect of FcγRIIb co-ligation on the DNA content of the living population of collagen-specific splenic mononuclear cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d8-d34).

Splenic mononuclear cells from CFA control or CFA/Collagen primed DBA/1 mice (1 x 10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml collagen or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total living population of cells analysed from a single experiment.

.

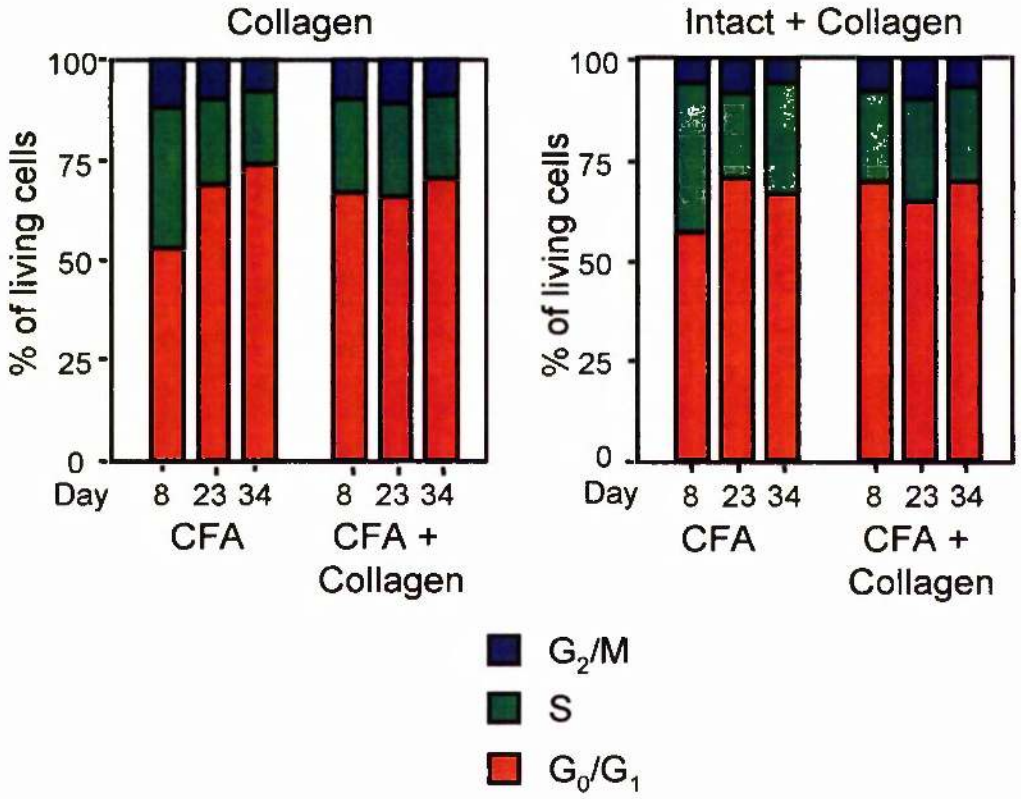

Collagen
Intact + Collagen
100
75
50
25
0
% of living cells
Day
8 23 34
8 23 34
CFA
CFA + Collagen
G_2/M
S
G_0/G_1

Figure 4.20 Mitochondrial membrane potential of splenic mononuclear cells from naïve, CFA control or CFA/Collagen primed DBA/1 mice post-sacrifice and following BCR, collagen or FcγRIIb stimulation of naïve splenic mononuclear cells, *ex vivo* (d8-d34).

The mitochondrial membrane potential of splenic mononuclear cells from naïve, CFA control and CFA/Collagen DBA/1 mice (1×10^6 cells) was analysed post-sacrifice. In addition, splenic mononuclear cells from naive DBA/1 mice (1×10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml collagen or 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. The mitochondrial membrane potential was determined by $DiOC_6(3)$ staining and FACS analysis, as described in the Materials and Methods. Data represents $DiOC_6\ (3)^{LOW}$ and $DiOC_6\ (3)^{HIGH}$ cell populations, as determined on a logarithmic FL-1 axis, and expressed as a percentage of the total number of cells analysed from a single experiment.

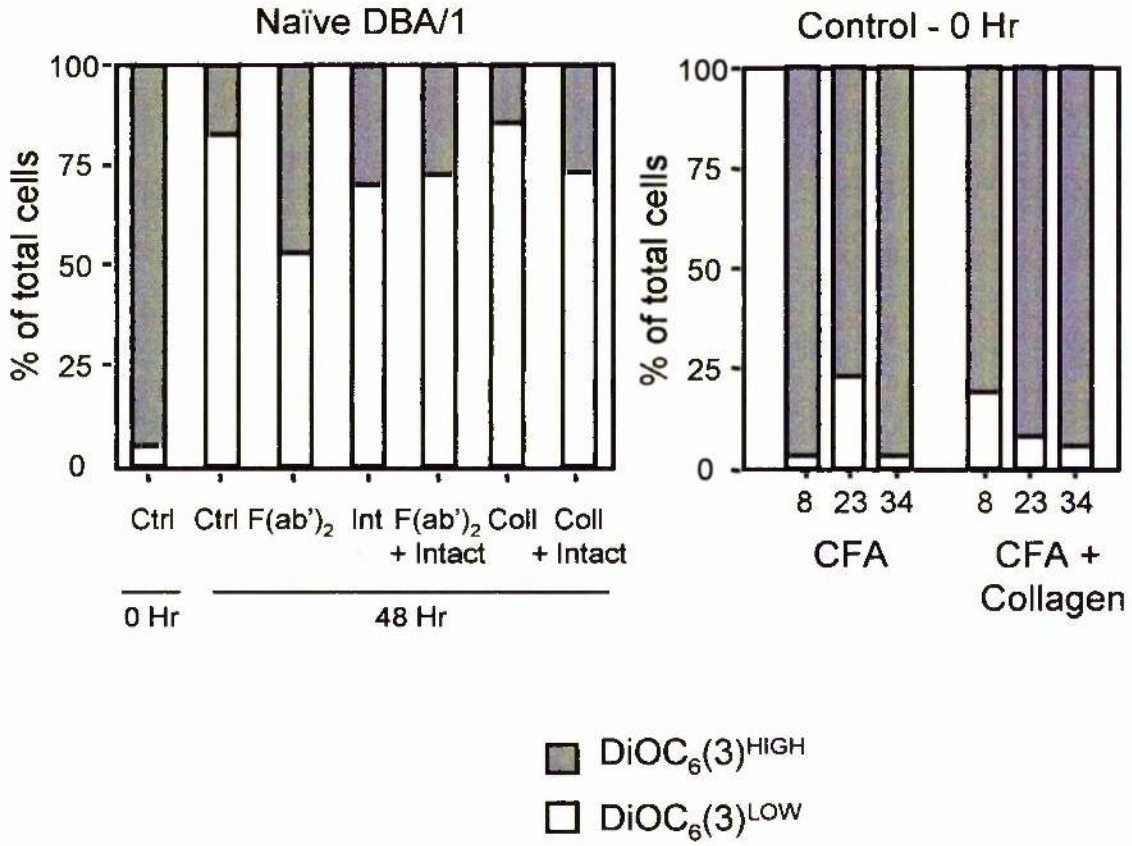

Naïve DBA/1
Control - 0 Hr
% of total cells
100
75
50
25
0
Ctrl
Ctrl
F(ab')2
Int
F(ab')2 + Intact
Coll
Coll + Intact
0 Hr
48 Hr
8 23 34
8 23 34
CFA
CFA + Collagen
DiOC6(3)HIGH
DiOC6(3)LOW

Figure 4.21 Effects of BCR and FcγRIIb co-ligation on the mitochondrial membrane potential of splenic mononuclear cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d8-d34).

Splenic mononuclear cells from CFA control or CFA/Collagen primed DBA/1 mice (1 x 10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. The mitochondrial membrane potential was determined by $DiOC_6(3)$ staining and FACS analysis, as described in the Materials and Methods. Data represents $DiOC_6(3)^{LOW}$ and $DiOC_6(3)^{HIGH}$ cell populations, as determined on a logarithmic FL-1 axis, and expressed as a percentage of the total number of cells analysed from a single experiment.

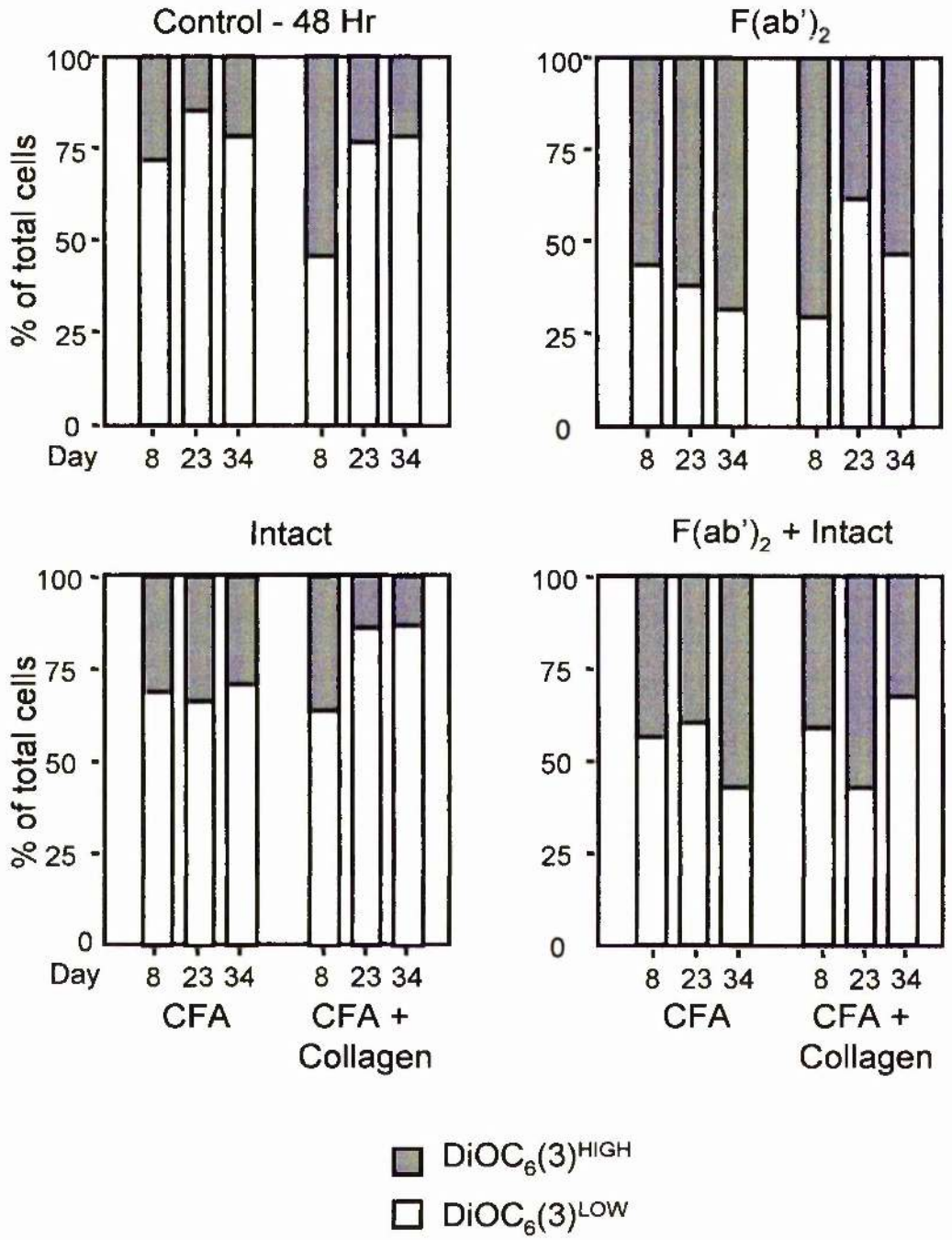

Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact
% of total cells
100
75
50
25
0
Day
8 23 34 8 23 34
CFA
CFA + Collagen
DiOC6(3)HIGH
DiOC6(3)LOW

Figure 4.22 Effects of FcγRIIb ligation on the mitochondrial membrane potential of collagen-specific splenic mononuclear cells from CFA control and CFA/Collagen primed DBA/1 mice, *ex vivo* (d8-d34).

Splenic mononuclear cells from CFA control or CFA/Collagen primed DBA/1 mice (1 x 10^6 cells) were stimulated for 48 hours in the presence of 50 μg/ml collagen or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. The mitochondrial membrane potential was determined by $DiOC_6(3)$ staining and FACS analysis, as described in the Materials and Methods. Data represents $DiOC_6(3)^{LOW}$ and $DiOC_6(3)^{HIGH}$ cell populations, as determined on a logarithmic FL-1 axis, and expressed as a percentage of the total number of cells analysed from a single experiment.

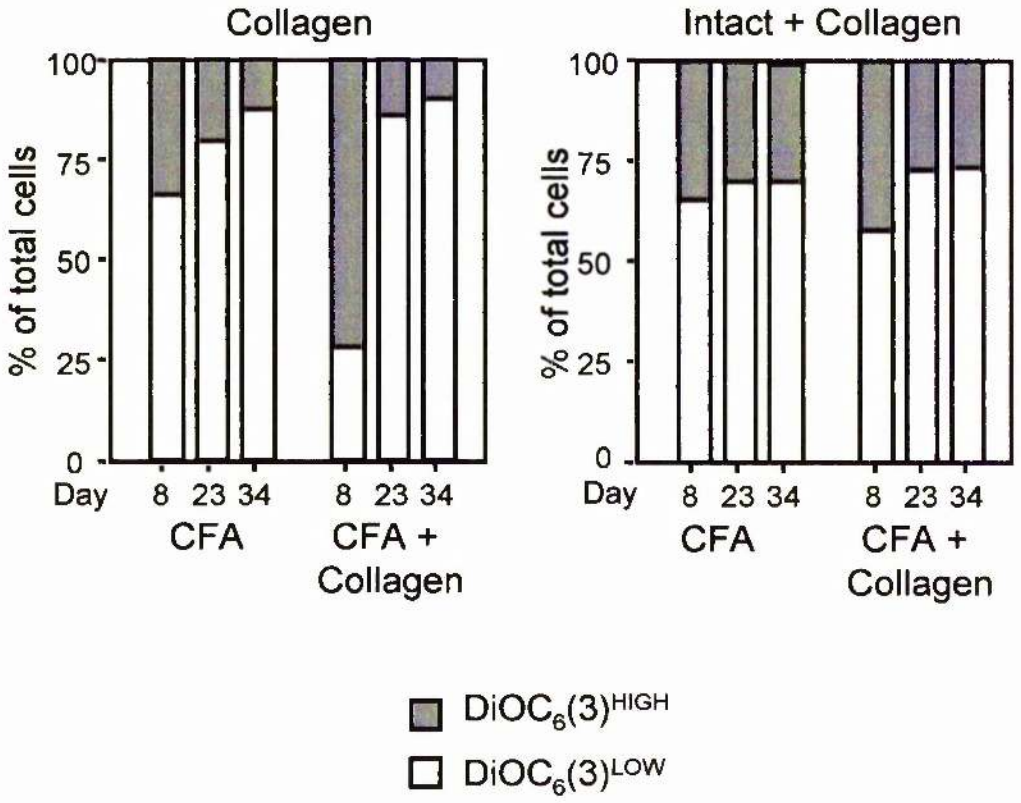

Collagen
Intact + Collagen
100
75
50
25
0
% of total cells
Day
8 23 34
8 23 34
CFA
CFA + Collagen
DiOC6(3)HIGH
DiOC6(3)LOW

Figure 4.23 Effect of FcγRIIb co-ligation on the BCR–mediated proliferative response of mononuclear cells from lymph nodes of CFA control and CIA mice, *ex vivo*, (d8-d34).

Mononuclear cells from lymph nodes of CFA or CFA/Collagen primed DBA/1 mice (5 x 10^5 cells) were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment. [^{3}H]-incorporation data from stimulation of mononuclear cells from lymph nodes of naïve DBA/1 mice was unavailable.

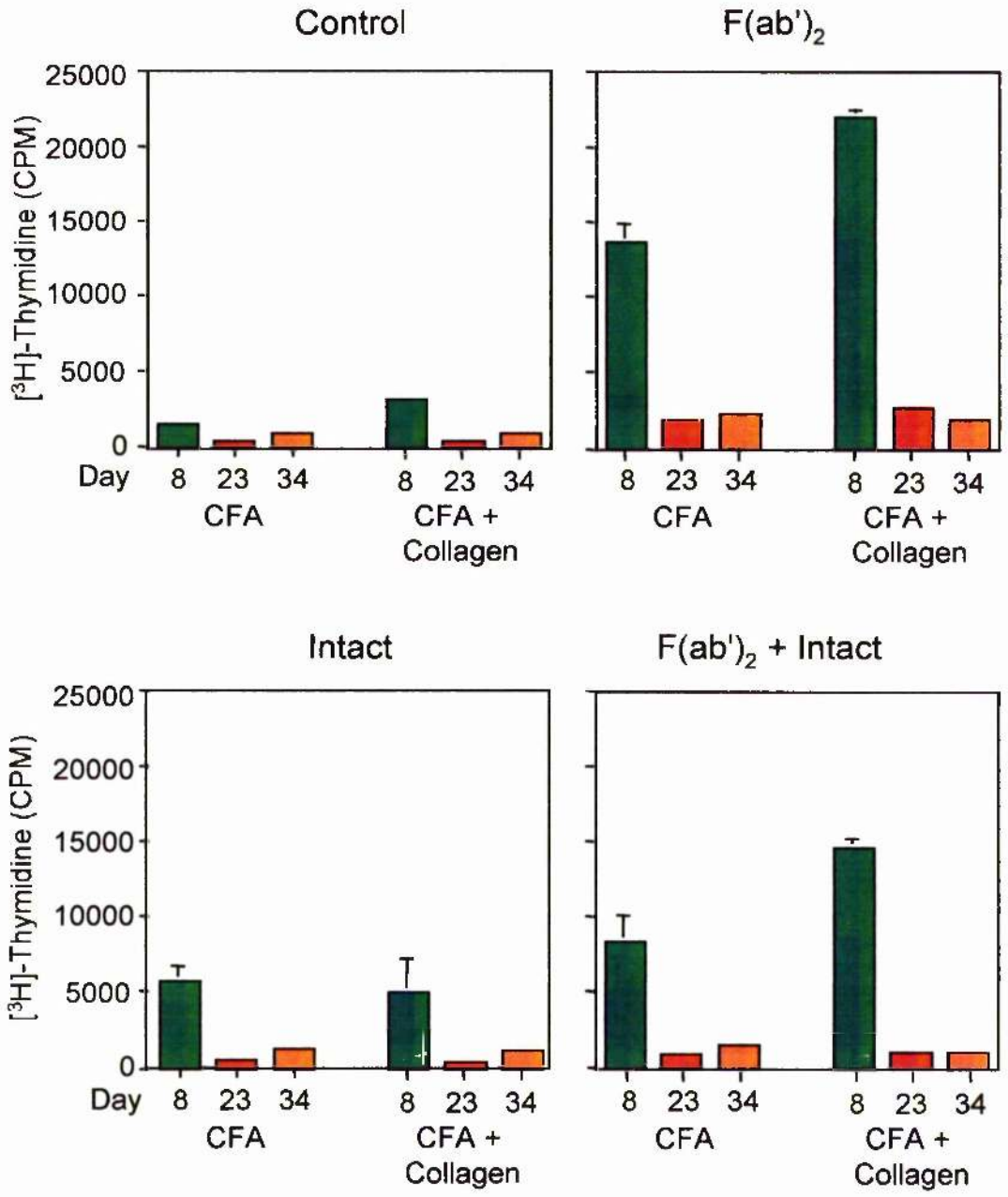

Control
F(ab')2
Intact
F(ab')2 + Intact
[3H]-Thymidine (CPM)
25000
20000
15000
10000
5000
0
Day
8 23 34
CFA
8 23 34
CFA + Collagen

Figure 4.24 Effects of FcγRIIb co-ligation on the collagen-specific proliferative response of mononuclear cells from lymph nodes of CFA control and CIA mice, *ex vivo*, (d8-d34).

Mononuclear cells from lymph nodes of CFA or CFA/Collagen primed DBA/1 mice (5 x10^5 cells) were stimulated for 48 hours in the presence of 50 μg/ml Collagen or 75μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment. [^{3}H]-incorporation data from stimulation of mononuclear cells from lymph nodes of naïve DBA/1 mice was unavailable.

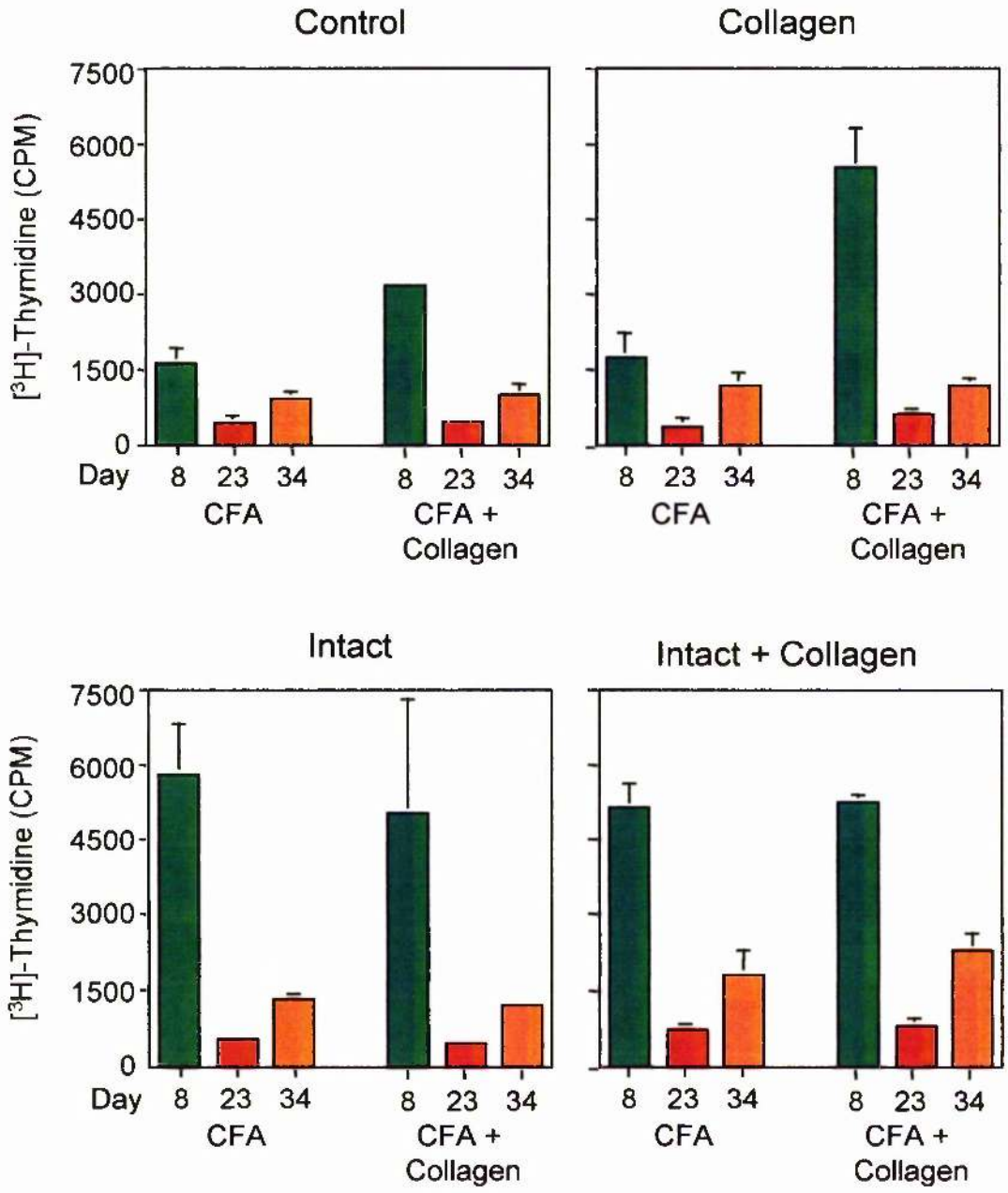

Control
Collagen
Intact
Intact + Collagen
[3H]-Thymidine (CPM)
7500
6000
4500
3000
1500
0
Day
8
23
34
CFA
CFA + Collagen

Figure 4.25 Effects of BCR and FcγRIIb co-ligation on the DNA content of mononuclear cells from lymph nodes of CFA control and CIA mice, *ex vivo* (d8-d34).

Mononuclear cells from lymph nodes of CFA control or CFA/Collagen primed DBA/1 mice (1 x 10^6 cells) were analysed for DNA content post-sacrifice or were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total number of cells analysed from a single experiment. n.d. = not done.

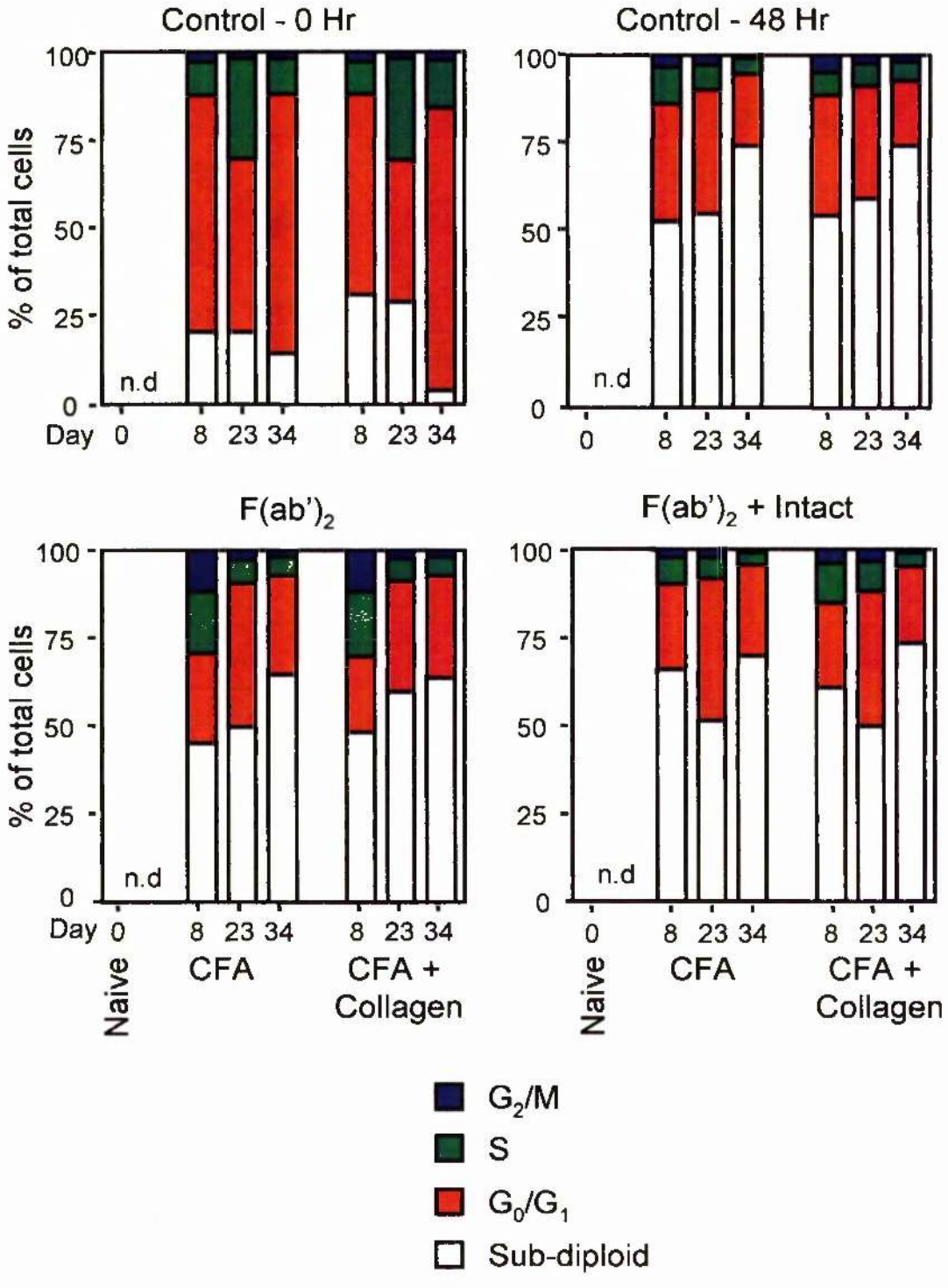

Control - 0 Hr
Control - 48 Hr
F(ab')2
F(ab')2 + Intact
% of total cells
100
75
50
25
0
n.d
Day 0 8 23 34 8 23 34
Naive
CFA
CFA + Collagen
G2/M
S
G0/G1
Sub-diploid

Figure 4.26 Effects of BCR and FcγRIIb co-ligation on the DNA content of the living population of mononuclear cells from lymph nodes of CFA control and CIA mice, *ex vivo* (d8-d34).

Mononuclear cells from lymph nodes of CFA control or CFA/Collagen primed DBA/1 mice (1 x 10^6 cells) were analysed for DNA content post-sacrifice or were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total living population of cells analysed from a single experiment. n.d. = not done.

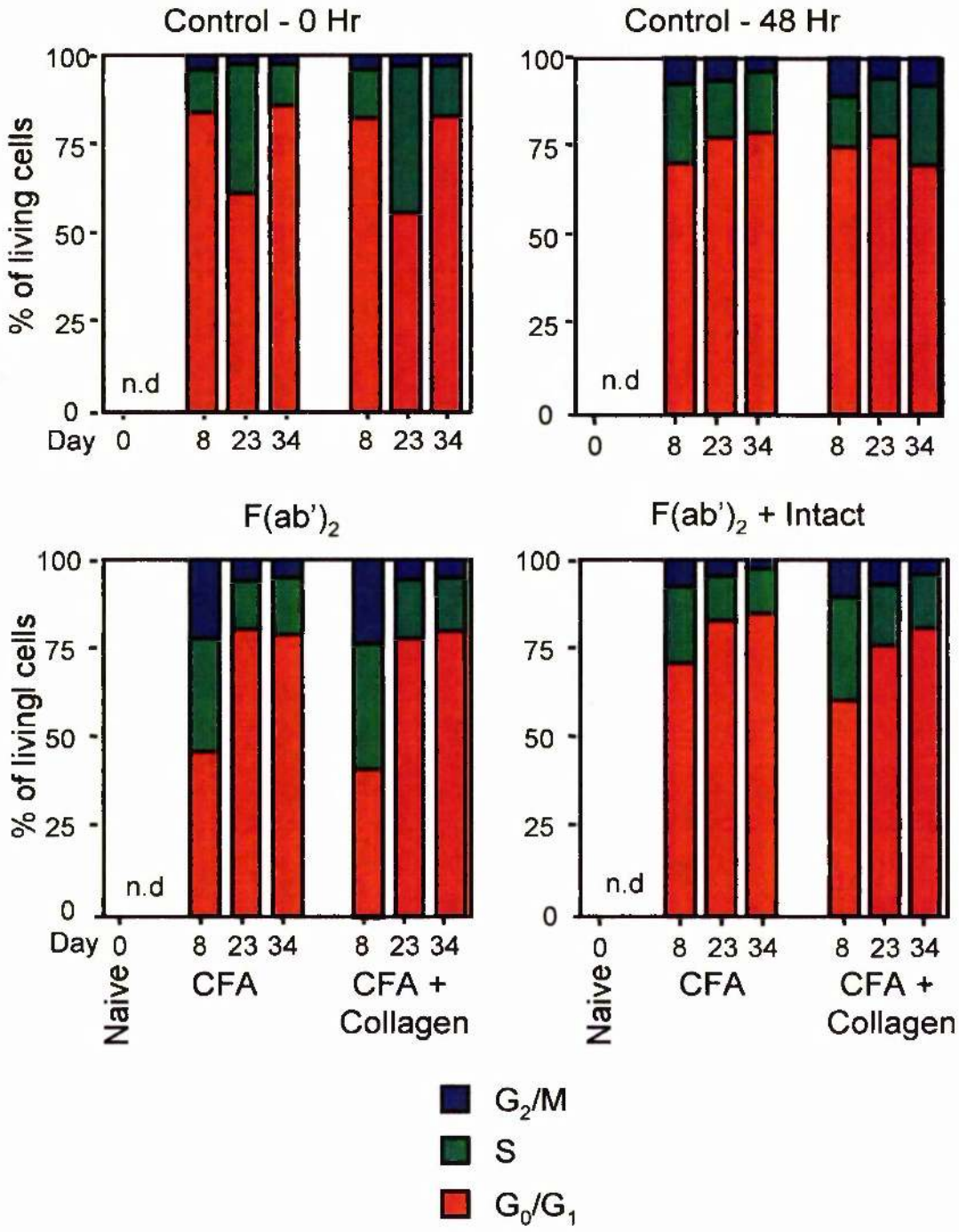

Control - 0 Hr
Control - 48 Hr
F(ab')2
F(ab')2 + Intact
% of living cells
% of livingl cells
100
75
50
25
0
n.d
Day 0 8 23 34 8 23 34
Naive
CFA
CFA + Collagen
G2/M
S
G0/G1

Figure 4.27 Effects of BCR and FcγRIIb co-ligation on the mitochondrial membrane potential of mononuclear cells from lymph nodes of CFA control and CIA mice, *ex vivo* (d8-d34).

The mitochondrial membrane potential of mononuclear cells from lymph nodes of CFA control or CFA/Collagen primed DBA/1 mice (1 x 10^6 cells) was analysed post-sacrifice of following stimulation for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. The mitochondrial membrane potential was determined by $DiOC_6(3)$ staining and FACS analysis, as described in the Materials and Methods. Data represents $DiOC_6(3)^{LOW}$ and $DiOC_6(3)^{HIGH}$ cell populations, as determined on a logarithmic FL-1 axis, and expressed as a percentage of the total number of cells analysed from a single experiment.

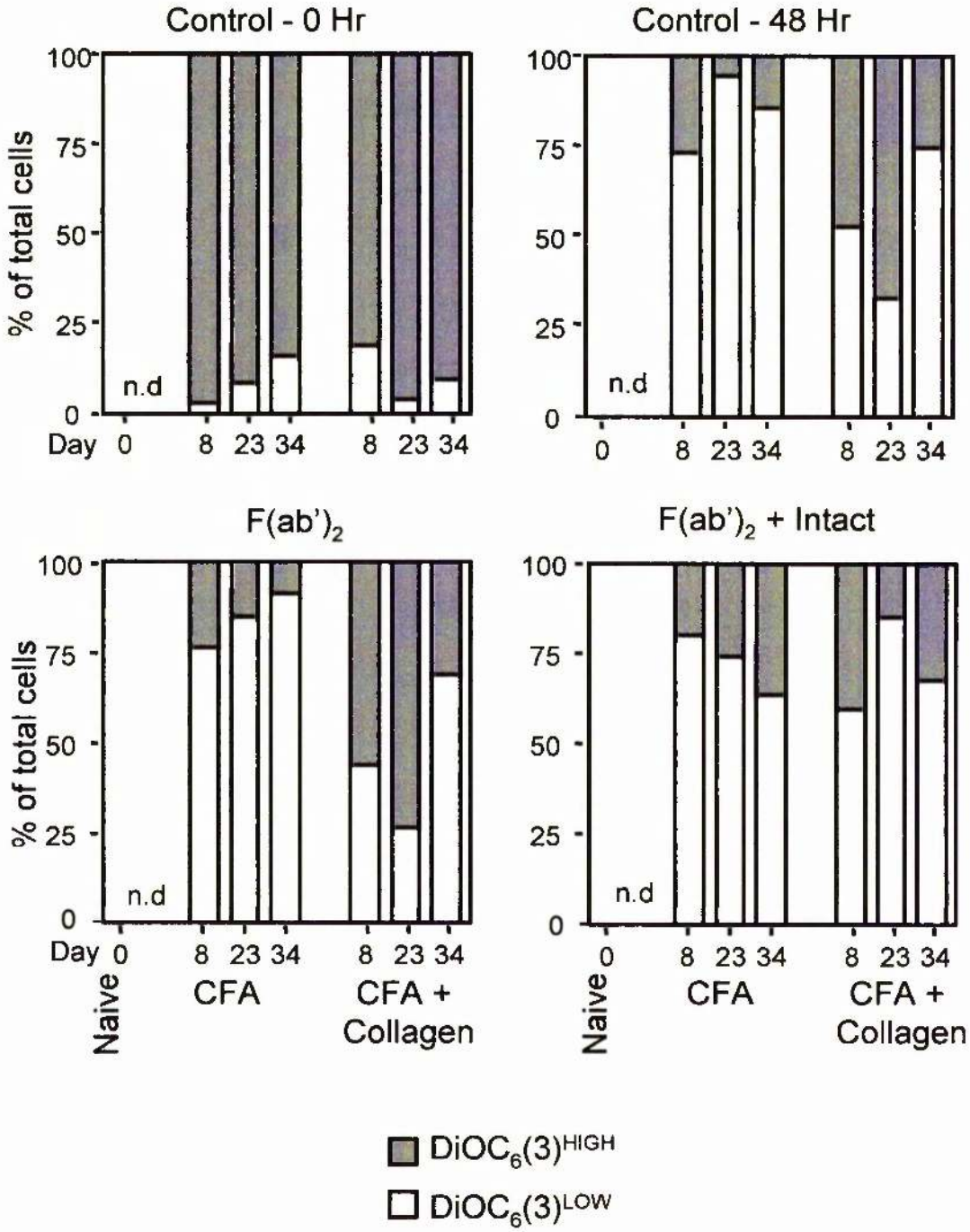

Control - 0 Hr
Control - 48 Hr
F(ab')2
F(ab')2 + Intact
% of total cells
100
75
50
25
0
n.d
Day 0 8 23 34 8 23 34
Naive
CFA
CFA + Collagen
DiOC6(3)HIGH
DiOC6(3)LOW

Figure 4.28 FcγRIIb1 and FcγRIIb2 mRNA expression levels in splenic B cells from CFA control and CFA/Collagen mice (d21-d25).

Total RNA was extracted from unstimulated, purified splenic B cells (5 x 10^6 cells) of CFA control or CFA/Collagen primed DBA/1 mice and was subjected to reverse transcription in the presence of 100 U Superscript II RT, as described in the Materials and Methods. FcγRIIb1 (upper panel) and FcγRIIb2 (lower panel) isoform transcripts were assayed for by real-time PCR (TaqMan). mRNA levels are expressed as a percentage relative to HPRT mRNA levels ± standard deviation of triplicate measurements from a single experiment.

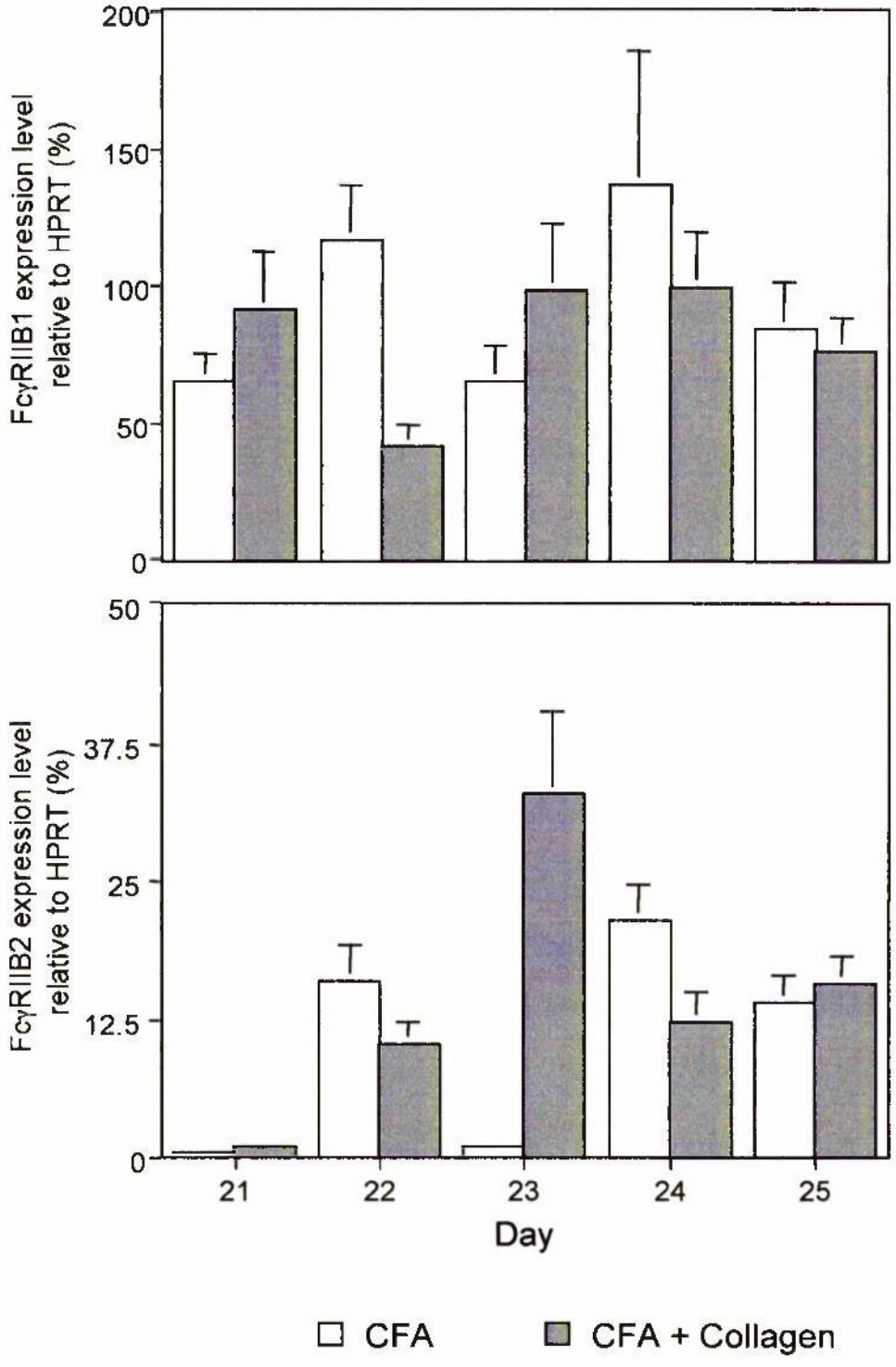

FcγRIIB1 expression level relative to HPRT (%)
200
150
100
50
0
FcγRIIB2 expression level relative to HPRT (%)
50
37.5
25
12.5
0
21
22
23
24
25
Day
CFA
CFA + Collagen

Figure 4.29 ES-62 desensitises coupling of the BCR to key proliferative signalling cascades.

Exposure of murine B cells to ES-62 selectively desensitises BCR-mediated proliferation by targeting key proliferative signalling pathways. Whilst, early sIg-coupled mediators such as the PTKs Lyn, Syk and Blk are relatively unaffected the subsequent PTK-mediated activation of downstream PI3-K and Ras-MAPK pathways is targeted. The nematode product also modulates the activity of certain PKC isoforms. The BCR is uncoupled from those pathways represented to the right of the arrow. (Adapted from Harnett and Harnett, 1999).

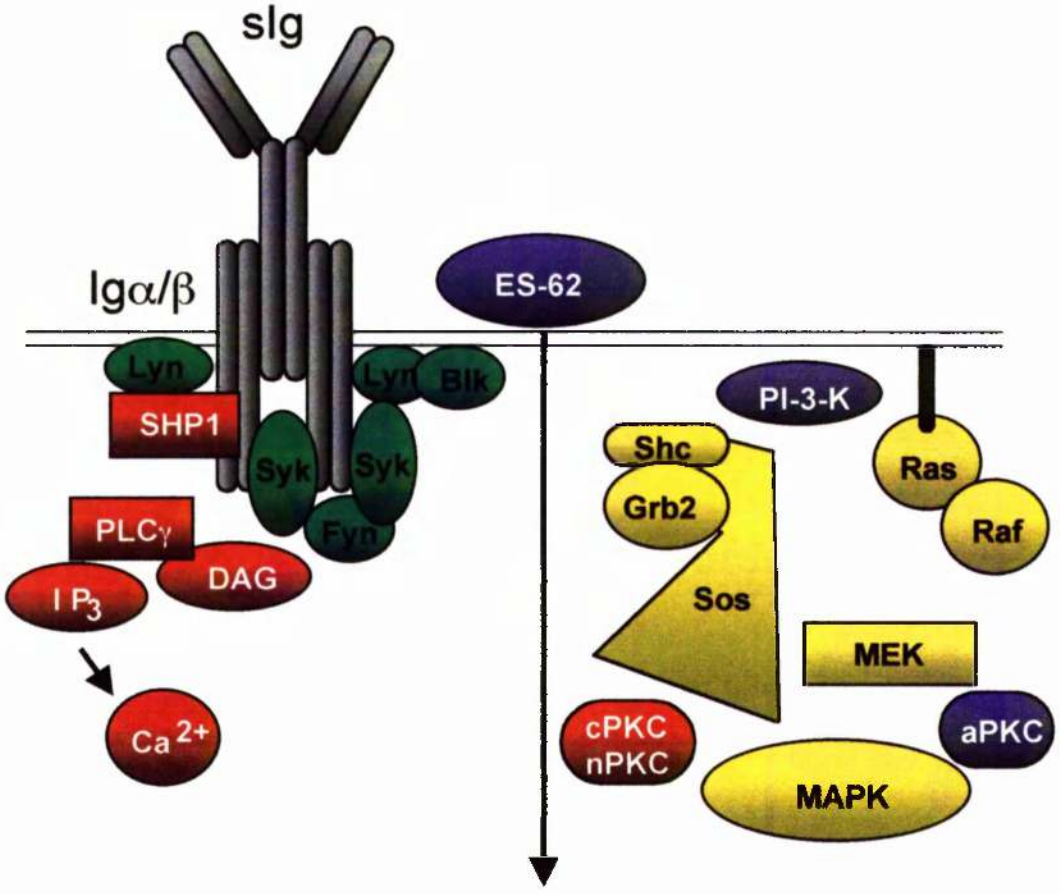

sIg
Igα/β
ES-62
Lyn
SHP1
Lyn
Blk
Syk
Syk
Fyn
PLCγ
DAG
I P3
Ca 2+
PI-3-K
Shc
Grb2
Sos
Ras
Raf
MEK
cPKC
nPKC
aPKC
MAPK
BCR desensitisation

Figure 4.30 ES-62 uncouples the BCR from ErkMAPK activation by priming B cells for BCR-mediated recruitment of SHP-1 and Pac-1.

ES-62 uncouples the BCR from ErkMAPK by (1) promoting the BCR recruitment of Lyn-SHP-1 protein tyrosine phosphatase complexes. (2) the dephosphorylation of Ig-β, preventing the recruitment of Shc-SOS complexes. (3) the formation of ErkMAPK/Pac-1 complexes (adapted from Deehan *et al.*, 2001).

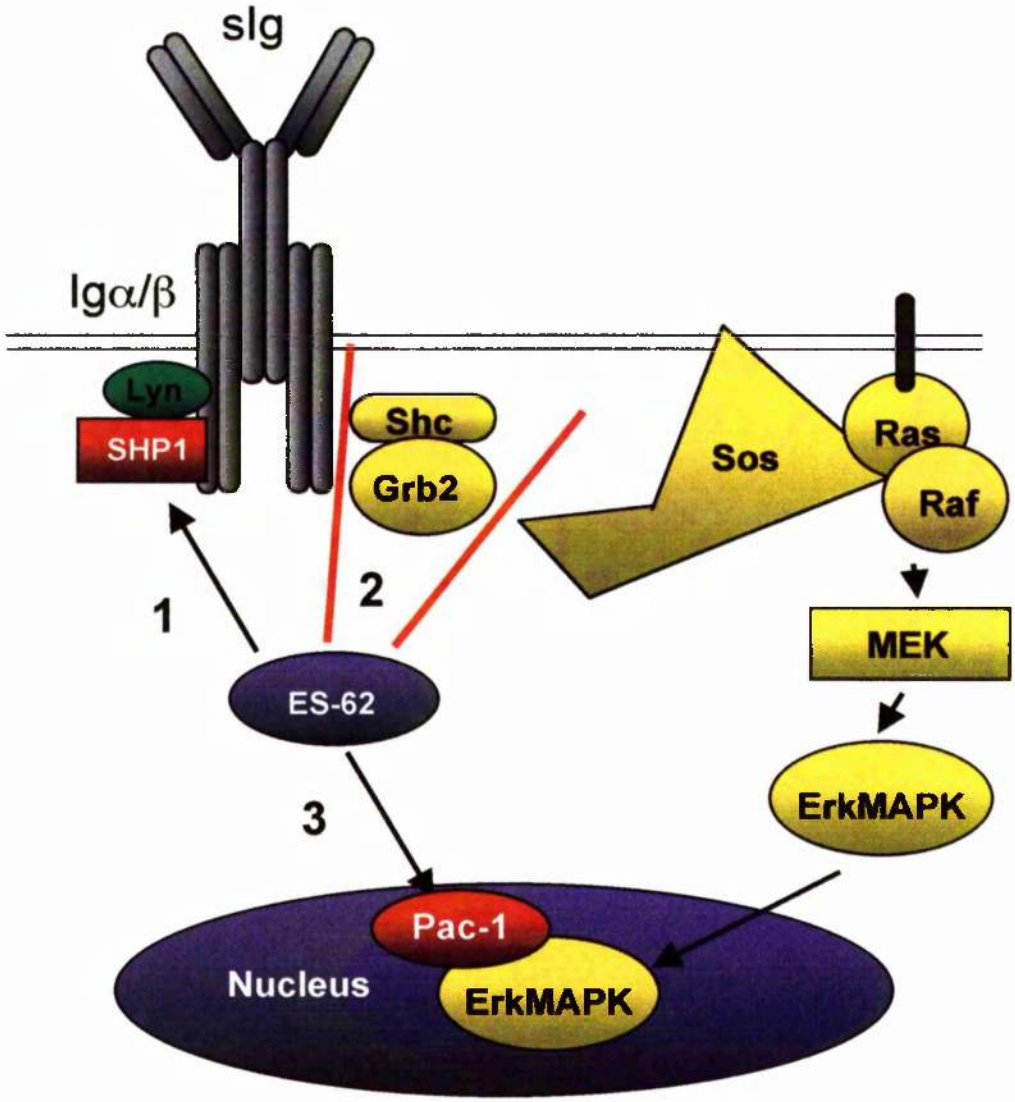

sIg
Igα/β
Lyn
SHP1
Shc
Grb2
Sos
Ras
Raf
MEK
ErkMAPK
1
2
3
ES-62
Pac-1
Nucleus
ErkMAPK

Figure 4.31 Effect of *in vivo* exposure to ES-62 on the FcγRIIb inhibition of BCR-mediated proliferative responses of splenic B cells and mononuclear cells from lymph nodes of Balb/c mice, *ex vivo*.

Balb/c mice were exposed to PBS or different concentrations of ES-62 *in vivo* by release from osmotic pumps for two weeks. Mononuclear cells from lymph nodes (upper panel) (5 x 10^5 cells) and purified splenic B cells (lower panel) (2 x 10^5 cells) were stimulated for 48 hours *in vitro* in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) alone or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells cultured in the presence of medium alone were included as a control. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment.

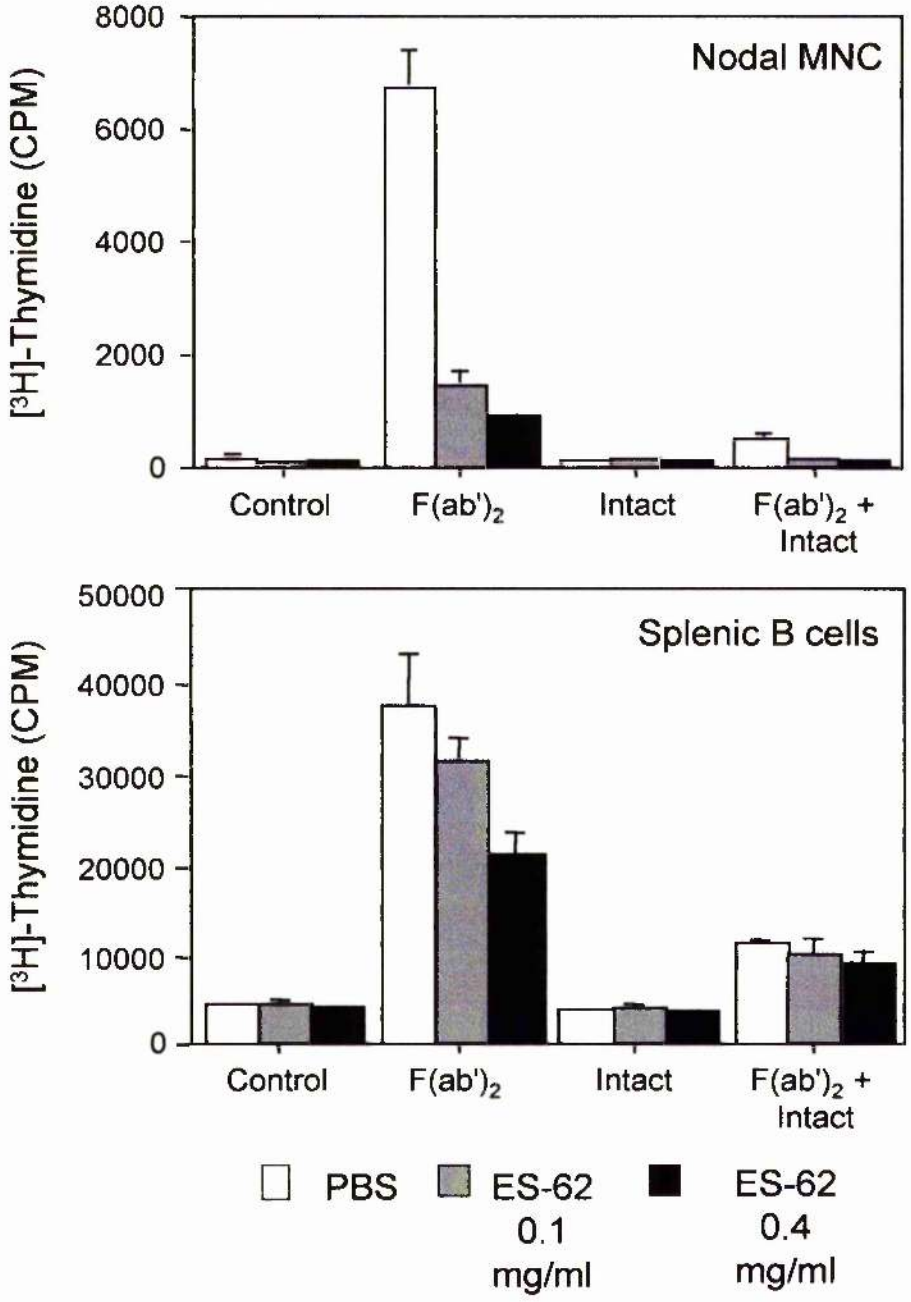

Nodal MNC
[3H]-Thymidine (CPM)
8000
6000
4000
2000
0
Control
F(ab')2
Intact
F(ab')2 + Intact
Splenic B cells
[3H]-Thymidine (CPM)
50000
40000
30000
20000
10000
0
Control
F(ab')2
Intact
F(ab')2 + Intact
PBS
ES-62 0.1 mg/ml
ES-62 0.4 mg/ml

Figure 4.32 Collagen-induced Arthritis/ES-62 prophylactic protocol.

↑ = Induction of CIA. ↑ = ES-62 treatment. Male DBA/1 mice (PBS 13 per group; ES-62 14 per group) were immunised intradermally at the tail base with a total of 100µl CFA emulsified bovine type II collagen (CII; 200µg) or CFA alone on day 0. An intraperitoneal CII challenge (CII, 200µg in PBS) was carried out on day 21. ES-62 (2 µg/animal, s.c.) was administered prior to induction of CIA at d-2, and concurrently with collagen challenges at d0 and d21. *ES-62: additional ES-62 treatments were administered d22-24 plus 1 treatment every three days until the end of the study (ES-62 Multi). PBS was used as the control. Animals were observed for up to 5 weeks for the development of arthritis.

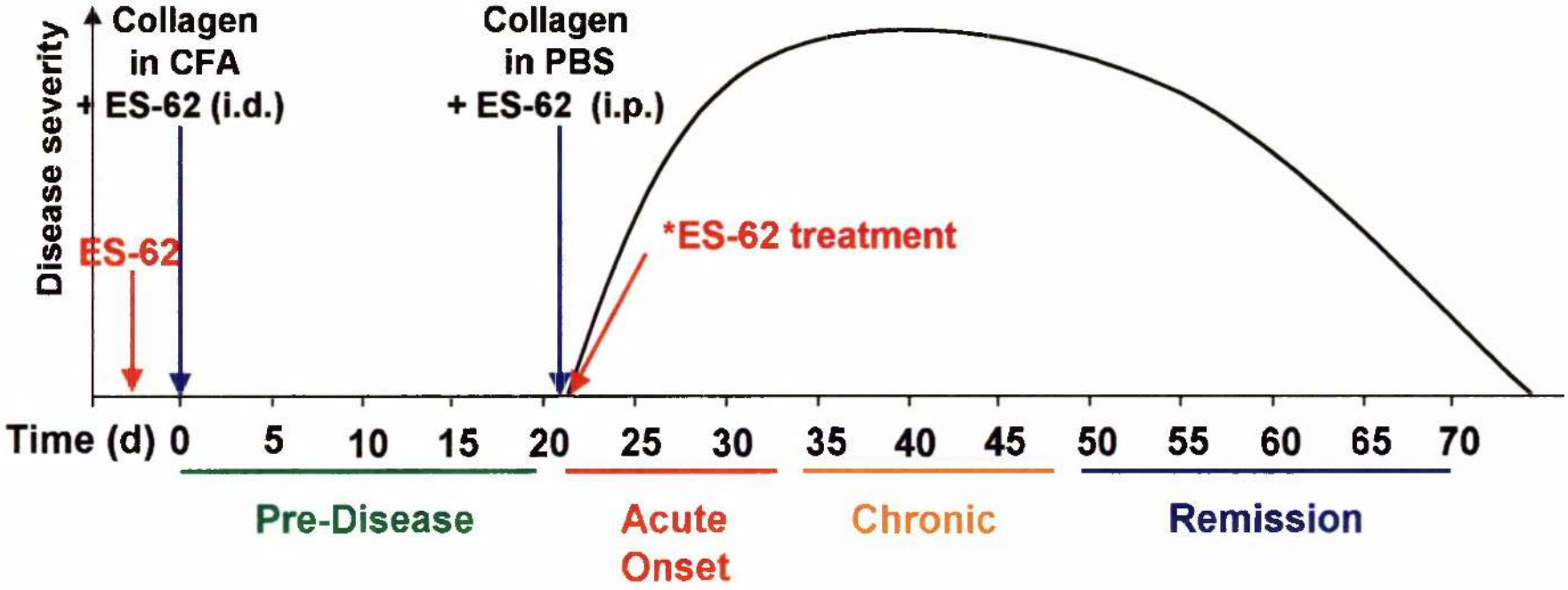

Disease severity
Collagen
in CFA
+ ES-62 (i.d.)
Collagen
in PBS
+ ES-62 (i.p.)
ES-62
*ES-62 treatment
Time (d) 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Pre-Disease
Acute
Onset
Chronic
Remission

Figure 4.33 Clinical measurements and histological assessments of collagen-induced arthritis mice following prophylactic treatment with ES-62.

Dr. Bernard Leung undertook all clinical measurements and histological assessments of the collagen-induced Arthritis/ES-62 prophylactic model. Data of individual measurements from the PBS, ES-62 and ES-62 Multi groups (PBS n=13 /group; ES-62 n=14/group) were collected from the time of the intraperitoneal CII challenge on day 21 until the termination of the study on day 50. Graphs represent the mean articular index and disease incidence rates of the arthritic groups only and the paw pad thickness of the entire study group. Data are the mean ± S.E.M from a single experiment.

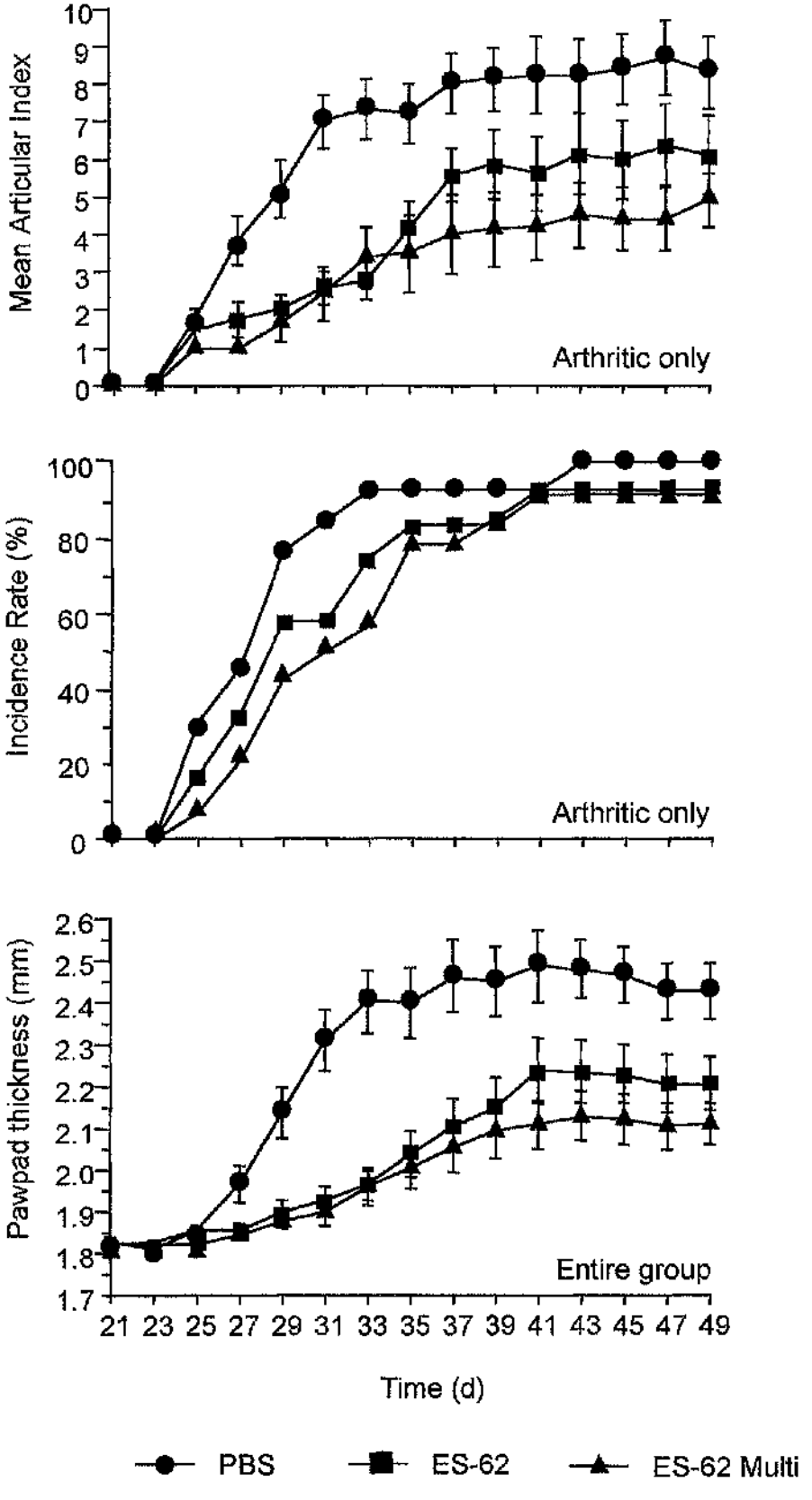

Mean Articular Index
10
9
8
7
6
5
4
3
2
1
0
Arthritic only
Incidence Rate (%)
100
80
60
40
20
0
Arthritic only
Pawpad thickness (mm)
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
Entire group
21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
Time (d)
PBS
ES-62
ES-62 Multi

Figure 4.34 Effect of FcγRIIb co-ligation on the BCR-mediated proliferative response of splenic B cells from collagen-induced arthritis model mice following prophylactic treatment with PBS or ES-62, *ex vivo* (d50).

On day 50 of the study, purified splenic B cells (2 x10^5 cells) from PBS, single-dose ES-62 or multiple dose ES-62 prophylactic treated CIA mice, were stimulated for 48 hours in the presence of 50 μg/ml collagen or 50 μg/ml F(ab')$_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells cultured in the presence of medium alone were included as a control. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment.

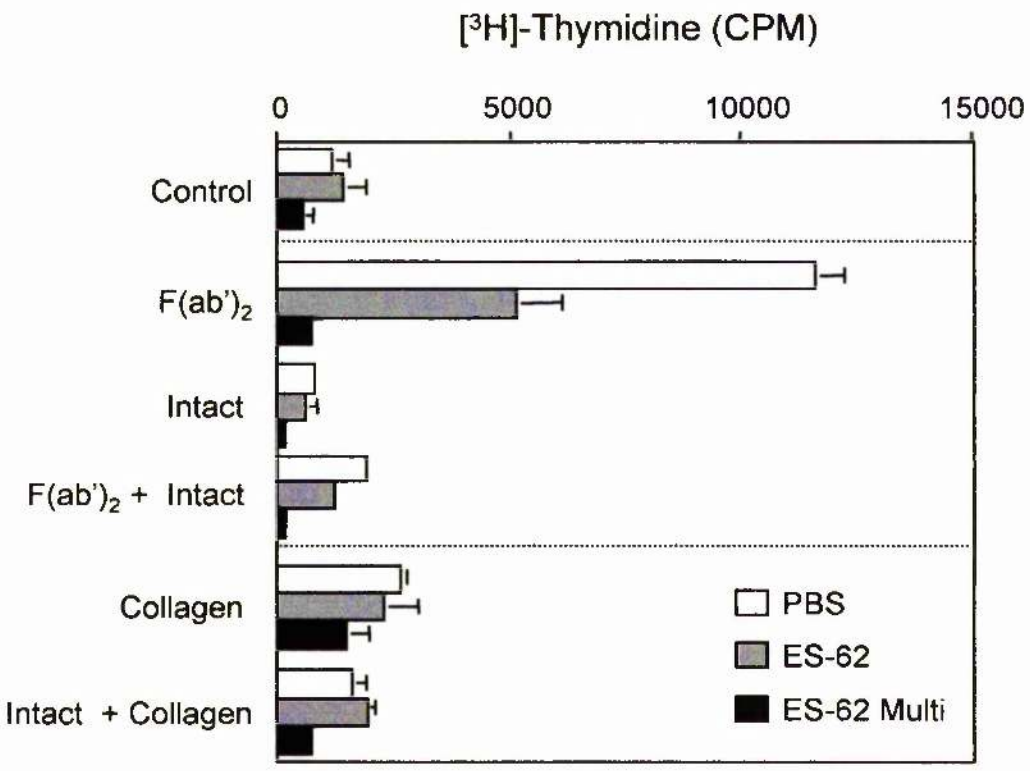

[3H]-Thymidine (CPM)
0
5000
10000
15000
Control
F(ab')2
Intact
F(ab')2 + Intact
Collagen
Intact + Collagen
PBS
ES-62
ES-62 Multi

Figure 4.35 Effect of FcγRIIb co-ligation on the BCR-mediated proliferative response of splenic B cells from a female MRL-*lpr/lpr* mouse, *ex vivo*.
Purified splenic B cells (2 x 10^5 cells) from a female MRL-*lpr/lpr* mouse, aged 18 weeks, were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgG/IgM or 75μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment. *$p<0.05$, Mann-Whitney test.

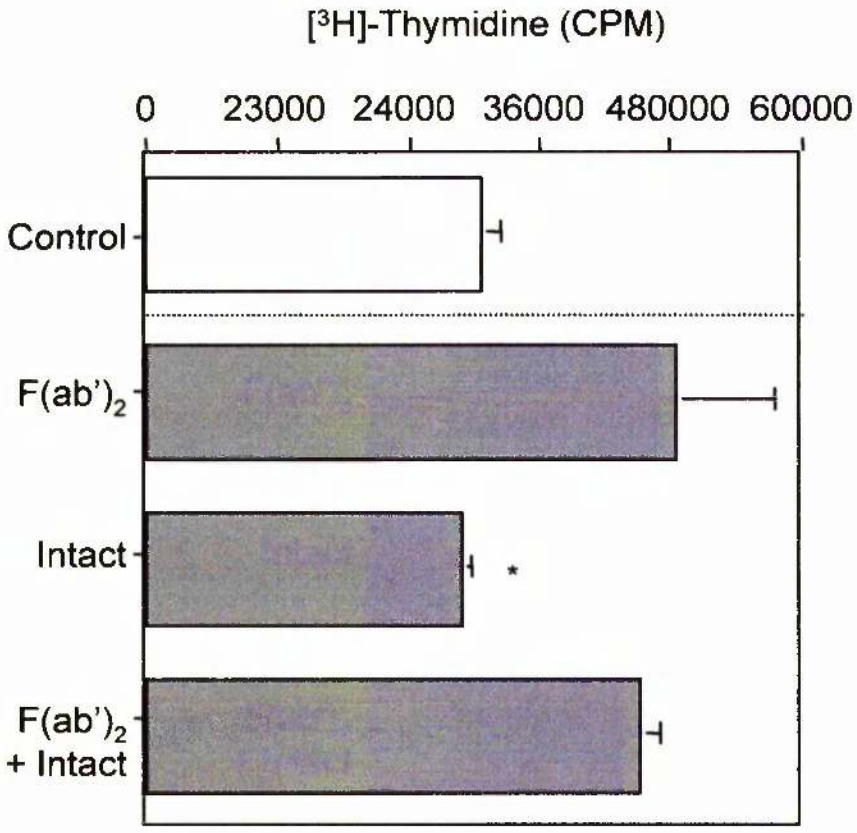

[3H]-Thymidine (CPM)
0
23000
24000
36000
480000
60000
Control
F(ab')2
Intact
*
F(ab')2
+ Intact

Figure 4.36 Effect of FcγRIIb co-ligation on the BCR-mediated proliferative response of splenic B cells from MRL-*lpr/lpr* mice, *ex vivo* (w14-w26).

Purified splenic B cells from male MRL-*lpr/lpr* mice (2 x10^5 cells) were stimulated for 48 hours in the presence of 50 μg/ml F(ab')$_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM or 10 U/ml murine IL-4 either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment.*$p<0.05$, Mann-Whitney test.

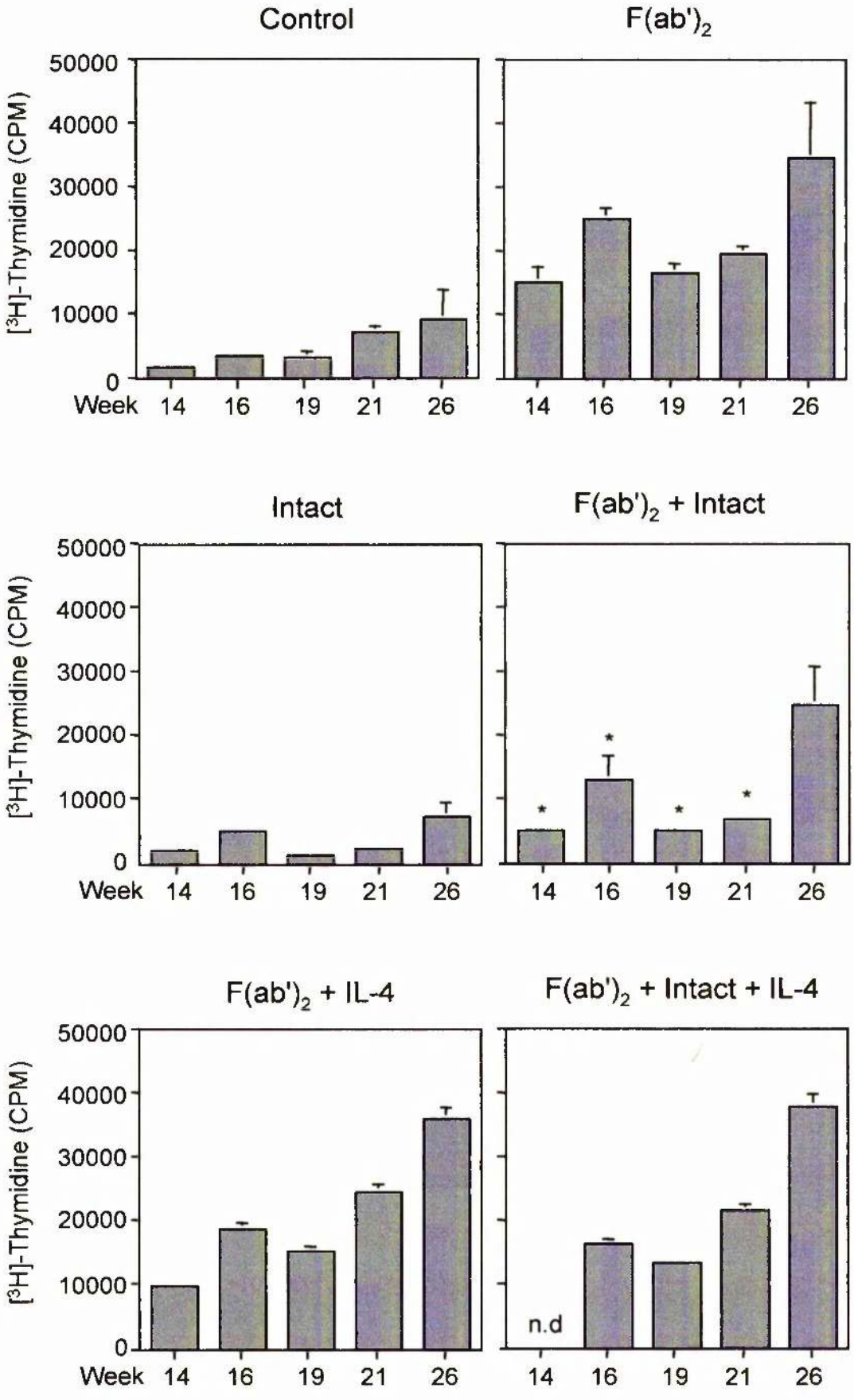

Control
F(ab')2
Intact
F(ab')2 + Intact
F(ab')2 + IL-4
F(ab')2 + Intact + IL-4
[3H]-Thymidine (CPM)
50000
40000
30000
20000
10000
0
Week
14
16
19
21
26
*
n.d

Figure 4.37 Effects of BCR and FcγRIIb co-ligation on the DNA content of splenic B cells from MRL-*lpr/lpr* mice, *ex vivo* (w14-w26).

Purified splenic B cells from male MRL-*lpr/lpr* mice (1 x 10^6 cells) were subjected to DNA content analysis post-sacrifice or were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total number of cells analysed from a single experiment.

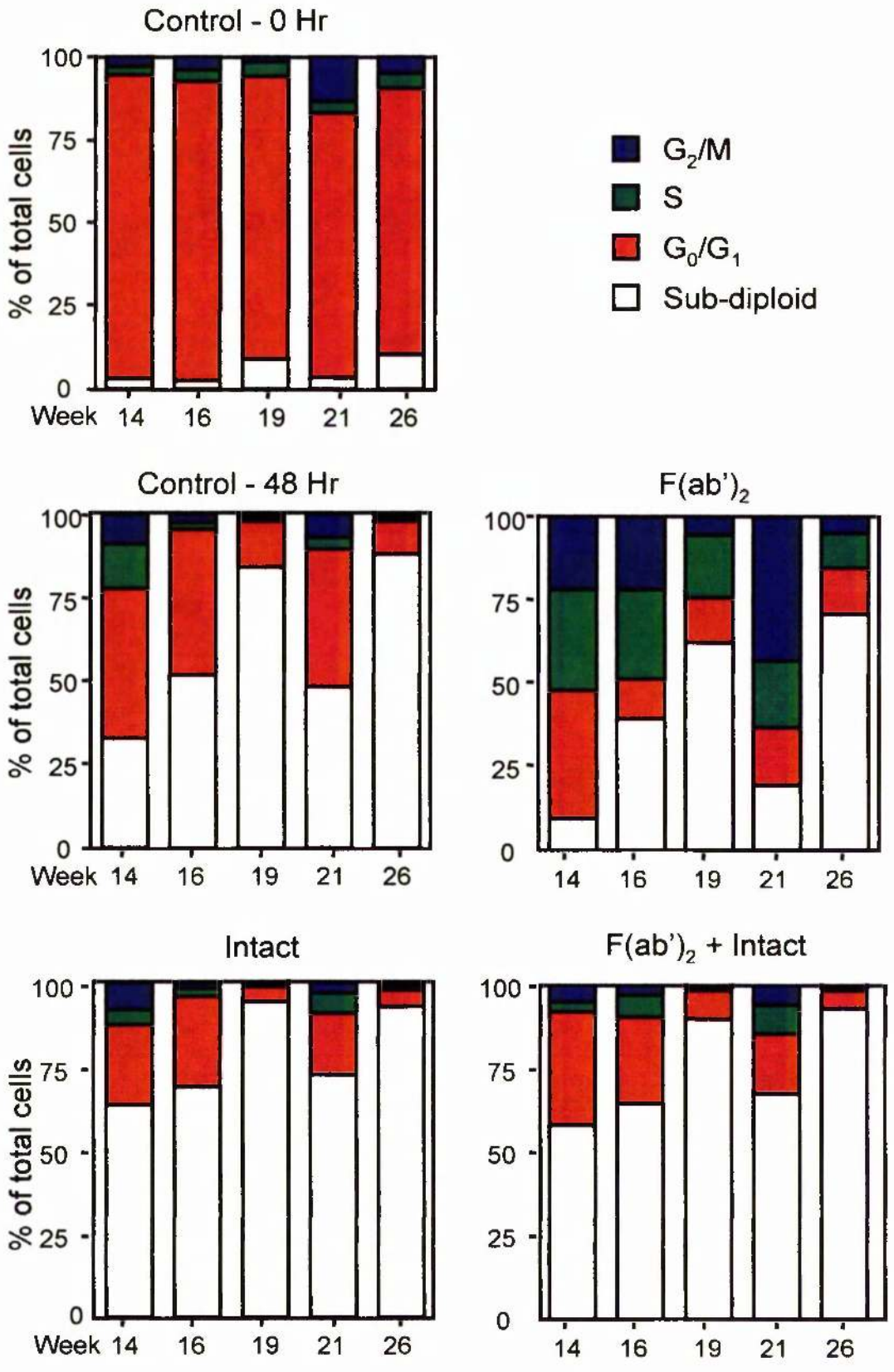

Control - 0 Hr
Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact
% of total cells
100
75
50
25
0
Week 14 16 19 21 26
14 16 19 21 26
G2/M
S
G0/G1
Sub-diploid

Figure 4.38 Effects of BCR and FcγRIIb co-ligation on the DNA content of the living population of splenic B cells from MRL-*lpr/lpr* mice, *ex vivo* (w14-w26).

Purified splenic B cells from male MRL-*lpr/lpr* mice (1 x 10^6 cells) were subjected to DNA content analysis post-sacrifice or were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in the Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total living population of cells analysed from a single experiment.

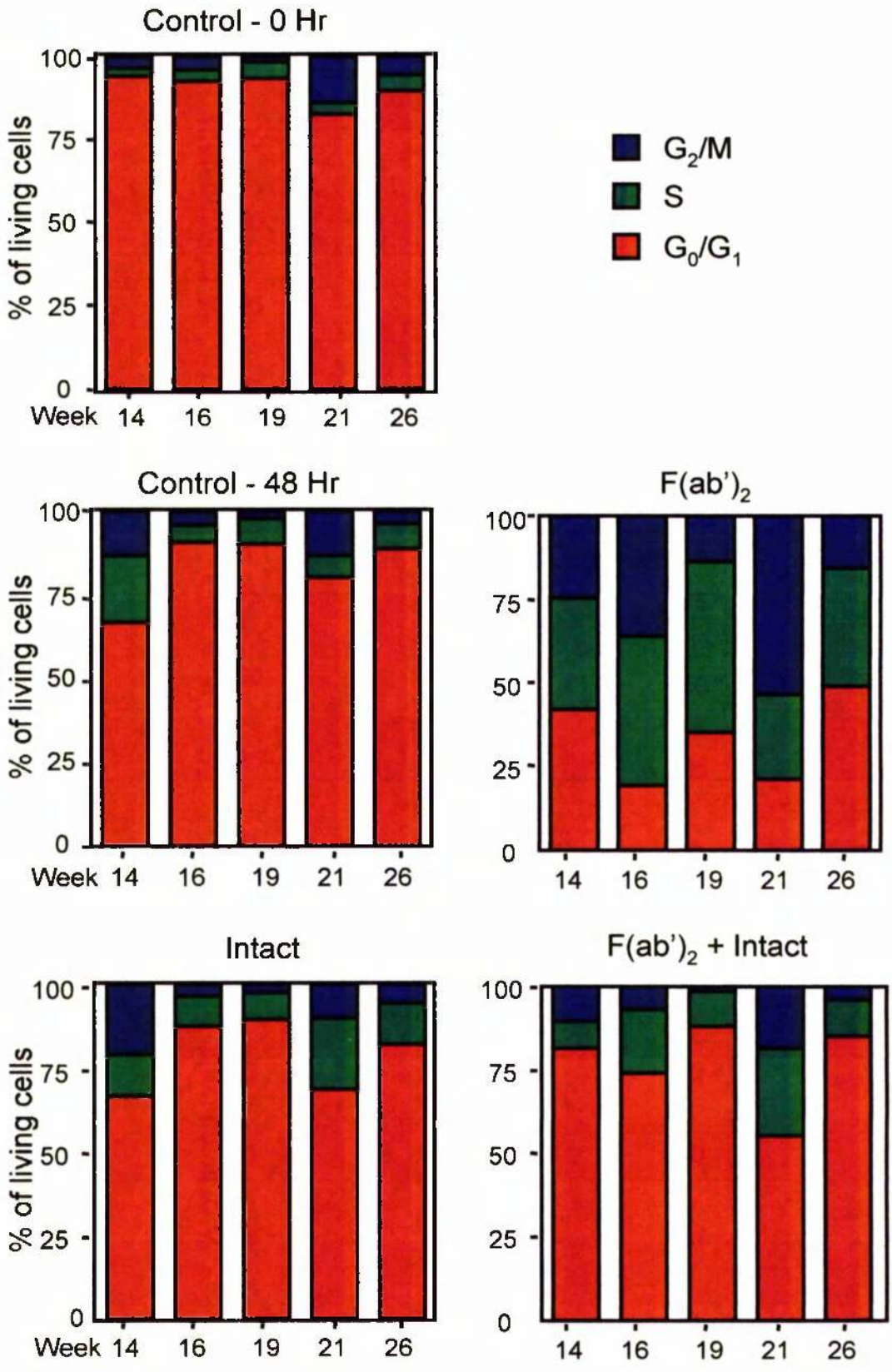

Control - 0 Hr
Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact
% of living cells
100
75
50
25
0
Week
14
16
19
21
26
G2/M
S
G0/G1

Figure 4.39 Effects of BCR and FcγRIIb co-ligation on the mitochondrial membrane potential of splenic B cells from MRL-*lpr/lpr* mice, *ex vivo* (w14-w26).

Purified splenic B cells from male MRL-*lpr/lpr* mice (1×10^6 cells) were assessed for mitochondrial membrane potential post-sacrifice or were stimulated for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. The mitochondrial membrane potential was determined by $DiOC_6(3)$ staining and FACS analysis, as described in the Materials and Methods. Data represents $DiOC_6(3)^{LOW}$ and $DiOC_6(3)^{HIGH}$ cell populations, as determined on a logarithmic FL-1 axis, and expressed as a percentage of the total number of cells analysed from a single experiment. (n.d. = not done).

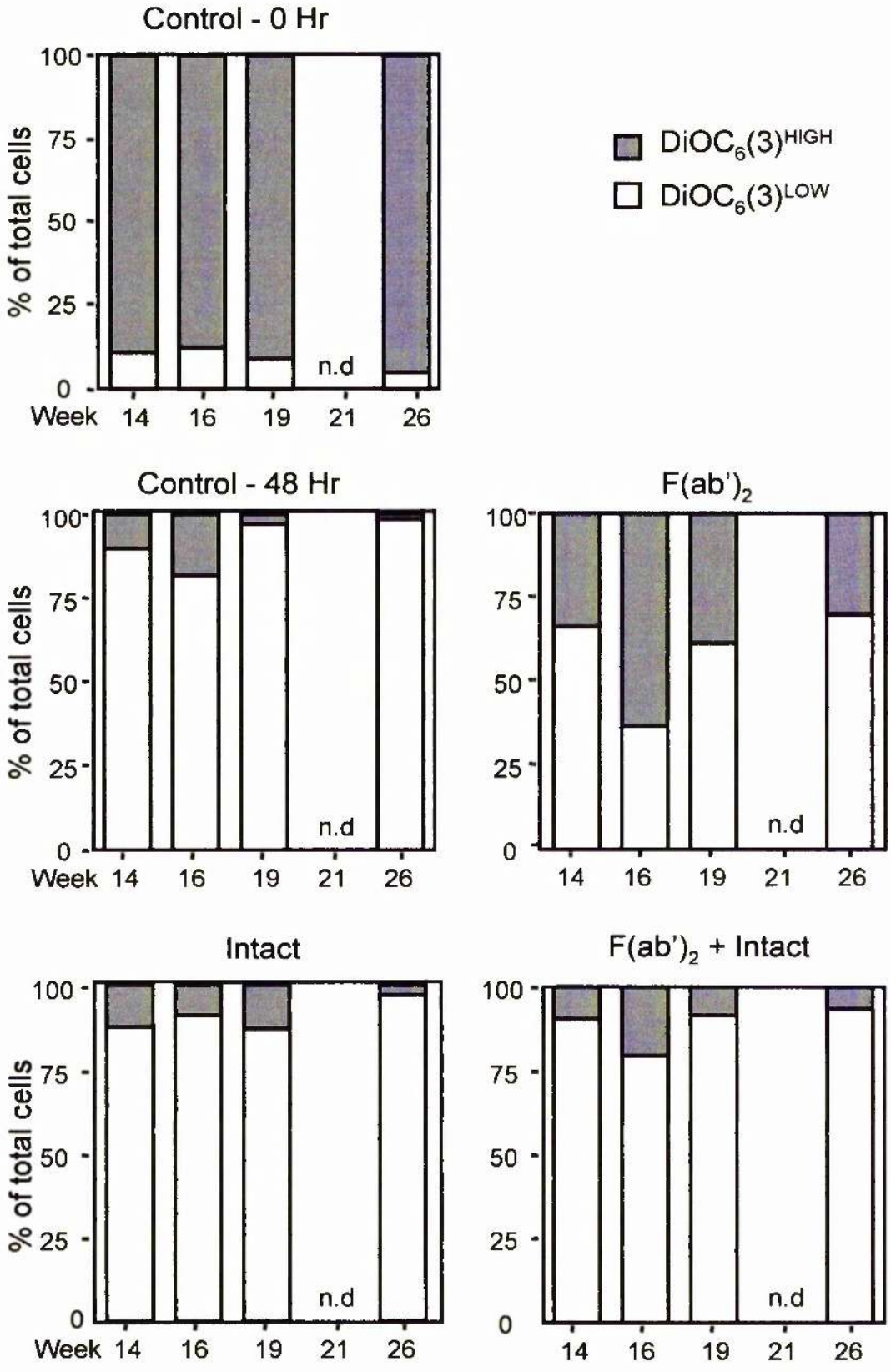
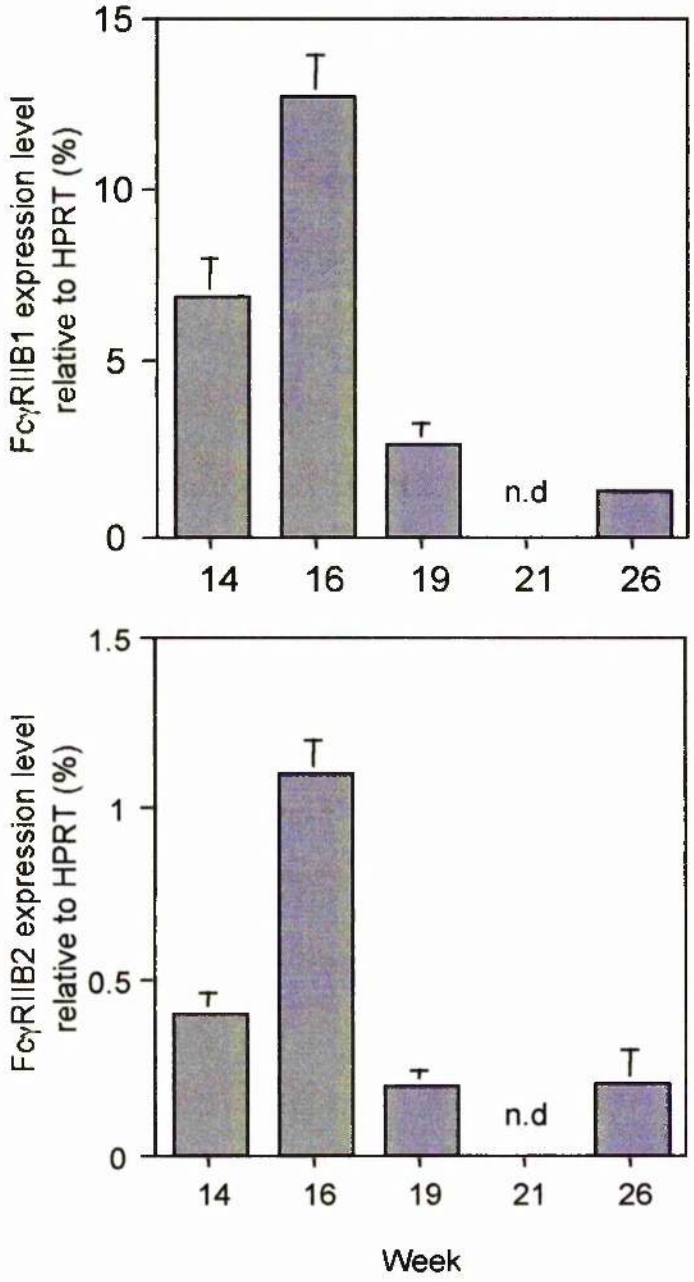

Control - 0 Hr
% of total cells
100
75
50
25
0
n.d
Week 14 16 19 21 26
DiOC6(3)HIGH
DiOC6(3)LOW
Control - 48 Hr
F(ab')2
Intact
F(ab')2 + Intact

Figure 4.40 Effects of BCR and FcγRIIb co-ligation on the FcγRIIb1 and FcγRIIb2 mRNA expression levels in splenic B cells from MRL-*lpr/lpr* mice, *ex vivo* (w14-w26).

Total cellular RNA was extracted from purified splenic B cells from male MRL-*lpr/lpr* mice (5 x 10^6 cells) post-sacrifice or following stimulation for 48 hours in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, as indicated. Cells stimulated in the presence of medium alone were included as a control. Total RNA was subjected to reverse transcription in the presence of 100 U Superscript RT II. FcγRIIb1 (upper panel) and FcγRIIb2 (lower panel) isoform transcripts were assayed for by real-time PCR (TaqMan). Levels are expressed as a percentage relative to HPRT mRNA levels ± standard deviation of triplicate measurements from a single experiment.


15
10
5
0
FcγRIIB1 expression level relative to HPRT (%)
n.d
14
16
19
21
26
1.5
1
0.5
0
FcγRIIB2 expression level relative to HPRT (%)
n.d
14
16
19
21
26
Week

Chapter 5 – Role of FcγRIIb in Human Autoimmune Disease

5.1 Human Autoimmune Disease

As discussed in the previous chapter autoimmunity is the inappropriate response of the immune system to self-antigens. Autoimmunity can lead to autoimmune diseases such as systemic lupus erythematosus (SLE) and Goodpasture's syndrome which are characterised by tissue damage, caused as a result of the formation of immune complexes and local inflammation (Ada, *et al.*, 1997). Clinical disease generally manifests as a result of damage induced in one or more organs via the inappropriate activation of immune-mediated inflammation. Collectively, autoimmune disease is estimated to affect 4-5% of the total human population, with females generally having a higher disease incidence than males (Vyse and Todd, 1996). Five of the most common autoimmune diseases are rheumatoid arthritis (RA), Graves' disease, insulin-dependent diabetes mellitus (IDDM), SLE and multiple sclerosis (MS). Together they represent almost 50% of all autoimmune diseases.

Despite numerous studies, the pathogenic mechanisms leading to a breakdown of self-tolerance and the initiation of autoimmunity remain poorly characterised. Many studies have attempted to associate autoimmunity with abnormal lymphocyte homeostasis (Theofilopoulos, *et al.*, 2001), focusing on abnormal T cell responses, production of T cell cytokines and/or defective control by regulatory T cells (Maloy and Powrie, 2000). Early investigations into the role of B cells focused on their ability to produce pathogenic autoantibodies and the formation of antigen-antibody complexes. However, recent studies of B cells have demonstrated that they display a variety of characteristics that could contribute towards the initiation and perpetuation of an autoimmune disease (Edwards, *et al.*, 1999). Whilst acting as the precursors for antibody-secretion, B cells play an essential role in the development of lymphoid architecture by influencing the differentiation of follicular dendritic cells in the secondary lymphoid organs. In addition, by acting as antigen-presenting cells, B cells can

co-ordinate T cell migration and differentiation. B cell-derived cytokine production may also upregulate the functions of a variety of other cells involved in immune responses, such as antigen-presenting cells (reviewed by Porakishvili, *et al.*, 2001). Together, these results indicate that B cells play an essential role in the initiation and regulation of both T and B cell responses. Thus, excessive or upregulated B cell activity may increase the likelihood of developing autoimmunity. Importantly B cells have been shown to play a crucial role in the initiation of autoimmune disease in a murine model of SLE (Schlomchik, *et al.*, 1994) and in patient studies of rheumatoid arthritis (Edwards and Cambridge, 2001).

5.1.1 Systemic lupus erythematosus

Systemic lupus erythematosus is considered to be the prototypic immune complex disease (reviewed by Kotzin, 1996). It is a chronic autoimmune disease characterised by the development of IgG hypergammaglobulinemia and antibody production directed at the nuclear components of cells. Principal targets include certain protein-DNA complexes and ribonucleic proteins. Since large quantities of self-antigen are available, large numbers of immune complexes are deposited in the walls of the small blood vessels in the renal glomerulus, joints and other organs. The tissue-deposition of these antigen-antibody complexes ultimately leads to the release of inflammatory mediators and infiltration of inflammatory cells. These factors cause further tissue damage and the release of cellular contents. The increase in concentration of available self-antigen results in the further production of nuclear-protein complexes and a cyclical exacerbation of the disease.

The clinical manifestations of SLE are extremely diverse with patients displaying a multitude of different disease phenotypes (reviewed by Kotzin and O'Dell, 1995). For example, whilst some patients demonstrate a skin rash and joint pain but undergo spontaneous remission and hence require little medication, others may suffer from severe and progressive glomerulonephritis that require high dose steroid therapy and cytotoxic drugs. As such, it remains unclear whether

SLE is a single disease with variable severity levels or a group of related conditions.

The exact causative factor of SLE is unknown but the emergence of disease appears to depend on environmental factors that initiate or contribute to autoimmunity in genetically prone individuals (reviewed by Vyse and Kotzin, 1998). Candidate factors include female sex hormones (the disease is predominantly found in females of childbearing age) and viral/bacterial infection. The mechanism by which most autoantibodies in SLE may cause disease is also uncertain as the formation of circulating immune complexes, resulting in the deposition of anti-nuclear autoantibodies, does not appear to mediate the renal damage commonly observed. It is thought that either the glomerulus binds DNA, which is subsequently recognised by anti-DNA antibodies leading to *in situ* complex formation or there is cross reaction between a subset of anti-DNA antibodies with glomerular structures that are not DNA in origin.

The main immunological characteristics of SLE are a number of profound B cell abnormalities that permit the generation, differentiation and positive selection of B cells that secrete pathogenic autoantibodies (reviewed by Lipsky, 2001). SLE patients may possess B cells that display abnormal antigen-receptor repertoires or undergo mechanisms contributing to somatic hypermutation. Intrinsic B cell hyperactivity to normal antigenic stimulation may also provide the drive for autoantibody production.

5.1.2 Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory joint disease, resulting from a dysregulation of the immune response (reviewed by Sturrock, 1987; Feldmann *et al.*, 1996). The disease is characterised by the infiltration of the synovial membrane by activated lymphocytes, neutrophils and macrophages and hyperplasia of the resident synoviocytes. Often the initial damage occurs in the small joints of the hands and the feet, although any joint can be affected. Articular damage arises due to deregulated cellular processes and the overproduction of inflammatory cytokines (IL-1β and TNF-α) and other soluble mediators. These cytokines mediate the further production of inflammatory

molecules and the secretion of metalloproteinases (MMP) to degrade type II collagen in cartilage. The synovium thus becomes progressively thickened and inflamed with further infiltration by lymphocytes and plasma cells. The cellular aggregates present in rheumatoid synovium histologically resemble an active lymphoid tissue, thus implying immunological processes are a causative factor.

Despite numerous investigative studies the aetiological agent of rheumatoid arthritis is, as yet, unknown (reviewed by Feldmann *et al.*, 1996). It is not known whether the disease is initiated by an unrestrained inflammatory response to a microbial antigen or by a loss of self-antigen tolerance resulting in the production of autoantibodies, or a combination of the two. Investigations in a variety of animal arthritis models have highlighted important roles for autoreactive T cells, presentation of self-antigens by dendritic cells, pro-inflammatory cytokine secretion by macrophages and fibroblast mediated-cartilage damage. All of these mechanisms are thought to contribute to the synovial hyperplasia via the hyper-activation of effector cells and the increased levels of soluble inflammatory mediators.

Despite being present in the inflamed synovium, the role of B cells in rheumatoid arthritis is less well established (reviewed by Edwards *et al.*, 1999). It is thought that the local production of autoantibodies by B cells leads to complement fixation and the deposition of immune complexes within the synovial membrane. Combined with a heavy infiltration of plasma cells, autoantibody production results in remitting, relapsing inflammation and the progressive, erosive destruction of the adjacent cartilage and bone. Importantly, approximately 80% of subjects with rheumatoid arthritis develop circulating autoantibodies to IgG Fc, referred to as rheumatoid factor (RF). Interestingly, rheumatoid factor producing B cell clones are present in a few normal individuals. The rheumatoid factor produced by these normal individuals is mostly IgM and low affinity, with very little evidence of gene mutation. Thus, a block in class switching to IgG and affinity maturation of rheumatoid factor may be occurring in these individuals or a protective mechanism may operate in the follicle centre, preventing the initiation of disease.

Normally B cells are activated upon interaction with their specific antigen in conjunction with co-stimulatory signals from T_H cells. In theory, autoreactive B cells should be unable to survive because of T cell anergy to self resulting in a lack of positive co-stimulatory signals. However, reactive B cell clones expressing RF-BCRs may be able to circumvent this activation route by binding IgG attached to a non-self antigen, that can subsequently be presented to a responsive cognate T cell (Roosnek and Lanzavecchia, 1991). At the sites of rheumatoid factor synthesis, as in rheumatoid synovium, higher concentrations of IgG rheumatoid factor leads to the formation of dimers. These multimers fix complement poorly and thus have the potential to escape clearance by platelet complement receptors. Once in circulation these complexes may be able to access tissue macrophages and rheumatoid factor-specific B cells, providing a positive survival signal whilst disturbing the regulation by T_H cells (section **5.2**) (Edwards *et al.*, 1999).

Treatment of rheumatoid arthritis currently relies on a combinatorial approach of disease-modifying anti-rheumatic drugs (DMARD) and non-steroidal anti-inflammatory drugs (NSAID). DMARDs (also referred to as second-line agents) partially suppress the chronic inflammation and may slow down the rate of joint destruction and loss of function, whilst NSAIDs control the initial inflammation and the patient's appreciation of pain (Sturrock, 1987; Dinarello and Moldawer, 2000). New arthritis therapies are being designed to target specific pathways known to be involved in disease pathogenesis, such as the p38/MAPKinase pathway. The stress-activated p38/MAPKinase plays a major role in the synthesis and activity of several pro-inflammatory cytokines, particularly IL-1 and TNF-α (Lee, *et al.*, 1994), and inhibitors of p38-MAPKinase are non-specific anti-inflammatory agents. Rheumatoid arthritis is a "T_H1-type" inflammatory disease therefore new therapies aim to target the production of cytokines associated with T_H1 development, such as IL-12 and IL-18. Therapeutic studies have also included the targeting of pro-inflammatory cytokine production (TNF-α and IL-1) (Feige, *et al.*, 2000), macrophage cytokine networks (IL-15 and IL-18) and promoting the production of anti-inflammatory cytokines (IL-4 and IL-10) (Keystone, *et al.*, 1998). Non-cytokine approaches aim to address the synovial

hyperplasia and bone destruction by modulating the cell-cycle or inducing apoptosis of the proliferating synovium (Dinarello and Moldawer, 2000). Overall, any effective treatment of arthritis will require the elimination of disease-perpetuating lymphocytes as well as the induction of tissue repair. Indeed, a recent study has shown a sustained improvement in rheumatoid arthritis patients that have undergone B cell depletion (Edwards and Cambridge, 2001). Supporting the hypothesis that B cells play a major role in the perpetuation of disease.

5.2 Role of FcγRs in human autoimmune disease

Since the formation of autoantibodies and immune-complexes is central to many of the described autoimmune diseases, recent studies have focused on the role of antibody binding receptors in the immune response. Results obtained in mice deficient in ether FcR or complement have revealed distinct functions for these two classes of molecules (reviewed by Ravetch and Clynes, 1998; Heyman, 2000). Complement and its receptors was found to be essential for the interaction with natural antibodies (IgM) to mediate protection against pathogens. In addition, FcγRs couple the interaction of IgG antibodies to effector cells to trigger inflammatory responses. Interestingly, B cells express a Fc receptor for immunoglobulin, which is known to play an important role in the maintenance of B cell homeostasis and tolerance to self-antigen. Indeed, FcγRIIb directly influences the signalling threshold of B cells and, in conjunction with other B cell co-receptors, is essential for maintaining the balance between immunity and autoimmunity (Bolland and Ravetch, 2000).

To date, there have been no reports of a deficiency in FcγRIIb function in antibody-mediated human disease. A total absence of specific isoforms of FcγR is extremely rare in humans but individuals lacking stimulatory receptors (FcγRI or FcγRIIIb) have been reported to be generally healthy (Ceupeens, 1988). Therefore, it may be possible that differences in the relative expression levels of activation (ITAM) and inhibition (ITIM) FcγR influence the overall risk of developing autoimmune disease. Studies in activatory FcγR-deficient mice

seem to indicate this, as they demonstrate significantly reduced autoimmune-mediated tissue damage in disease models of systemic lupus erythematosus and rheumatoid arthritis (Clynes, *et al.*, 1998).

Inflammatory cytokines elicited during an immune response may also alter the expression and functional capacity of FcγRs, thereby modulating the signalling threshold of the FcγR bearing cell. For example, IFN-γ, a Th1-type cytokine, has been shown to decrease FcγRIIb2 expression on circulating human monocytes whilst IL-4, a T_H2-type cytokine increases levels resulting in the enhancement of phagocytic capacity and the rapid clearance of immune complexes (Pricop *et al.*, 2001). Thus, FcγRIIb may function to establish the threshold for activation of macrophages by immune complexes in an inflammatory response. Interestingly, the production of the anti-inflammatory cytokine, IL-4, is known to overcome FcγRIIb-mediated inhibition of B cell activation by providing a positive, survival and proliferative signal (O'Garra *et al.*, 1987).

Fc expressing effector cells also play an important role in rheumatoid arthritis, with the binding of IgG dimers by macrophages preceding the accumulation of T cells in the synovium. Expression levels of FcγRIIa on macrophages are also upregulated in the presence of the inflammatory cytokine, IFN-γ, enhancing the positive signal. Moreover, supporting evidence for a role of FcγRs in autoimmune diseases is demonstrated by the finding that negative signalling is blocked in neutrophils of rheumatoid arthritis patients by an anti-FcγRII antibody (Robinson, *et al.*, 1994). In addition, rheumatoid factor may compete with FcγRIIb for large antigen-antibody complexes, thus blocking FcγRIIb-mediated negative signalling in B cells (Edwards *et al.*, 1999).

Recently two groups have shown in gene linkage studies an association between the two alleles of the FcγRIIA gene and a susceptibility gene for SLE (reviewed by Vyse and Kotzin, 1998). The FcγRIIa-H131 allele is the only human FcγR that can recognises IgG_2 efficiently and a decrease in the prevalence of this allele has been reported in patients with systemic lupus

erythematosus. This implies that modulation of the expression of individual FcγRIIa alleles is linked to abnormal downregulation of endocytic capacity of FcγRIIa bearing cells, which may lead to insufficient immune-complex clearance. In addition, the nephritis loci in NZ hybrid mice maps to a region that contains FcγRIIb, implicating a possible genetic linkage between disease and a FcγR (reviewed by Vyse and Kotzin, 1998). Overall, ample evidence exists to suggest that antibody-binding receptors in humans contribute to autoimmune disease (reviewed by Guarnotta, *et al.*, 2000). Whether the results reflect a defect in the clearance of immune complexes or in the regulation of the antibody response is unclear.

5.3 Aims and objectives of this study

Human B cells encode two isoforms of the low affinity IgG receptor, FcγRIIb, FcγRIIb1 and FcγRIIb2. Both isoforms of FcγRIIb possess an inhibitory ITIM motif in their cytoplasmic domains. *In vitro* and *in vivo* studies have shown that FcγRIIb acts as a negative regulator of immune complex-triggered activation of B cells and, by directly influencing the signalling threshold of B cells, is essential for maintaining B cell homeostasis.

FcγRIIb2 is capable of mediating rapid IgG endocytosis whilst an insertion of 19 amino acids in the cytoplasmic tail of FcγRIIb1 disrupts the cytoskeletal attachment domain responsible for modulating receptor internalisation. Therefore, it may be possible that differences in the relative expression levels of the two isoforms of FcγRIIb, may influence T cell-mediated help and thus the regulation of B cell tolerance by immune-complexes and hence an individual's overall risk of developing autoimmune disease.

The objectives of this study were therefore to investigate the expression levels of the specific FcγRIIb isoforms using RT-PCR methods, to:

- define whether peripheral blood mononuclear cells from rheumatoid arthritis and systemic lupus erythematosus patients show aberrant FcγRIIb isoform expression levels relative to controls.

- examine whether FcγRIIb isoform expression levels are related to, or indicative of, disease status and progression in these rheumatoid arthritis and systemic lupus erythematosus patients.

In addition, the nuclear phosphatase, Pac-1 has been shown to be recruited during FcγRIIb mediated negative signalling in murine B cells. Thus, a further objective was to examine whether peripheral blood mononuclear cells from autoimmune patients display aberrant FcγRIIb-mediated recruitment of Pac-1, by investigating the relative expression levels in the same patient samples.

5.4 Results

5.4.1 RT-PCR analysis of FcγRIIb1 and FcγRIIb2 mRNA expression levels in human cell lines.

Due to the high percentage of identity between the extracellular domains of FcγRIIb1 and FcγRIIb2, commercial antibodies are unable to discriminate between the two isoforms on the cell surface due to cross-reaction. Thus, RT-PCR analysis was used to determine the mRNA message expression levels of the two FcγRIIb isoforms. To optimise the PCR conditions and cycle number for the primer sets, prior to the analysis of primary human samples, RT-PCR was performed on total RNA from human monocytic and B cell lines (**Figure 5.1A**). For PCR a single primer pair, cross-reactive for both FcγRIIb1 and FcγRIIb2 was used. The primers were designed to flank the splice region which gives rise to the two splice variants. Thus, the PCR product for FcγRIIb1 would be 365 base pairs in length and FcγRIIb2 would be 308 base pairs in length, FcγRIIb2 possessing a shorter C-terminal domain. A second primer pair was designed to amplify the internal insert present exclusively in the cytoplasmic domain of FcγRIIb1, producing a product of 54 base pairs.

Products for both FcγRIIb1 and FcγRIIb2 were detectable in the human monocytic cell line, U937 and the human mature B cell line, Daudi. Interestingly, whilst FcγRIIb1 is thought to be preferentially expressed by B cells, the level of FcγRIIb1 message is higher in U937 cells compared to the level in Daudi B cells. These data indicate that both cells types express mRNA for both isoforms whilst highlighting the potential differences between monocytic and B cell expression of FcγRIIb. Furthermore, the monocytic cell line, THP1 and the B cell line, Ramos were shown not to express the full length message, despite possessing sequences for the insert region of FcγRIIb1. Thus the relevance of the constant level of the internal FcγRIIb1 insert in all four samples is unclear as an absence of product would be expected in THP1 and Ramos samples whilst U937 and Daudi samples should demonstrate enhanced expression. Therefore, the presence of insert in the THP1 and Ramos samples may indicate aberrant splicing of FcγRIIb transcripts.

Following positive expression with the primer pairs in human cell lines, RT-PCR was used to determine the expression of the differentially spliced isoforms of FcγRIIb in human peripheral blood mononuclear cells (PBMCs). Details of the normal subjects assessed are shown in **Table 5.1**. **Figure 5.1B** demonstrates detectable levels of both isoforms in primary PBMCs from normal individuals. With the exception of increased expression of FcγRIIb1 in subject 0834, the levels of both isoforms detected were approximately constant in all samples relative to β-actin control levels.

5.4.2 Patient subject data

Peripheral blood samples were obtained from rheumatoid arthritis patients attending the Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow, who satisfied the 1987 American College of Rheumatology diagnostic criteria, (Arnett, *et al.,* 1988). Details of the subjects assessed are shown in **Table 5.2.** Disease status of arthritis patients was assessed by disease duration, full blood count, rheumatoid factor (positive or negative), extra-articular conditions and drug therapy, including non-steroidal anti-inflammatory drugs (NSAID), disease-modifying anti-rheumatic drugs (DMARD) and other non-related drugs. All clinical data was discussed in consultation with Dr Iain McInnes, a rheumatology clinician.

5.4.2.1 RT-PCR analysis of FcγRIIb1 and FcγRIIb2 mRNA expression levels in peripheral blood mononuclear cells from rheumatoid arthritis subjects.

To determine the mRNA expression levels of the two FcγRIIb isoforms in PBMCs from arthritis subjects, a random selection of PBMC total RNA was subjected to RT-PCR analysis using the primer pairs described above. **Figure 5.2** demonstrates minor differences in FcγRIIb isoform mRNA expression between individual patients. However, quantitation of isoform expression levels was prevented by non-linearity between the amount of PCR product and the obtained gel-image. Absolute values for the amount of PCR product could be determined by semi-quantitative PCR, however this method is not a realistic approach for the analysis of a large cohort of patient samples. Thus,

quantitative real-time PCR technology was utilised to quantify levels of isoform message in comparison to the message levels of Hypoxanthine-guanine phosphoribosyltransferase (HPRT).

5.4.2.2 Quantitative TaqMan® analysis of FcγRIIb1 and FcγRIIb2 mRNA expression levels in peripheral blood mononuclear cells from control and rheumatoid arthritis subjects.

TaqMan® primer and probe pairs were designed with the help of Dr. Carol Campbell (Dept. of Immunology, University of Glasgow) to amplify the individual FcγRIIb isoforms. Primers were based on the published sequences for human CD32 isoforms (Brooks, *et al.*, 1989). The FcγRIIb1-specific primers were designed to amplify the sequences specific for the cytoplasmic insert encoded for by the cytoplasmic exon 1, whilst primers for the FcγRIIb2 isoform were specific for areas across the splice site. Product sequences from the primer and probe pairs **(Table 2.3** and **Table 2.4** and **Figure 2.7)** were tested against Genebank sequences to ensure specificity and exclude cross-hybridisation with CD16 (FcγRIIIa or b), CD32 (FcγRIIa1 and FcγRIIc) and CD64 (FcγRIa1, b1 and b2).

All statistical analysis was performed with the help of Dr. Charlie McSharry (Dept. of Immunology, University of Glasgow) on Minitab software (State College, Pennsylvania, USA) using Pearson correlation and Mann-Whitney analysis as appropriate. A total of 47 arthritis and 13 control subjects were assessed for the relative expression of FcγRIIb isoforms and the statistical analysis is shown in **Table 5.3**. In the control group, no significant difference was demonstrated between the genders in their median ages, or in the expression levels of FcγRIIb1 or FcγRIIb2. Furthermore, the relative expression levels of FcγRIIb1 were found not to correlate with the expression levels FcγRIIb2 (**Figure 5.3**).

The arthritis subjects were significantly older than the control subjects, however no relation was found between FcγRIIb expression levels and age or gender. In

the arthritis group, FcγRIIb expression was not related to disease duration, the presence of rheumatoid factor or the individual levels of white blood cells. No significant difference was demonstrated between the genders in the expression levels of FcγRIIb1 or FcγRIIb2. Furthermore, no correlation was found between the relative expression levels of FcγRIIb1 or FcγRIIb2 (**Figure 5.4).** Interestingly, arthritis subject 908 demonstrated high levels of FcγRIIb2 however, the standard error was also high for this patient. Due to the lack of a suitable cohort of arthritis subjects with single drug therapy treatments or extra-articular conditions, no statistical conclusions can be drawn concerning the expression of FcγRIIb isoforms in relation to drug therapy and extra-articular disease. Importantly, expression levels of FcγRIIb2 were similar in both groups however, the expression levels of FcγRIIb1 were significantly lower in arthritis subjects (arthritis 4.9% vs. control 17.2%, $p < 0.003$) (**Figure 5.5**). These data clearly indicate that a downregulation of FcγRIIb1 mRNA levels is found in arthritis subjects and could represent a mechanism that predisposes an individual to disease onset and/or progression.

5.4.2.3 Quantitative TaqMan® analysis of Pac-1 mRNA expression levels in peripheral blood mononuclear cells from control and arthritis subjects.

The nuclear phosphatase, Pac-1 has been shown in this study (Chapter 3) to be recruited during FcγRIIb-mediated negative signalling in murine B cells. To examine whether peripheral blood mononuclear cells from the same autoimmune patients displayed aberrant expression of Pac-1, the relative expression levels of Pac-1 were determined. A primer and probe pair was designed with the help of Dr. Carol Campbell (Dept. of Immunology, University of Glasgow) to amplify Pac-1 specific sequences in the samples (**Table 2.3** and **Table 2.4**). To prevent the possible amplification of contaminating genomic DNA, TaqMan® probes are normally designed to bind directly over intron/exon junctions. A lack of binding to genomic DNA during the amplification process prevents probe cleavage and production of a positive signal. Due to the strict design requirements of the TaqMan® system it was not possible to design a probe and primer set that spanned an intron/exon boundary for Pac-1.

Therefore to exclude the possibility of a false-positive, due to the amplification of genomic sequences, matching RNA samples that had not been reverse transcribed were run in parallel to the cDNA samples. Values from control samples, which amplified genomic sequences, were taken as base line controls and values subtracted from the Pac-1 expression levels to obtain a normalised Pac-1 expression value.

Statistical analysis of Pac-1 expression levels in control and arthritis subjects is shown in **Table 5.3**. Perhaps, surprisingly, given the downregulation of FcγRIIb1 expression in RA patients, no relationship was found between Pac-1 expression levels and any selected criteria in control or arthritis subjects. Thus, it is unlikely that modulation of Pac-1 message levels in peripheral blood mononuclear cells predisposes an individual to arthritis.

5.4.2.4 Quantitative TaqMan® analysis of FcγRIIb1 and FcγRIIb2 mRNA expression levels in peripheral blood mononuclear cells from control and systemic lupus erythematosus subjects.

Total RNA from peripheral blood mononuclear cells of systemic lupus erythematosus patients and age matched controls was kindly provided by Dr. H. Siew (Dept. of Rheumatology, Allergy and Immunology, Tan Tock Seng Hospital, Singapore). A total of 15 SLE and 10 control subjects (**Tables 5.4** and **5.5**) were assessed for the relative expression of FcγRIIb isoforms. All the subjects were ethnically matched and female, as the disease is predominantly displayed in females of childbearing age. The same TaqMan® primer and probe pairs, as described above, were used to amplify up the individual FcγRIIb isoforms and the statistical analysis is shown in **Table 5.3**. Graphical representations of the data are shown in **Figures 5.6** and **5.7**. In contrast to the arthritis patients, no relationship was found between the expression levels of the individual FcγRIIb isoforms and any selected criteria in control or SLE subjects. Whilst not significant at a level of $p<0.05$, FcγRIIb1 expression levels in SLE patients were reduced in comparison to control subjects and a larger cohort of subjects may provide a statistically relevant significance between the two groups (see above).

5.4.2.5 Quantitative TaqMan® analysis of Pac-1 mRNA expression levels in peripheral blood mononuclear cells from control and systemic lupus erythematosus subjects.

To examine whether peripheral blood mononuclear cells from the same cohort of SLE patients and controls displayed aberrant expression of Pac-1, the relative expression levels of Pac-1 were determined. The same Pac-1 primer and probe pair, as described above, was used to amplify up a Pac-1 specific sequence in the samples. Matching RNA samples that had not been reverse transcribed were also run in parallel to the cDNA samples, to exclude the possibility of amplification of genomic sequences.

Statistical analysis of Pac-1 expression levels in control and SLE subjects is shown in **Table 5.6**. Graphical representations of the data are shown in **Figures 5.6** and **5.7**. Similar to the arthritis subjects and controls, no relationship was found between Pac-1 expression levels and any selected criteria in control or SLE subjects. Thus, it is unlikely that modulation of Pac-1 message levels in peripheral blood mononuclear cells predisposes an individual to SLE.

5.5 Discussion

Differences in the relative expression levels of the two isoforms of FcγRIIb, FcγRIIb1 and FcγRIIb2, may influence the regulation of B cell responses by immune-complexes and thus may represent a mechanism that initiates autoimmunity. An investigation of the mRNA message levels of the two FcγRIIb isoforms in peripheral blood cells from autoimmune patients, demonstrated that FcγRIIb1 expression levels were lower in rheumatoid arthritis patients compared to normal controls. In contrast, mRNA levels of the two isoforms were similar in patients with systemic lupus erythematosus. These preliminary results indicate that progression of rheumatoid arthritis in humans may be perpetuated by a downregulation of FcγRIIb expression, which may remove the negative feedback regulation of autoreactive B cells, by IgG-containing immune complexes.

5.5.1 FcγRIIb and rheumatoid arthritis

Rheumatoid arthritis is a complex autoimmune disease and, similar to other autoimmune diseases, displays a polygenic phenotype. Disease susceptibility factors have been proposed to include MHC class II antigen HLA-DR4 and related haplotypes and a variety of non-MHC genes. Whilst FcγRIIb has been implicated as a risk factor for the initiation and propagation of disease in murine models of arthritis, a similar role has not been attributed to this receptor in human rheumatoid arthritis. An investigation of FcγRIIb isoform expression in PBMCs from 47 arthritis patients demonstrated a significant reduction in the mRNA expression levels of FcγRIIb1 in arthritis patients compared to controls. However, no differences in the expression of FcγRIIb2 or Pac-1 were demonstrated.

Rheumatoid factor (RF) antibodies recognise the Fc portion of IgG and occur commonly in rheumatoid arthritis. High affinity rheumatoid factors accumulate in the synovium and contribute to the disease pathogenesis by forming immune complexes and inducing activation of complement. Moreover, B cells that express RF-BCRs can obtain help without requiring IgG-responsive T cells as, by taking up IgG attached to a non-self antigen, they can present that antigen to responsive T cells. Of interest to this study, RF-BCRs may compete with FcγRIIb for large complexes, reducing the negative signals from this receptor.

Interestingly, only the expression of FcγRIIb1 appeared to be modulated in arthritis patients whilst expression of the endocytosis competent isoform, FcγRIIb2, remained constant. Thus FcγRIIb2 may be the isoform predominantly utilised by B cells for antigen uptake and processing whilst modulation of FcγRIIb1 expression dictates the threshold for BCR signalling. By downregulating expression of FcγRIIb1, whilst keeping FcγRIIb2 levels constant, an autoreactive B cell might remain responsive to stimulation by immune complexes and co-stimulatory T cell help. CD40 ligand (CD40-L) expressing, activated T cells, in conjunction with T cell derived IL-4, will induce proliferation, antibody production and isotype switching by the RF specific B cells in addition to overcoming the inhibitory effects of FcγRIIb2. Furthermore, the cytokine

profile present in inflammatory disease will affect not just B cells but also FcγRIIb-bearing phagocytic cells (see below) thus promoting the initiation and propagation of disease.

Sarmay *et al.*, 1991 have observed in their studies that whilst both isoforms are present, the mRNA levels of FcγRIIb2 exceed that of FcγRIIb1 in resting and activated normal human tonsil B cells. In contrast, results from this study demonstrated higher levels of FcγRIIb1 expression in control subjects, in comparison to FcγRIIb2. Indeed, this relationship was similar in all groups studied. It would therefore be of interest to examine the relative isoform expression in purified peripheral blood lymphocytes to examine whether the expression levels are similar when compared to the whole blood population.

However, peripheral blood contains only 1 x 10^6 lymphocytes/ml and, not accounting for losses during purification, at least 20 ml of blood would be required to purify enough B cells for a TaqMan® study. Peripheral blood samples from patients are uniformly only 10 mls, thus further studies may have to rely on FACS analysis with whole blood samples and specific antibodies to the intracellular domains of the two isoforms (currently not available commercially) in combination with 2.4G2 staining of FcγRIIb on the cell surface.

Indeed, this further study would also exclude the contribution made to the expression levels by other FcγRIIb-bearing cells, since differential isoform expression by B cells may be masked by FcγRIIb-expressing myeloid cells and/or inappropriate tissue samples. The role of other FcγRIIb-expressing cells is important to consider since the disease phenotype may not be solely attributable to the effect of FcγRIIb on the production of autoantibodies but may well reflect aberrant non-inflammatory immune complex clearance by the phagocytic system. Indeed, studies in FcγRIIb-deficient mice indicate that FcγRIIb has a dual function in the spontaneous development of lupus-like disease (see below) involving both the production of autoantibodies and the clearance of immune complexes (Bolland and Ravetch, 2000), thus implying that both of these events may be required for the initiation of autoimmune

disease. However, in agreement with murine FcγRIIb-deficiency models the patient results suggest that downregulation or deficiency of the negative receptor may result in the exacerbation of disease. Similar to the study of FcγRIIb promoter polymorphisms in germinal B cells of SLE mice (Jiang *et al.*, 1999, 2000) abnormal downregulation of FcγRIIb1 expression upon autoantigen stimulation might enable autoreactive B cells to escape suppressive signalling.

Future studies should also examine FcγRIIb isoform expression in samples of synovial membrane from arthritis subjects, since it is uncertain whether PBMCs are reflective of the overall immune status. Indeed, preliminary studies in synovial membrane digests from six arthritic subjects correlate with the low levels of FcγRIIb1 expression observed in PBMCs (average expression level of 17.2% in PBMCs of control subjects versus 6.2% in synovial membrane of patients, relative to HPRT levels; results not shown). However, FcγRIIb2 expression in the same digests was found to be enhanced (expression level of 40% relative to HPRT levels; results not shown) and may reflect the high percentage of FcγRIIb2-expressing myeloid cells in the synovial population.

Interestingly, FcγRIIb expression was found to be independent of age, gender, presence of rheumatoid factor, disease duration and white blood cell levels (results not shown). Due to the small cohort of patients analysed for this pilot study, no correlation could be drawn between individual drug therapies and FcγRIIb expression. Since the majority of arthritis therapies modulate the synthesis of pro-inflammatory cytokines and signal transduction by inflammatory cell, it is likely that their therapeutic actions will modulate the expression of FcγRIIb. Indeed, studies by Sarmay *et al.*, 1991 found that activation of human B cells, by IgM crosslinking or the presence of IL-4, induces alternative splicing of FcγRIIb1 mRNA with simultaneous suppression of FcγRIIb2 mRNA. Their data clearly indicates that B cell activation promotes differential FcγRIIb isoform expression and in RA patients this may modulate activation of autoreactive B cells.

Corticosteroids suppress gene expression and synthesis of pro-inflammatory cytokines whilst levels of anti-inflammatory cytokines are relatively unaffected (reviewed by Dinarello and Moldawer, 2000). Other observed effects include modulation of immunoglobulin synthesis and programmed cell death of lymphocytes, contributing to an overall suppression of the immune system. In contrast, non-steroidal anti-inflammatory drugs (NSAIDs) exert their primary influence on inflammatory disease by inhibiting prostaglandin synthesis. Disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate, inhibit the inflammatory response on many levels acting as anti-inflammatory agents, immunosuppressive therapies and anti-proliferative drugs. Whilst DMARDs non-specifically target the production of pro-inflammatory cytokines, recent therapeutic studies have specifically targeted the production of TNF-α, with the use of anti-TNF-α antibodies (Infliximab) or soluble TNF-α receptor fusion proteins (sTNF-RII:Fc; Etanercept). Neither of these drugs were currently being administered to any of the patients studied however, it would be of interest in future studies to investigate the role of these suppressive agents on the functionality and expression levels of FcγRIIb in patients.

Many of these therapeutic drugs act, in part, to reduce the T_H1 inflammatory disease phenotype thus patients on DMARD or NSAID therapy may express higher levels of FcγRIIb1. Unfortunately, due to the complexity of involvement of co-morbid conditions and the fact that most patients are on combination therapy, it proved impossible to generate statistically meaningful comparative groupings. However, a small cohort of patients on single drug therapy appeared to demonstrate reduced or similar levels of FcγRIIb1 message when compared to the group as a whole (average expression level of 4.2% in PBMCs of single drug therapy patients versus 4.9% in PBMCs of total patient cohort; results not shown). These preliminary results implicate a potential role for pro-inflammatory cytokines in the differential expression of FcγRIIb1 and FcγRIIb2 in arthritis.

5.5.2 FcγRIIb and systemic lupus erythematosus

The main immunological characteristics of systemic lupus erythematosus (SLE) are a number of profound B cell abnormalities that permit the generation,

differentiation and positive selection of B cells that secrete pathogenic autoantibodies (reviewed by Lipsky, 2001). Thus, it is possible that a loss of FcγRIIb negative regulation, results in the production of B cells that are hyperactive to normal antigenic stimulation and provides the drive for autoantibody production. However, neither functional defects in FcγRIIb nor polymorphic variants of this receptor have been described in patients with SLE thus far.

An investigation of FcγRIIb expression in PBMCs from 15 SLE patients demonstrated no significant difference in the mRNA expression levels of FcγRIIb1 and FcγRIIb2, compared to the expression levels of 10 matched controls. Furthermore, no correlation was found between the relative expression levels of FcγRIIb1 or FcγRIIb2. Whilst not statistically significant, FcγRIIb1 expression levels in SLE patients were reduced in comparison to control subjects (SLE 10.1 vs. Control 16.3) and this may be of relevance in a clinical environment. A larger cohort of subjects may therefore provide a statistically relevant significance between the two groups. In addition, no significant difference in the mRNA expression levels of Pac-1 were found when comparing the two groups. Thus, it is unlikely that aberrant Pac-1 expression or function is a risk factor associated with susceptibility to SLE in humans.

These results were somewhat surprising in light of the results from the MRL-*lpr/lpr* mice, which suggested that FcγRIIb-mediated negative regulation might be dysregulated in this model. However, since functional expression of the receptor was not examined in this human study it is difficult to draw comparisons between the two studies. Furthermore, whilst a central role for hyperactive B cells and autoantibodies has been implicated in SLE, the role for autoantibodies in the development of disease has been challenged by a genetically manipulated MRL-*lpr/lpr* model in which the mice are able to develop nephritis despite being unable to secrete autoantibodies (Chan *et al.*, 1999). However, as B cell deficient MRL-*lpr/lpr* mice do not develop nephritis (Schlomchik *et al.*, 1994), these latter results may suggest a more important

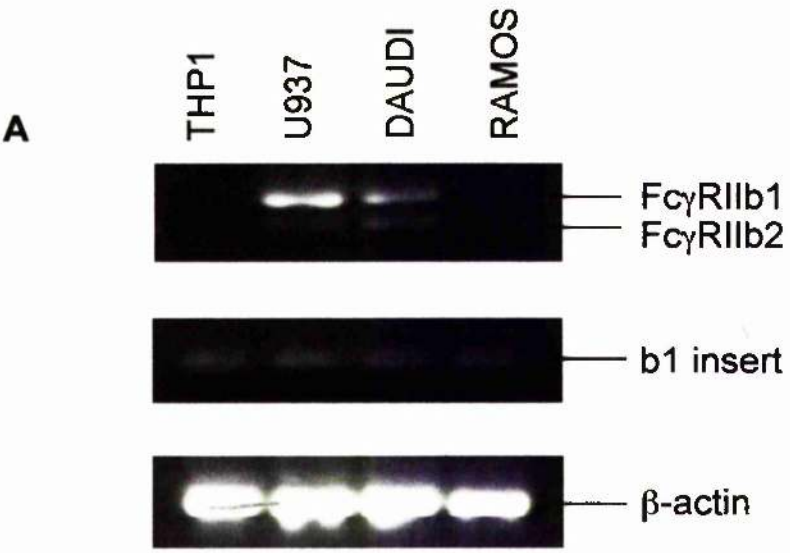
role for B cells in the activation of T cells but not necessarily for the production of autoantibodies in this murine model of lupus-like disease.

The diversity of clinical manifestations and disease phenotypes in humans, together with a lack of a human counterpart to the murine *lpr* phenotype, has made the study of FcγRs in human SLE very difficult. However, a skewing of FcγRIIa allotype expression has been reported in SLE patients and linked with nephritis in African-Americans (Duits *et al.*, 1995). The differential expression results in the downregulation of the high responder allele of FcγRIIa, implying that the skewing may lead to the downregulation of endocytic capability of phagocytes and insufficient clearance of immune complexes. Polymorphisms in these FcγR genes may be important susceptibility factors for SLE nephritis. Thus, the diversity of disease phenotypes may be as a result of the genetically determined ability of FcγR to bind and clear the immune complexes.

In view of the ability of B cells to regulate many aspects of immune reactivity it is likely that dysregulated B cell functions contribute significantly to the initiation and progression of human SLE. Thus, whilst a role for FcγRIIb in SLE is, as yet, undetermined future studies may reveal that aberrant expression or function of this receptor may predispose an individual to SLE.

Figure 5.1 RT-PCR analysis of FcγRIIb1 and FcγRIIb2 mRNA expression levels in human cell lines and peripheral blood mononuclear cells from normal individuals.

Total RNA was extracted from **(A)** human monocytic (U937 and THP-1) and B cell lines (Daudi and Ramos) or **(B)** human peripheral blood mononuclear cells from normal individuals (5 x 10^6 cells) and was reverse transcribed in the presence of 100 U Superscript II RT, as described in the Materials and Methods. PCR was performed using a single primer pair, cross-reactive for both FcγRIIb1 and FcγRIIb2, producing PCR products of 365 and 308 base pairs in length, respectively. A second primer pair was designed to amplify the internal insert present exclusively in the cytoplasmic domain of FcγRIIb1, producing a product of 54 base pairs. Human β-actin primers were used to assess the relative quantity of mRNA.


A
THP1
U937
DAUDI
RAMOS
FcγRIIb1
FcγRIIb2
b1 insert
β-actin

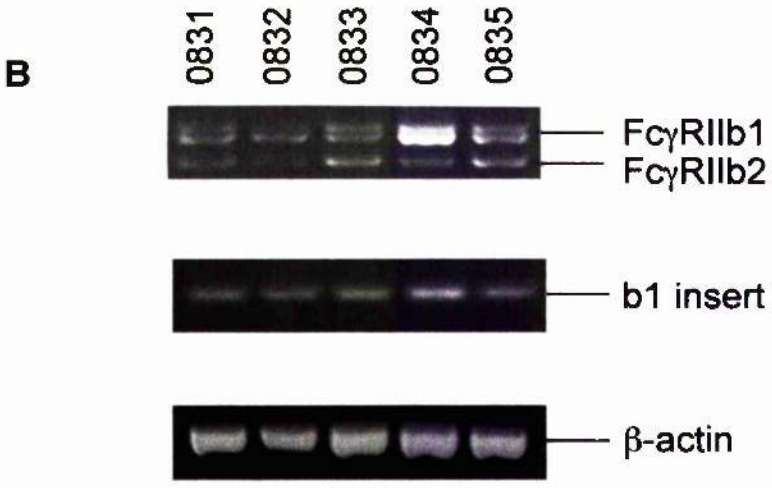

B
0831
0832
0833
0834
0835
FcγRIIb1
FcγRIIb2
b1 insert
β-actin

Figure 5.2 RT-PCR analysis of FcγRIIb1 and FcγRIIb2 mRNA expression levels in peripheral blood mononuclear cells from arthritis subjects.

Total RNA was extracted from human peripheral blood mononuclear cells (5 x 10^6 cells) from arthritis subjects and was reverse transcribed in the presence of 100 U Superscript II RT, as described in the Materials and Methods. PCR was performed using a single primer pair, cross-reactive for both FcγRIIb1 and FcγRIIb2, producing PCR products of 365 and 308 base pairs in length, respectively. A second primer pair was designed to amplify the internal insert present exclusively in the cytoplasmic domain of FcγRIIb1, producing a product of 54 base pairs. Human β-actin primers were used to assess the relative quantity of mRNA.

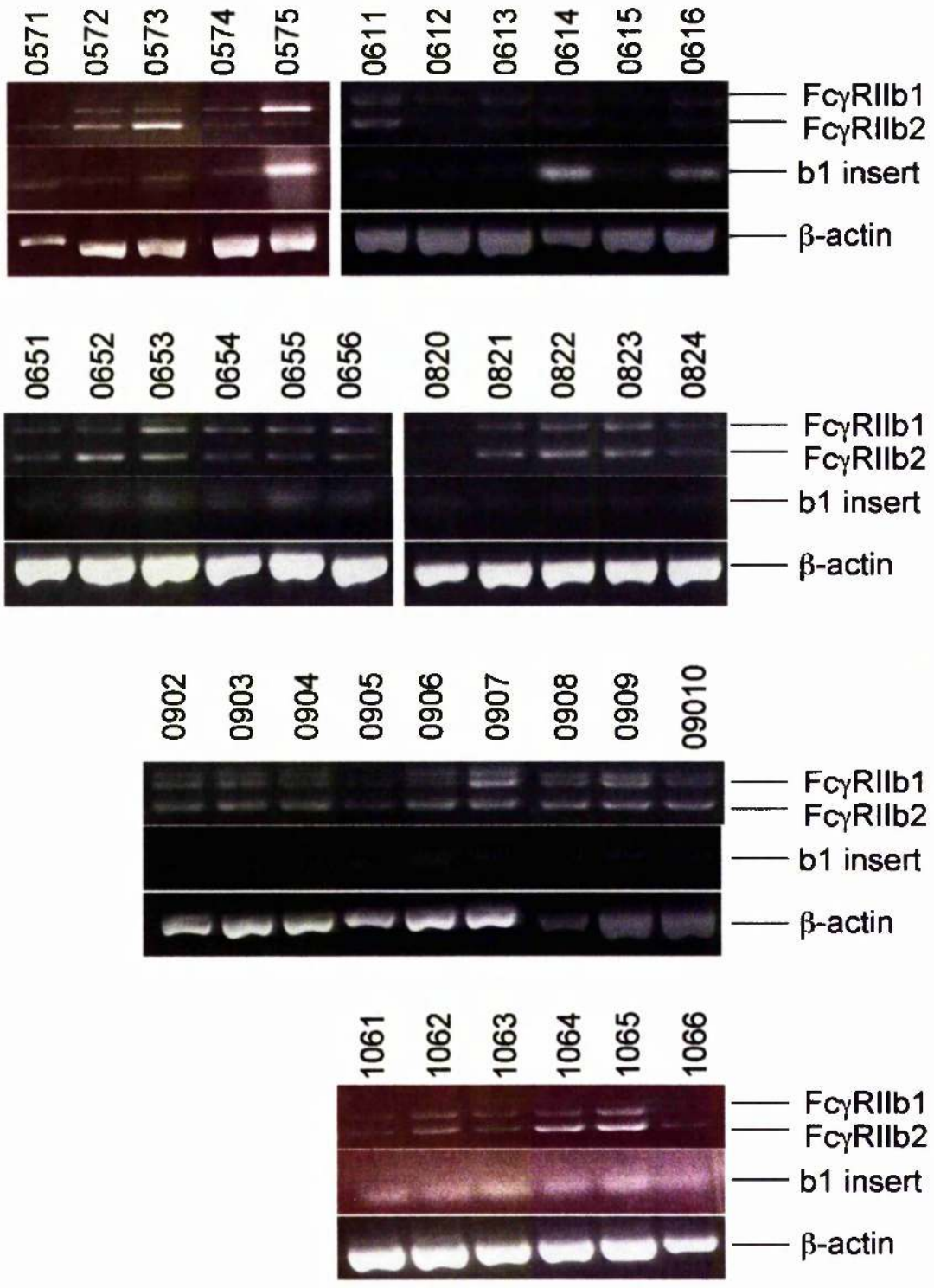

0571
0572
0573
0574
0575
0611
0612
0613
0614
0615
0616
FcγRIIb1
FcγRIIb2
b1 insert
β-actin
0651
0652
0653
0654
0655
0656
0820
0821
0822
0823
0824
FcγRIIb1
FcγRIIb2
b1 insert
β-actin
0902
0903
0904
0905
0906
0907
0908
0909
09010
FcγRIIb1
FcγRIIb2
b1 insert
β-actin
1061
1062
1063
1064
1065
1066
FcγRIIb1
FcγRIIb2
b1 insert
β-actin

Table 5.1 Details of control subjects at time of study

Peripheral blood samples were obtained from 13 normal individuals. FcγRIIb1, FcγRIIb2 and Pac-1 transcripts were assayed for by real-time PCR (TaqMan®). mRNA levels are expressed as a percentage relative to HPRT mRNA levels ± S.E for three experiments.

CONTROL SUBJECTS (n = 13)

No.	GENDER	AGE	FcγRIIb1	± S.E	FcγRIIb2	± S.E	Pac-1	± S.E
4226	F	23	33	4.8	25.5	3.7	17.84	2.50
4221	F	24	1.8	0.5	3.4	1.7	0.00	0.00
4223	F	24	21	5.2	5	2.8	14.39	3.50
833	F	31	8.8	0.6	8.0	0.9	0.00	0.00
835	F	43	10.4	1.8	7.0	0.8	0.01	0.00
4224	M	24	34	11.8	21	1.93	0.14	0.12
4346	M	31	20.1	4.6	14.2	2.6	0.02	0.00
4283	M	32	4.6	0.9	4.3	1	24.83	2.51
4345	M	32	17.2	4.8	3.5	1.1	3.77	0.00
4225	M	36	36	7	54	14	8.34	2.03
834	M	47	13.9	2.9	4.8	1.4	0.21	0.04
832	M	55	17.3	2.5	0.1	0.0	26.73	2.04
831	M	58	12.1	1.1	1.7	0.4	0.02	0.00

Table 5.2 Details of arthritis subjects at time of study

Peripheral blood samples were obtained from 47 arthritis patients attending the Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow, who satisfied the 1987 American College of Rheumatology diagnostic criteria, (Arnett, *et al.,* 1988).

Disease duration is determined in years from the first clinical confirmation of arthritis. The **drug profile** is segregated into non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) and other non-related drugs.

NSAID; Nap, Naproxen. **Para**, Paracetamol. **Co-pro,** Co-proxamol. **Asp,** Aspirin. **Nab,** Nabumetone. **Indo,** Indomethacin. **Diclo,** Diclofenac. **Etod,** Etodolac. **Vol,** Voltarol.

DMARD; Pen, Penicillamine. **Sulph,** Sulphasalazine. **Metho,** Methotrexate. **Hydroxy,** Hydroxychloroquine. **Pred,** Prednisolone. **Aza,** Azathioprine.

OTHER; Frus, Frusemide. **Omer,** Omerprazole. **Glu,** Gluclazide.

Full blood count; WBC, white blood cells. **LYMPH,** lymphocytes. **NEUTRO,** neutrophils. **MONO,** monocytes. **EOSIN,** eosinophils. **BASO,** basophils.

RF; rheumatoid factor assessed as either positive (+) or negative (-).

Total RNA was extracted from human peripheral blood mononuclear cells (5 x 10^6 cells) from the 47 arthritis subjects and was reverse transcribed in the presence of 100 U Superscript II RT, as described in the Materials and Methods. FcγRIIb1, FcγRIIb2 and Pac-1 transcripts were assayed for by real-time PCR (TaqMan®). mRNA levels are expressed as a percentage relative to HPRT mRNA levels ± S.E for three experiments.

ARTHRITIS SUBJECTS (n = 47)

No.	GENDER	AGE	PRIMARY DIAGNOSIS	DISEASE (YRS)	DRUG PROFILE NSAID	DMARD	OTHER	CO-MORBID CONDITIONS	CELLS ($x10^9/l$) WBC	LYMPH	NEUTRO	MONO	EOSIN	BASO	RF	FcγRIIb1 ± S.E		FcγRIIb2 ± S.E		Pac-1 ± S.E	
4342	F	39	n/k	1	None	None	None	Mixed connective tissue disease	5.5	1.8	3.2	0.4	0.1	0	-	7.6	2.2	6.8	3	0.1	0.0
4282	F	42	Soft-tissue rheumatism	<1	Diclo.	None	None	Arthralgia, obesity, hypertension, hypercholesterolaemia	6.1	1.8	3.7	0.4	0.2	0	-	4.8	0.3	2.9	0.4	0.0	0.0
617	F	47	Erosive RA	15	Froben	None	None	Carpal tunnel syndrome	6.8	1.3	3.4	0.5	0.4	0	+	2.9	0.9	7.3	0.4	0.4	0.1
614	F	53	RA	10	None	Sulph., Hydroxy.	Prepulsid, Ranitidine,	Stomach cancer	12.1	1.7	8.5	1.5	0.3	0.1	+	1.4	1.1	6.6	0.8	0.7	0.1
324	F	54	Erosive RA	40	Asp.	Metho.	Dihydrocodeine, Omer.	Gastritis, anaemia, Rh lung disease, thrombocytosis, osteoporosis	16.8	1.3	17.4	0.5	0	0	+	8.7	1.9	7.4	1.9	0.0	0.0
612	F	56	RA	n/k	Nap., Asp.	Metho., Sulph., Hydroxy	Cocodamol	Pneumonia	11.7	2.1	6.9	0.6	0.1	0.1	-	1.2	0.3	4.3	0.5	0.9	0.0
619	F	57	RA	23	Nab.	Sulph., Hydroxy.	Misoprostol	breast cancer, hypertension, GI	4.7	1.7	3.3	0.4	0.2	0	+	1.4	0.2	6.1	0.6	0.4	0.0
652	F	57	RA	30	Indo.	Sulph.	None	None	8.1	1	5.8	0.9	0.3	0.1	+	5.9	1.3	12.2	2.8	2.3	0.6
1056	F	57	Erosive RA	31	None	Sulph., Minocycline	IV Ig, Warfarin, Thyroxin	Amyloid, rheumatic heart disease	6	0.6	4.1	1	4	0	+	4.9	1.9	17.6	8.4	7.7	2.8
909	F	57	RA	2	Diclo.	Hydroxy.	Omer.	Inflammatory arthropathy, thyroid abnormalities	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	8.3	0.9	8.2	1.1	17.6	3.9
655	F	59	Erosive RA	30	Nap.	Pen.	None	Proteinuria	6.6	1.6	3.2	0.6	0.2	0	+	15.4	3.0	5.1	0.9	7.8	1.4
656	F	60	Erosive RA	32	Relifex	Pen.	None	Proteinuria	5.9	0.7	4.8	0.3	0	0	+	5.2	0.5	1.6	0.2	3.8	1.2
907	F	60	RA	32	Ketoprofen	Gold	Omer., Didronel	Heart disease, Hep C, Osteopenia, hypogammaglobulinemia	5.6	1.3	3.6	0.6	0.1	0	-	10.8	2.4	3.7	0.9	0.0	0.0
615	F	61	Juvenile onset RA	50	Co-pro.	Sulph.	None	None	7.8	1.4	3.5	0.5	0.1	0	-	1.7	0.4	3.5	0.5	1.3	0.3
6110	F	65	RA	20	Nap.	Gold	Bendrofluazide	Osteoporosis, GI upset	5.4	1.6	3.3	0.4	0.1	0	+	4.2	0.8	12.4	2.8	0.5	0.1
901	F	65	Erosive RA	10	Nab.	Cyclosporin	Omer., Codydramol, Calcitrol,	Asthma, Osteoporosis	7.9	1.8	5.5	0.5	0.1	0	+	14.0	3.0	11.6	2.6	0.1	0.0
657	F	66	RA	13	Vol., Co-pro.	Sulph.	n/k	Osteoporosis, proteinuria	9.1	1.7	6.3	0.5	0.3	0.1	+	1.4	0.3	11.6	1.8	0.0	0.0
1064	F	68	RA	n/k	n/k	n/k	n/k	n/k	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	u/k	8.3	2.3	6.1	1.7	290.1	76.8
1063	F	68	RA	n/k	n/k	n/k	n/k	n/k	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	u/k	3.3	0.6	3.8	1.0	8.2	1.4
902	F	69	RA	11	Nap.	Salazopyrin, Hydroxy.	None	Hypertension, Osteoporosis	4.8	1.4	5.3	0.4	0	0	-	12.0	2.2	16.9	7.3	0.1	0.0
572	F	69	Erosive RA	11	None	Gold	Para.	Asthma, Osteoporosis, anaemia, peptic ulcer	10.2	1.6	12.7	0.8	1.1	0	+	8.8	2.3	6.8	1.3	2.7	0.6
903	F	71	Erosive RA	11	Nab.	Metho., Pred.	None	Osteoarthritis, Inflammatory arthropathy, Sjogrens syndrome	16.1	4	10.6	1.3	0.3	0.1	+	3.5	0.5	3.6	0.5	24.9	4.6
821	F	71	Erosive RA	13	None	Sulph.	Omer., Frus., Glu.	NIDDM, Proteinuria	5.4	2.3	2.7	0.4	0	0	-	0.6	0.1	1.8	0.3	0.0	0.0
618	F	72	Erosive RA	15	None	Gold	None	Liver function abnormal	5.6	1.7	3.2	0.8	0.5	0.1	+	2.1	0.2	2.9	0.7	0.2	0.0
654	F	72	RA	n/k	n/k	n/k	n/k	n/k	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	u/k	7.7	1.5	2.3	0.7	17.3	4.5
4281	F	77	RA	2	Diclo., Etod.	None	Dihydrocodiene	Hypertension, osteoarthritis,	6	1.3	3.1	0.4	0.1	0	+	3.3	1.48	3.4	0.8	0.3	0.0
1062	F	78	RA	n/k	n/k	n/k	n/k	n/k	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	4.9	1.4	2.8	0.8	27.4	8.1
908	F	85	RA	11	Asp., sulindac	Gold	None	Eczema, Hypertension, Proteinuria	6.3	1.4	4.2	0.4	0.2	0.1	-	23.0	4.2	209.0	109.0	0.3	0.1
673	F	n/k	RA	n/k	n/k	n/k	n/k	None	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	21.7	6.6	7.7	2.2	10.2	2.9
651	F	n/k	RA	n/k	n/k	n/k	n/k	n/k	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	1.2	0.8	2.8	0.8	17.7	5.0
4284	M	34	Fibromyalgia	1	None	None	Anti-depressants, Zydol	Back pain, osteoarthritis	7.8	2.8	4.2	0.6	0.1	0.1	-	5.8	1.6	0.9	0	0.0	0.0
4341	M	35	Psoriatic Arthritis	1	Rofecoxib	None	None	Psoriatic spondyloarthropathy, psoriasis	5.5	1.8	3.3	0.6	0.1	0	-	12.5	1	4.2	1.4	0.1	0.0
611	M	43	RA	18	None	Sulph., Hydroxy.	None	None	9.2	1.3	7.1	0.6	0.1	0	+	2.7	0.6	4.7	0.9	0.7	0.1
904	M	44	RA	n/k	n/k	n/k	n/k	n/k	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	3.6	0.7	8.0	1.9	9.5	1.0
4344	M	44	RA	n/k	Etod.	Sulph.	None	Psoriatic arthritis	5.6	1.2	3.9	0.3	0.2	0	-	15	2.9	7.6	2.28	0.2	0.0
4343	M	53	RA	n/k	n/k	n/k	n/k	n/k	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	u/k	5.2	1.2	2.5	0.5	0.1	0.0
906	M	56	RA	11	Etod.	Metho.	None	Osteoporosis, gleno-humaral shoulder disease	7.3	1.7	4.5	0.4	0.2	0	+	4.2	0.7	18.5	15.7	0.0	0.0
6111	M	58	Erosive RA	17	Asp.	Pen.	Frus., Glu., Lansoprazole	Heart disease, pneumonia, osteoporosis, anaemia, NIDDM	10	1.2	8.2	0.6	0	0	+	1.8	0.5	4.7	0.6	0.3	0.1
616	M	60	Erosive RA	10	Nap.	Pen.	Diazepam, Bendrofluazide	Peptic ulcer	8.4	1.8	5.5	0.8	0.2	0	-	1.5	0.2	1.9	0.3	1.1	0.1
823	M	60	RA	30	Nap.	Dapsone	Ranitidine	Dermatitis herpetiformis	8.3	1.7	4.9	0.6	0.2	0	-	5.4	1.5	6.2	3.3	0.0	0.0
653	M	62	Erosive RA	15	Nap.	Sulph.	None	Thrombocytopenia	5.5	1.3	3.5	0.5	0.1	0	+	9.1	1.1	2.6	0.6	1.7	0.3
905	M	63	RA	22	Diclo.	Gold	None	None	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	1.4	0.2	2.2	0.3	2.1	0.4
1061	M	64	Erosive RA	4	Arthrotec	Gold	Co-dydramol	NIDDM, anaemia, weight loss	12.5	1.1	10.7	0.5	0.1	0	-	39.0	4.8	0.4	0.1	172.9	11.1
1065	M	66	Erosive RA	34	Indo.	Gold	None	Vasculitis, renal failure, obesity, ulcers	9.8	1.4	6.5	0.5	0.3	0	+	5.9	0.9	15.2	2.6	2.4	3.4
613	M	72	Erosive RA	19	Co-pro.	Sulph.	Para.	Stroke, anaemia	7	1.5	4.7	0.6	0.1	0	–	0.7	0.2	3.6	0.8	0.2	0.1
575	M	n/k	n/k	n/k	n/k	n/k	n/k	n/k	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	u/k	0.8	0.4	5.2	1.5	4.6	2.9
4285	M	n/k	n/k	n/k	n/k	n/k	n/k	n/k	n.d	n.d.	n.d.	n.d	n.d.	n.d	-	3.3	0.8	2.05	0.4	0.0	0.0

Table 5.3 Statistical analysis of FcγRIIb1, FcγRIIb2 and Pac-1 expression levels in peripheral blood mononuclear cells from control and arthritis subjects

All statistical analyses were performed on Minitab software (State College, Pennsylvania, USA) using Pearson correlation and Mann-Whitney analyses as appropriate. FcγRIIb1, FcγRIIb2 and Pac-1 transcripts were assayed for by real-time PCR (TaqMan®). mRNA levels are expressed as a percentage relative to HPRT mRNA levels ± S.E for three experiments.

	Arthritis subjects (n = 47)	Control subjects (n = 13)	Mann-Whitney Significance
Male	17 (35 %)	8 (62 %)	-
Female	30 (65 %)	5 (38 %)	-
Age range (yr)	34-85	23-58	-
Age median (yr)	60	32	p = 0
Disease duration range (yr)	1-50	-	-
Median disease duration (yr)	15	-	-
B1 expression range	0.6-30	1.8-36	-
Median B1 expression	4.9	17.2	p = 0.0003
B2 expression range	0.4-209	0.1-54	-
Median B2 expression	4.7	5	n/s
PAC-1 expression range	0-290*	0-26.3	-
Median PAC-1 expression	0.5	0.2	n/s

	Arthritis subjects (n = 47)		
	MALE (n = 17)	**FEMALE (n = 30)**	**Mann-Whitney Significance**
Age range (yr)	34-72	39-85	-
Age median (yr)	58	65	p = 0.03
Disease duration range (yr)	1-34	1-50	-
Median disease duration (yr)	13	15	n/s
B1 expression range	0.7-30	0.6-23	-
Median B1 expression	4.2	4.9	n/s
B2 expression range	0.4-18.50	1.6-209	-
Median B2 expression	4.2	6.1	n/s
PAC-1 expression range	0-172.9	0-290	-
Median PAC-1 expression	0.1	0.6	n/s

	Control subjects (n = 13)		
	MALE (n = 8)	**FEMALE (n = 5)**	**Mann-Whitney Significance**
Age range (yr)	24-58	23-43	-
Age median (yr)	34	24	n/s
B1 expression range	4.6-36	1.8-33	-
Median B1 expression	17.3	10.4	n/s
B2 expression range	0.1-54	3.4-25.5	-
Median B2 expression	4.55	7	n/s
PAC-1 expression range	0.02-26.7	0-17.8	-
Median PAC-1 expression	1.99	0.01	n/s

Figure 5.3 TaqMan® quantitative RT-PCR of FcγRIIb1, FcγRIIb2 and Pac-1 mRNA expression levels in peripheral blood mononuclear cells from control subjects.

Total RNA was extracted from peripheral blood mononuclear cells (5 x 10^6 cells) from normal individuals and was subjected to reverse transcription in the presence of 100 U Superscript II RT, as described in the Materials and Methods. FcγRIIb1, FcγRIIb2 and Pac-1 transcripts were assayed for by real-time PCR (TaqMan®). Individual mRNA levels are expressed as a percentage relative to HPRT mRNA levels with the median indicated by the horizontal bar.

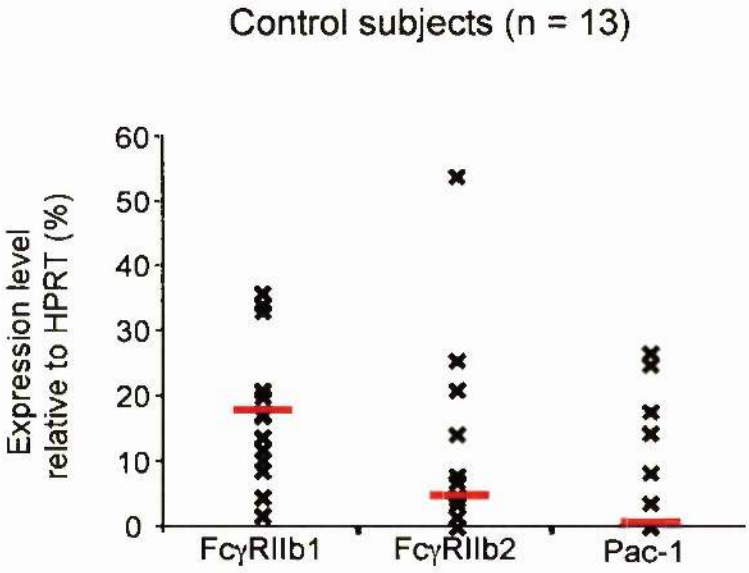

Control subjects (n = 13)
Expression level relative to HPRT (%)
60
50
40
30
20
10
0
FcγRIIb1
FcγRIIb2
Pac-1

Figure 5.4 TaqMan® quantitative RT-PCR of FcγRIIb1, FcγRIIb2 and Pac-1 mRNA expression levels in peripheral blood mononuclear cells from arthritis subjects.

Total RNA was extracted from peripheral blood mononuclear cells (5 x 10^6 cells) from arthritis subjects and was subjected to reverse transcription in the presence of 100 U Superscript II RT, as described in the Materials and Methods. FcγRIIb1, FcγRIIb2 and Pac-1 transcripts were assayed for by real-time PCR (TaqMan®). Individual mRNA levels are expressed as a percentage relative to HPRT mRNA levels with the median indicated by the horizontal bar.

A) Individual mRNA levels of FcγRIIb1, FcγRIIb2 and Pac-1 as determined by real-time PCR (TaqMan®) for the total arthritis group (n = 47).

B) Individual mRNA levels of FcγRIIb1, FcγRIIb2 and Pac-1 as determined by real-time PCR (TaqMan®) for the total arthritis group minus the three highest expressing individuals (n = 44).

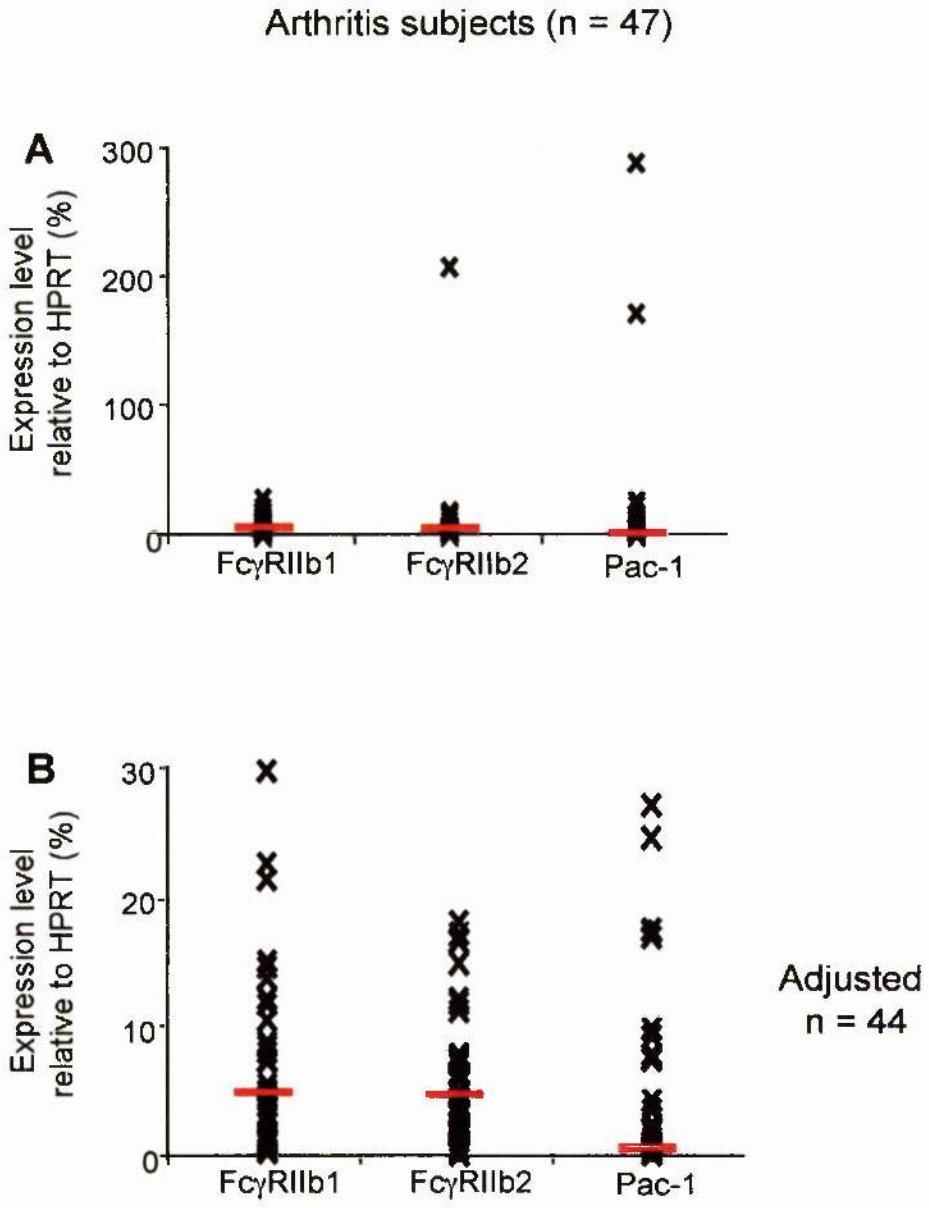

Arthritis subjects (n = 47)
A
Expression level relative to HPRT (%)
300
200
100
0
FcγRIIb1
FcγRIIb2
Pac-1
B
Expression level relative to HPRT (%)
30
20
10
0
FcγRIIb1
FcγRIIb2
Pac-1
Adjusted n = 44

Figure 5.5 Comparison of FcγRIIb1, FcγRIIb2 and Pac-1 mRNA expression levels in peripheral blood mononuclear cells from control and arthritis subjects.

A comparison of the mRNA expression levels of FcγRIIb1, FcγRIIb2 and Pac-1 transcripts in peripheral blood mononuclear cells from control and arthritis subjects, as determined by real-time PCR (TaqMan®). Individual mRNA levels are expressed as a percentage relative to HPRT mRNA levels with the median indicated by the horizontal bar.

A) Comparison of the individual mRNA levels of FcγRIIb1, FcγRIIb2 and Pac-1 as determined by real-time PCR (TaqMan®) for the control versus the total arthritis group (n = 47).

B) Comparison of the individual mRNA levels of FcγRIIb1, FcγRIIb2 and Pac-1 as determined by real-time PCR (TaqMan®) for the control versus the total arthritis group minus the three highest expressing individuals (n = 44).

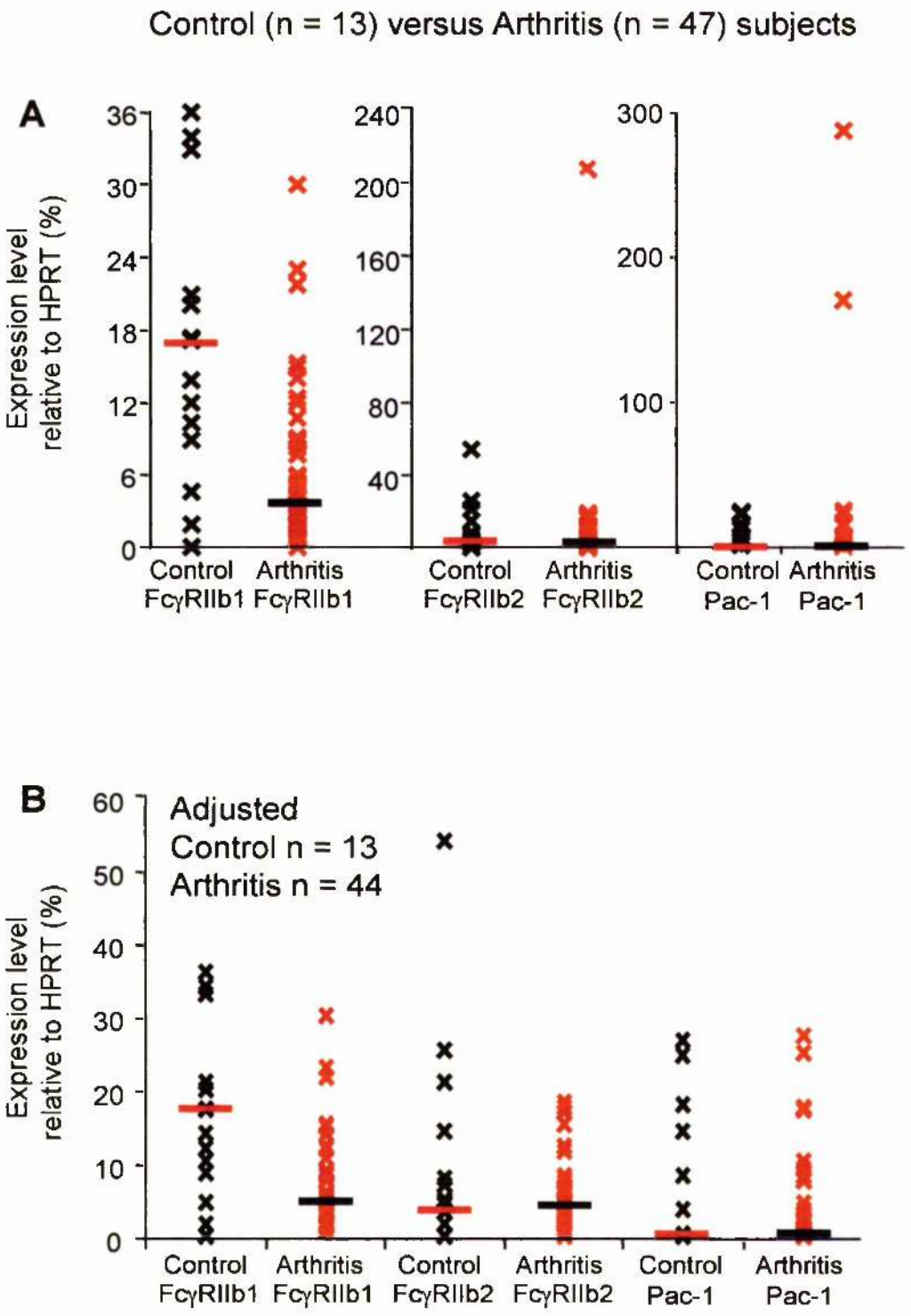

Control (n = 13) versus Arthritis (n = 47) subjects
A
Expression level relative to HPRT (%)
36
30
24
18
12
6
0
240
200
160
120
80
40
300
200
100
Control FcγRIIb1
Arthritis FcγRIIb1
Control FcγRIIb2
Arthritis FcγRIIb2
Control Pac-1
Arthritis Pac-1
B
Adjusted
Control n = 13
Arthritis n = 44
Expression level relative to HPRT (%)
60
50
40
30
20
10
0
Control FcγRIIb1
Arthritis FcγRIIb1
Control FcγRIIb2
Arthritis FcγRIIb2
Control Pac-1
Arthritis Pac-1

Table 5.4 Details of SLE control subjects at time of study

Total RNA from peripheral blood samples was obtained from 10 normal individuals. FcγRIIb1, FcγRIIb2 and Pac-1 transcripts were assayed for by real-time PCR (TaqMan®). mRNA levels are expressed as a percentage relative to HPRT mRNA levels ± S.E for three experiments.

SLE CONTROL SUBJECTS (n = 10)

No.	GENDER	AGE	FcγRIIb1	± S.E	FcγRIIb2	± S.E	Pac-1	± S.E
44316	F	22	17	3.7	8.2	2.05	0.91	0.21
44317	F	30	5	0.9	4.07	0.5	2.73	0.35
44324	F	31	16.9	5	5.2	1.7	0.56	0.18
44322	F	33	17.3	3.8	5.9	1.9	0.10	0.03
44325	F	36	16.8	7	2.25	1.36	0.07	0.03
44319	F	37	13.8	3.05	11.5	3.1	0.14	0.03
44318	F	39	7	1.4	4.2	0.9	0.13	0.04
44320	F	44	4.7	0.7	4.4	0.6	0.04	0.02
44323	F	44	15.4	2.8	2.8	1.5	0.03	0.02
44321	F	45	30.9	10	12.2	4.5	0.16	0.07

Table 5.5 Details of SLE subjects at time of study

Total RNA from peripheral blood samples was obtained from 15 SLE subjects. FcγRIIb1, FcγRIIb2 and Pac-1 transcripts were assayed for by real-time PCR (TaqMan®). mRNA levels are expressed as a percentage relative to HPRT mRNA levels ± S.E for three experiments.

SLE SUBJECTS (n = 15)

No.	GENDER	AGE	FcγRIIb1	± S.E	FcγRIIb2	± S.E	Pac-1	± S.E
44314	F	19	41	9.3	11.1	6.4	0.37	0.20
4436	F	20	7.5	1	3.1	0.9	0.00	0.00
44315	F	20	14	2.4	10.8	2.3	0.06	0.05
4433	F	28	29	7.6	21	6.7	0.18	0.08
4434	F	31	5.2	1.2	6.5	1.3	0.27	0.09
4431	F	34	6.4	1.2	4.9	1.5	0.59	0.36
4438	F	34	20.2	4.9	9.4	3.12	0.01	0.00
4437	F	38	16.2	4.2	23.2	6.3	3.52	0.94
44310	F	39	9.2	2.5	7.4	4.06	0.11	0.02
44313	F	39	8	1	4.9	1.22	1.17	0.41
4439	F	41	14.9	5	9.6	4.18	0.09	0.06
4432	F	44	10.3	1.9	5.5	1.16	0.48	0.09
44312	F	47	19.5	3.4	5.6	2.03	0.03	0.02
4435	F	50	7.2	1.8	6.3	1.2	2.18	0.20
44311	F	52	7.2	0.6	3.1	0.41	0.01	0.00

Table 5.6 Statistical analysis of FcγRIIb1, FcγRIIb2 and Pac-1 expression levels in peripheral blood mononuclear cells from control and SLE subjects.

All statistical analysis was performed on Minitab software (State College, Pennsylvania, USA) using Pearson correlation and Mann-Whitney analyses as appropriate. FcγRIIb1, FcγRIIb2 and Pac-1 transcripts were assayed for by real-time PCR (TaqMan®). mRNA levels are expressed as a percentage relative to HPRT mRNA levels ± S.E for three experiments.

	SLE subjects (n = 15)	SLE Control subjects (n = 10)	Mann-Whitney Significance
Male	0 (0 %)	0 (0 %)	-
Female	15 (100 %)	10 (100 %)	-
Age range (yr)	19-52	22-45	-
Age median (yr)	38	36.5	n/s
B1 expression range	5.2-41	4.7-30.9	-
Median B1 expression	10.3	16.1	n/s
B2 expression range	3.1-23.2	2.3-12.2	-
Median B2 expression	6.5	4.8	n/s
Pac-1 expression range	0-3.5	0-2.7	-
Median Pac-1 expression	0.18	0.2	n/s

Figure 5.6 TaqMan® quantitative RT-PCR of FcγRIIb1, FcγRIIb2 and Pac-1 mRNA expression levels in peripheral blood mononuclear cells from control subjects and SLE subjects.

Total RNA was extracted from peripheral blood mononuclear cells (5 x 10^6 cells) from **(A)** control and **(B)** SLE subjects and was subjected to reverse transcription in the presence of 100 U Superscript II RT, as described in the Materials and Methods. FcγRIIb1, FcγRIIb2 and Pac-1 transcripts were assayed for by real-time PCR (TaqMan®). Individual mRNA levels are expressed as a percentage relative to HPRT mRNA levels with the median indicated by the horizontal bar.

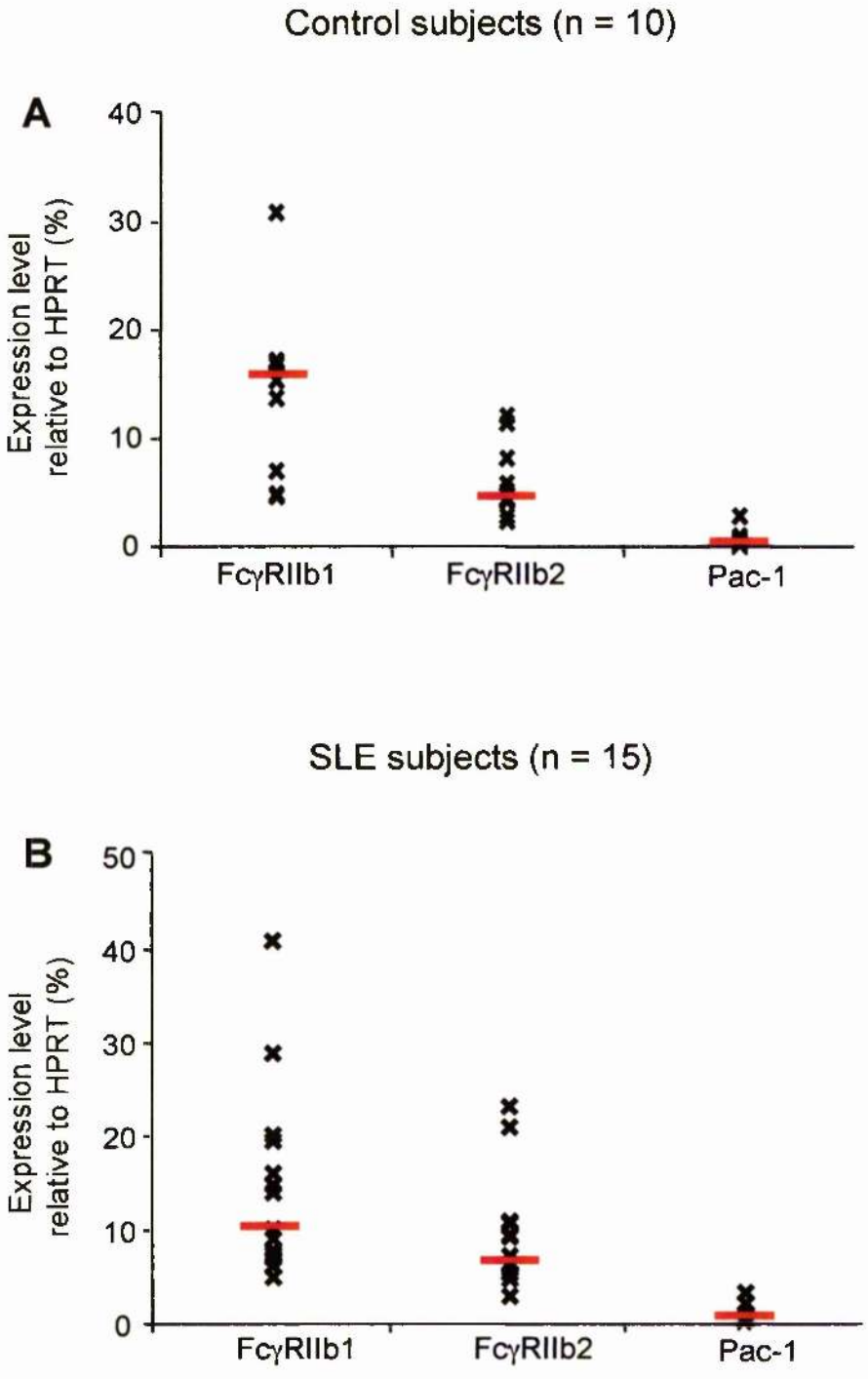
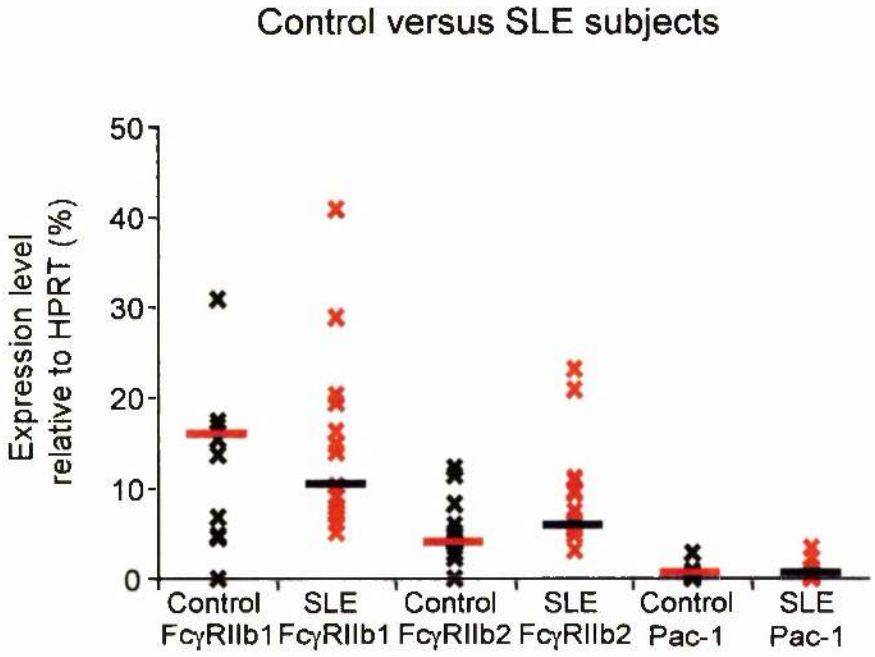

Control subjects (n = 10)
A
Expression level relative to HPRT (%)
40
30
20
10
0
FcγRIIb1
FcγRIIb2
Pac-1
SLE subjects (n = 15)
B
Expression level relative to HPRT (%)
50
40
30
20
10
0
FcγRIIb1
FcγRIIb2
Pac-1

Figure 5.7 Comparison of FcγRIIb1, FcγRIIb2 and Pac-1 mRNA expression levels in peripheral blood mononuclear cells from control and SLE subjects.

A comparison of the mRNA expression levels of FcγRIIb1, FcγRIIb2 and Pac-1 transcripts in peripheral blood mononuclear cells from control and SLE subjects, as determined by real-time PCR (TaqMan®). Individual mRNA levels are expressed as a percentage relative to HPRT mRNA levels with the median indicated by the horizontal bar.


Control versus SLE subjects
Expression level relative to HPRT (%)
50
40
30
20
10
0
Control FcγRIIb1
SLE FcγRIIb1
Control FcγRIIb2
SLE FcγRIIb2
Control Pac-1
SLE Pac-1

Chapter 6 - General Discussion

The work presented in this thesis has focused on identifying the important signalling mechanisms underlying the negative regulation of B cells by FcγRIIb (**Figure 6.1**) and how dysregulation of FcγRIIb expression or signalling may provide a mechanism for the initiation of autoimmune disease in predisposed individuals. Co-ligation of the BCR and FcγRIIb by cognate antigen-antibody complexes leads to the inhibition of BCR-triggered B cell activation and the arrest of cellular proliferation. In contrast, aggregation of FcγRIIb alone, via non-cognate antigen-antibody complexes, generates a pro-apoptotic signal. This study has not addressed the signalling mechanisms initiated following non-cognate aggregation of FcγRIIb but instead has focused on FcγRIIb-induced anergy of resting B cells and the switching off of activated B cells by cognate antigen-antibody complexes.

In Chapter 3, investigations centred on the molecular mechanisms initiated following BCR-FcγRIIb co-ligation which might lead to the induction of growth-arrest and/or the commitment to apoptosis in primary B cells. Chapter 4 set out to ascertain whether aberrant FcγRIIb-mediated negative regulation of B cells is important for the onset and progression of autoimmunity in established murine models of autoimmune disease. This was accomplished by investigating the expression and function of FcγRIIb in B cells from collagen-induced arthritis (CIA) and murine systemic lupus erythematosus (MRL-*lpr/lpr)* murine models. Finally, in Chapter 5, studies focused on determining whether the findings established in the *in vitro* signalling studies and/or the murine models of autoimmune disease translated to explaining the lesions underlying human autoimmune diseases. In particular, this chapter focused on analysis of whether differential expression of the two isoforms of FcγRIIb, FcγRIIb1 and FcγRIIb2, is related to, or indicative of, disease susceptibility and progression in patients with autoimmune disease, such as rheumatoid arthritis or systemic lupus erythematosus.

6.1 Molecular mechanisms of FcγRIIb signalling

Previous published studies had highlighted an important role for the inositol 5'-phosphatase, SHIP-1, in the inhibition of BCR-mediated activation by FcγRIIb and the induction of growth-arrest mechanisms in B cells. An investigation of the downstream signalling mechanisms activated by this phosphatase therefore represented a starting point for the determination of FcγRIIb-induced signalling events in primary B cells.

Initial studies investigated the expression and activation kinetics of SHIP following BCR and/or FcγRIIb ligation, in order to confirm that SHIP is exclusively and transiently activated under conditions of negative regulation. Interestingly, in agreement with recent studies by Ingham *et al.*, 1999 our data suggests that both BCR and FcγRIIb-mediated signalling events are capable of inducing the phosphorylation of SHIP. However, SHIP-mediated inositol phosphatase activity was only active under conditions of FcγRIIb co-ligation. Furthermore, phosphorylation under these conditions results in the formation of SHIP-Shc complexes. Whilst Shc has been shown to be dispensable for BCR coupling to the RasMAPK pathway in DT40 cells (Hashimoto *et al.*, 1998), many other groups have demonstrated an essential role for Shc in the regulation of BCR-coupled ErkMAPKinase in mammalian B cells (Campbell, 1999; Ingham *et al.*, 1999; Kelly and Chan, 2000) Thus, low affinity SHIP-Shc complexes may occur in BCR stimulated cells that promote activation of the RasMAPK pathway. Following FcγRIIb co-ligation these complexes may undergo dissociation, following SHIP recruitment to FcγRIIb, and reform under conditions that promote inactivation of the RasMAPK pathway. The recent identification of new isoforms of SHIP suggests that the differential roles previously described for SHIP may actually be due to the action of alternative forms, which are differentially regulated within the cell.

Examination of Ras activity indicated that whilst FcγRIIb-ligation resulted in the formation of SHIP-Shc complexes and the abrogation of ErkMAPKinase activity, Ras activity was unaffected. This finding is in contrast to recently published

studies that have shown that SHIP-dependent recruitment of p62Dok is important for the downregulation of Ras activity following FcγRIIb-ligation (Tamir *et al.*, 2000a). Moreover, this novel finding questions the proposed mechanism of SHIP-mediated sequestration of Shc in FcγRIIb-mediated uncoupling of the BCR from ErkMAPKinase and suggests that inhibitory mechanism may act at or downstream of Ras recruitment of the Raf-MEK-ErkMAPKinase cascade. To investigate this proposal, we examined the mechanisms that inhibit ErkMAPKinase activity at the level of, or below Raf. Initial studies ruled out the involvement of Rap-1, RKIP and PP2A, so we examined whether the nuclear phosphatase, Pac-1, was involved in the suppression of ErkMAPKinase following co-ligation of FcγRIIb with the BCR. Our results suggested that FcγRIIb ligation increased the association of Pac-1 with ErkMAPKinase. Further studies to confirm co-localisation of these molecules would confirm a role for Pac-1 in FcγRIIb-mediated responses. It would also be interesting to examine which transcription factors are inhibited following FcγRIIb-inhibition of B cell proliferation, with the previously described ErkMAPKinase targets of c-Myc and Elk-1 (Kolch, 2000) as possible candidates.

Whilst FcγRIIb co-ligation induced the abrogation of ErkMAPKinase activity, investigation of the other members of the MAPKinase family, JNK and p38, showed that JNK activity was also reduced following FcγRIIb co-ligation so that surprisingly, JNK was more activated in BCR stimulated cells. These results are in contrast to other studies which implicate a possible role for JNK in the induction of FcγRIIb-induced apoptosis via Btk (Kawakami *et al.*, 1997; Bolland *et al.*, 1998) and thus warrants further investigation. However, activation of JNK may only occur under conditions of apoptosis and therefore may not be required for FcγRIIb-mediated growth-arrest, as demonstrated in our studies.

In addition to inhibiting BCR-proliferative signals, we have also shown that following co-ligation, FcγRIIb mediates pro-apoptotic signals by inhibiting the activation of the pro-survival molecule Akt. Our results confirm a potential role for this protein in FcγRIIb-induced apoptosis (Pearse *et al.*, 1999). Although published results suggest that SHIP inhibits the activation of Akt by hydrolysing

PtdIns(3,4,5)P_3, to PtdIns(3,4)P_2, Akt is now known to bind with higher affinity to this hydrolysis product, than to PtdIns(3,4,5)P_3 (Downward, 1998) suggesting that SHIP activation is not responsible for the FcγRIIb-mediated inhibition of Akt. Therefore, we examined whether the inositol 3' phosphatase, PTEN, was activated following co-ligation of FcγRIIb with the BCR. Our results demonstrated that FcγRIIb co-ligation increased the phosphatase activity of PTEN, with kinetics to suggest that it acts downstream of SHIP, to generate PtdIns(3,4)P_2 and PtdIns(3)P which do not recruit Akt and hence terminate ongoing BCR signalling.

In conclusion, our studies suggest that B cell unresponsiveness following FcγRIIb ligation may result from the inhibition of proliferative signalling cascades and the promotion of apoptotic signals. This was further confirmed by studies on the activation of cell-cycle regulators and nuclear transcriptional proteins. For example, in agreement with studies by Malbec *et al.* and others we have shown that FcγRIIb-mediated growth-arrest leads to the modulation of cell-cycle regulatory proteins. In particular, signals derived from FcγRIIb partially suppressed BCR-induced Rb phosphorylation and thus potentially prevent the dissociation of E2F and transcription of S-phase genes resulting in cell cycle arrest in G0/G1 phase. Further studies, utilising RNA protection assays may enable us to identify the gene targets of E2F modulated by FcγRIIb. Similarly, we have shown that FcγRIIb ligation results in the disruption of BCR-mediated NF-κB activation which is required both for the induction of immunoregulatory genes and for the regulation of cell fate (Ghosh *et al.*, 1998). Recent reports have implicated a role for PTEN in the regulation of such NF-κB activation and hence it would be interesting to determine whether FcγRIIb signalling employed PTEN in a dual pronged mechanism (switch off of Akt-mediated survival signals and switch on of NF-κB-mediated apoptotic signals) to effect B cell tolerance. Finally, FcγRIIb ligation also results in the rapid phosphorylation of the tumour suppressor protein, p53, on serine 15: a modulation, which correlates with disruption of the mitochondrial membrane potential and commitment to apoptosis. Furthermore, as, in addition to its role in promoting apoptosis, p53 has been reported to influence regulation of cell cycle progression by promoting

the upregulation of cyclin-dependent kinase inhibitors, such as p27, it will be interesting to determine whether FcγRIIb ligation utilises such signals in driving B cell growth arrest. It would therefore be interesting to examine whether p53 regulation promotes the disruption of NF-κB DNA binding complexes.

This study has therefore identified a number of novel features in the regulation of B cell activation by FcγRIIb. In particular, this study is this first to describe the coupling of FcγRIIb to PTEN and Pac-1 activation, highlighting a novel role for these signalling pathway regulators in the modulation of B cell fate by FcγRIIb. Additionally it has highlighted the differential mechanisms of FcγRIIb-mediated growth arrest versus commitment to apoptosis during negative regulation of B cells by immune complexes. A working model of how these inhibitory signals are integrated is shown in **Figure 6.2**.

6.2 FcγRIIb and Murine Autoimmune Disease Models

Disruption of the inhibitory FcγR, FcγRIIb, by gene targeting is known to disrupt the homeostasis of the immune system and have a pro-inflammatory effect. Thus FcγRIIb deficient mice display augmented hypersensitivity and anaphylactic responses (Takai *et al.*, 1996) whilst certain strains of FcγRIIb$^{-/-}$ mice are able to develop an autoimmune syndrome resulting in the production of autoantibodies, accelerated antibody-induced glomerulonephritis and immune complex mediated alveolitis (Suzuki *et al.*, 1998; Clynes *et al.*, 1999; Nakamura, A. *et al.*, 2000). An investigation of the role of FcγRIIb in the regulation of the development of collagen induced arthritis (CIA) in susceptible (DBA/1, H-2^{q}) strains and in the spontaneous murine systemic lupus erythematosus model, MRL-*lpr/lpr* demonstrated differential requirements for FcγRIIb expression and function.

In contrast to the FcγRIIb knockout studies, the demonstration that FcγRIIb-mediated B cell inhibition appears to be intact throughout development of CIA disease in a naturally susceptible strain of the CIA murine model was somewhat surprising. However, in light of recent studies proposing that aberrant FcγRIIb

expression only pre-disposes certain strains of mice to autoimmune disease, these findings might suggest that this regulatory pathway is not important in the induction or progression of CIA in H-2^q susceptible strains of mice. Furthermore, these findings suggest that FcγRIIb is not a uniform risk factor for this disease.

In contrast to the CIA data, FcγRIIb-mediated B cell inhibition was found to be dysregulated in the later stages of lupus-like disease in MRL-*lpr/lpr* mice. Furthermore, this progression was accompanied by a downregulation in FcγRIIb isoform expression. Whilst FcγRIIb deficiency may not generally predispose the immune system to mediate an autoimmune response, it suggests that FcγRIIb may be a susceptibility factor which contributes to the genetic and environmental background that is required for the emergence of lupus-like disease in mice. Indeed, investigations of the FcγRIIb promoter region in strains of autoimmune-prone mice, have highlighted that deletions in this area are associated with reduced surface expression and function of FcγRIIb on macrophages and B cells (Luan *et al.*, 1996; Pritchard *et al.*, 2000). Furthermore, studies in autoimmune disease-prone mice have shown a correlation between FcγRIIb promoter polymorphisms and the subsequent down-regulation of surface expression and a concomitant up-regulation of IgG antibody responses and promotion of lupus-like disease (Jiang *et al.*, 1999, 2000). Suggesting that FcγRIIb plays an essential role in the regulation of B cell mediated immune responses in the initiation of disease in these models.

Since FcγRIIb is known to play an important role in the maintenance of B cell homeostasis and tolerance to self-antigen, by directly influencing the BCR signalling threshold, it is interesting that a recent study has highlighted a role for different B cell receptor signaling thresholds in the spontaneous development of lupus-like disease in hybrid NZB/NZW F_1 (NZB/W) mice (Wellmann *et al.*, 2001). This study demonstrated that whilst central tolerance to dsDNA was intact in these autoimmune disease-prone mice, the NZB/W B cells underwent an altered selection process. A lack of receptor editing and deletion of DNA reactive B cells, presumably due to an aberrantly high threshold for the induction of apoptosis, produced a higher frequency of anti-DNA B cells

amongst the follicular B cell population, compared to the control strain. In conjunction with aberrant FcγRIIb expression, these results could explain the qualitative differences in the B cell repertoire of NZB/W mice as well as the hyperactivity of the B cells in such a repertoire.

6.3 Role of FcγRIIb in Human Autoimmune Disease

B cells are known to play an essential role in the initiation and regulation of both T and B cell responses. Thus, excessive or upregulated B cell activity may increase the likelihood of developing autoimmunity and subsequent autoimmune disease. Since the formation of autoantibodies and immune-complexes is central to many autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, recent studies have focused on the role of antibody binding receptors in the initiation and progression of disease.

Since B cells are known to express two different isoforms of FcγRIIb, that engage in differential responses to immune-complexes, an investigation of the expression of these isoforms in relation to human disease was undertaken. Using TaqMan® quantitative RT-PCR technology we examined the relative expression levels of the two FcγRIIb isoforms in peripheral blood mononuclear cells from rheumatoid arthritis and systemic lupus erythematosus patients. Furthermore, having demonstrated in Chapter 3 the FcγRIIb-mediated recruitment of the inducible dual (Thr/Tyr) phosphatase, Pac-1 played a key role in the uncoupling of the BCR from ErkMAPKinase and hence B cell proliferation, we also investigated whether the same patients demonstrated aberrant expression of this molecule. This dual approach allowed the assessment of whether aberrant FcR expression and/or functionality correlated with induction and/or progression of autoimmune disease in humans.

Consistent with the theory that FcγRIIb expression is important for maintenance of self-tolerance, the most important finding of this study was that the expression of FcγRIIb1 mRNA was down-regulated in PBMCs of rheumatoid arthritis patients compared to controls. In contrast, expression levels of the

endocytosis competent isoform FcγRIIb2 and Pac-1 mRNA were unaffected. Thus, this display of differential isoform expression provides a potential mechanism by which aberrant FcγRIIb expression by autoreactive B cells may promote autoimmunity (**Figure 6.3**). Further studies need to investigate whether FcγRIIb2 is the isoform predominantly utilised by B cells for antigen uptake and processing whilst modulation of FcγRIIb1 expression dictates the threshold for BCR signalling. Furthermore, whether differential FcγRIIb expression is promoted by the inflammatory cytokine profile and thus modulated by administration of anti-inflammatory drugs in rheumatoid arthritis patients.

Interestingly, systemic lupus erythematosus patients demonstrated similar mRNA expression levels of both FcγRIIb isoforms and Pac-1 compared to controls. These results were somewhat surprising with respect to the essential role of immune-complex deposition in the initiation of lupus disease and following the study of FcγRIIb function in MRL-*lpr/lpr* mice in Chapter 4. The differences in results may simply reflect the requirement of further genetic susceptibility factors for the initiation of disease in the separate systems or the masking of B cell FcγRIIb expression by other FcγRIIb-expressing cells in the PBMC samples. In addition, only a small cohort of patients of uniform ethnicity were studied, thus investigations of FcγRIIb expression in B cells from a larger cohorts of SLE patients, potentially from varying ethnic backgrounds, may provide statistically significant data to support a similar role for aberrant FcγRIIb expression in the initiation of SLE in certain populations.

Alternatively, although FcγRIIb and Pac-1 expression were found to be similar in control and patient samples, it was possible that rather than FcγRIIb-MAPKinase signalling being dysfunctional that another of the FcγRIIb-mediated inhibitory signals was deregulated. Interestingly, a deficiency in the PTK, Lyn, has been identified in patients with systemic lupus erythematosus (Liossis *et al.*, 2001). Semi-quantitative PCR analysis of Lyn mRNA expression levels revealed a significant decrease in the levels of Lyn in peripheral B cell of lupus patients that were reflected in a decrease of Lyn protein. Due to the essential role of Lyn

in the phosphorylation of FcγRIIb, down-regulation of Lyn expression may result in a lack of FcγRIIb activation following co-ligation with immune-complexes and thus prevent negative feedback regulation of potentially autoreactive B cells. Furthermore, Lyn deficiency may also prevent FcγRIIb-mediated recruitment of negative regulators, such as PTEN, thus enabling aberrant activation of pro-survival signals by Akt.

Overall, this study is this first to describe the deregulation of differential expression of FcγRIIb isoforms in relation to rheumatoid arthritis. Moreover, this study provides a potential mechanism for the modulation of B cell homeostasis whilst supporting the theory that an imbalance in FcγR interactions might underlie the severity of inflammation in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.

Figure 6.1 Regulation of the humoral immune response by FcγRIIb.
FcγRIIb plays an important role in the regulation of the humoral immune response by immune-complexes. Following co-ligation with the BCR, FcγRIIb suppresses the activation of B cells and controls antibody production by inducing growth-arrest. In contrast, self-ligated FcγRIIb results in the commitment of B cells to apoptosis and thus provides a mechanism for the maintenance of peripheral tolerance to self-antigen.

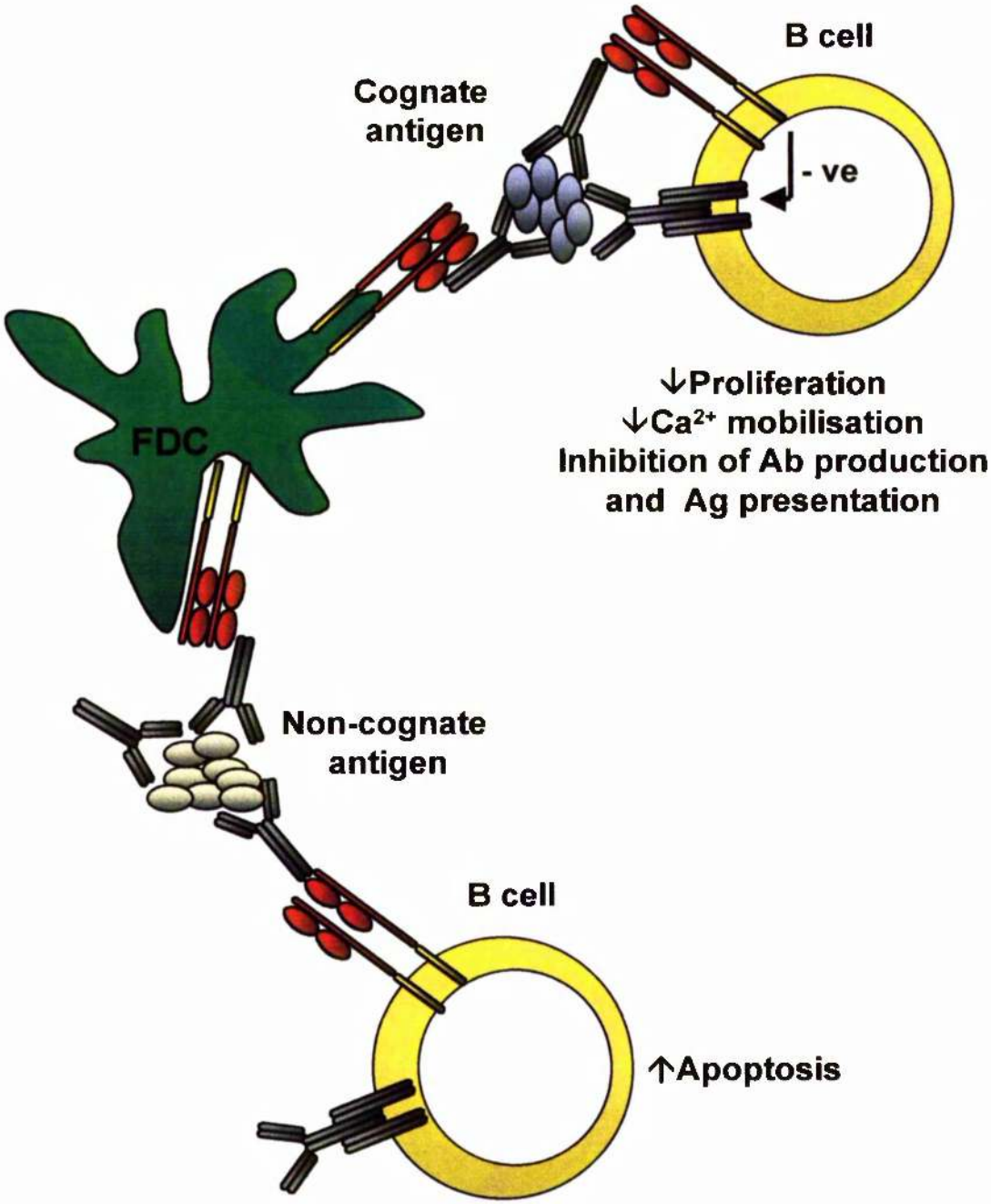

B cell
Cognate antigen
- ve
FDC
↓Proliferation
↓Ca^{2+} mobilisation
Inhibition of Ab production and Ag presentation
Non-cognate antigen
B cell
↑Apoptosis

Figure 6.2 A working model for the integration of FcγRIIb-mediated inhibitory signals in B cells.

Co-ligation of the BCR and FcγRIIb by cognate antigen-antibody complexes leads to tyrosine phosphorylation of the FcγRIIb cytoplasmic ITIM motif by the Src-family kinase Lyn, recruitment of SHIP and the inhibition of BCR-triggered calcium mobilisation and arrest of cellular proliferation. FcγRIIb uncouples the BCR from ErkMAPK by (1) the sequestration of Shc by SHIP, preventing the recruitment of Shc-Grb2-SoS complexes (2) recruiting a Ras GTPase activating protein (RasGAP) via p62Dok, in a SHIP-dependent manner (3) the dephosphorylation of MEK and (4) the formation of ErkMAPK/Pac-1 complexes.

SHIP is also able to inhibit calcium mobilisation by hydrolysing the membrane inositol phosphate, PtdIns(3,4,5)P_3 preventing the recruitment of Btk and PLC-γ, thus blocking a sustained calcium signal by inhibiting the influx of extracellular calcium. Co-ligation of the BCR with FcγRIIb also results in the termination of the pro-survival Akt signal, through the conversion of PI(3,4,5)P_3 to PI(3,4)P_2 by the inositol 5'-phosphatase, SHIP and the conversion of PI(3,4,5)P_3 and PI(3,4)P_2, by PTEN, thus counteracting the activities of PI-3-Kinase. Inhibition of these signals may also modulate the DNA binding activity of NF-κB.

FcγRIIb co-ligation inhibits BCR-mediated G_1-S phase progression by the partial suppression of BCR-induced Rb phosphorylation, potentially preventing the release of E2F and the transcription of S phase genes. FcγRIIb ligation also disrupts BCR-mediated cell-cycle progression by the rapid phosphorylation of p53 on Ser^{15} which can lead to cell cycle arrest at the G_1-S and G_2-M transition points and DNA repair or apoptosis. p53 transactivation also correlates with FcγRIIb-mediated disruption of the mitochondrial membrane potential.

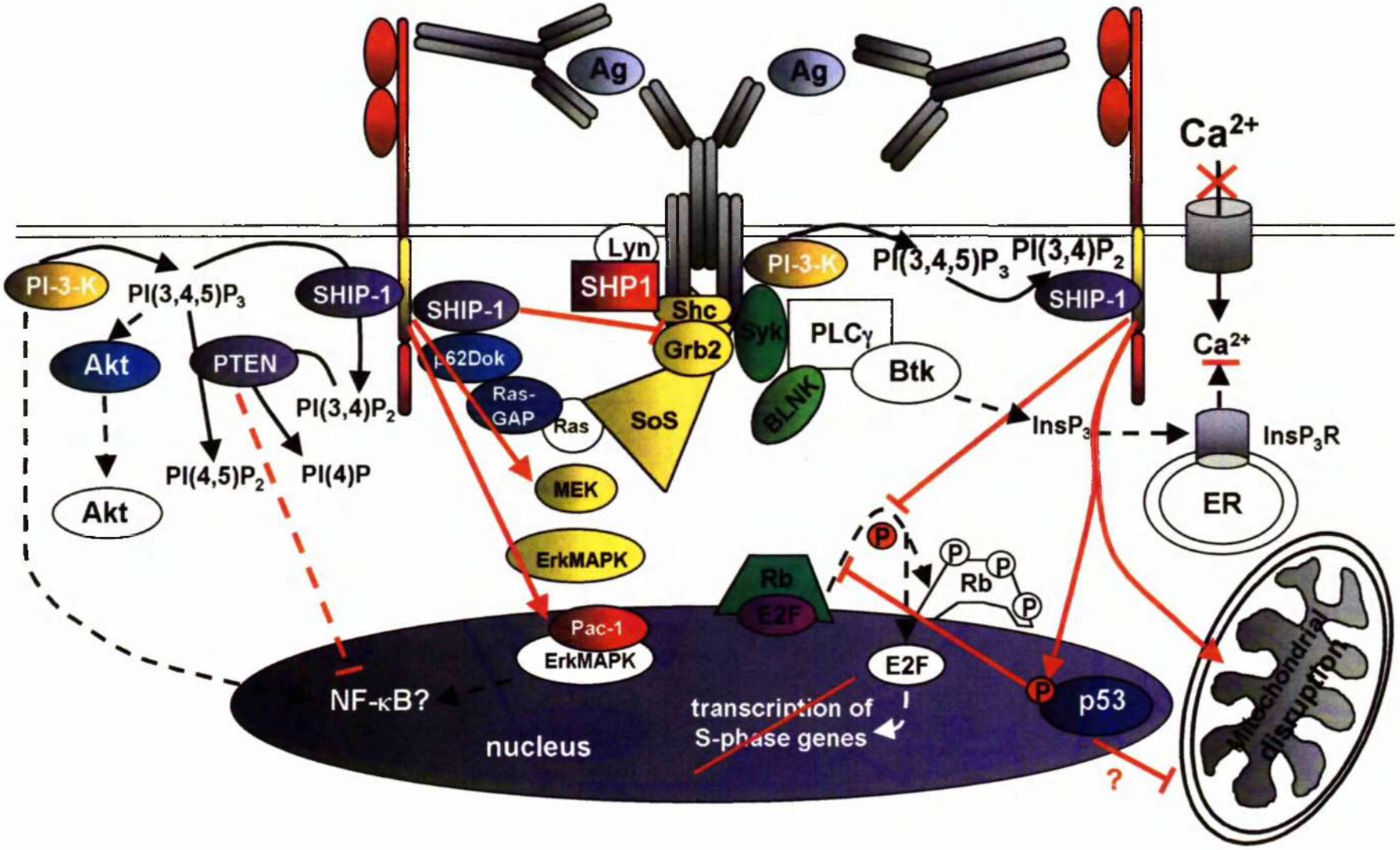

Ag
Ag
Ca2+
Lyn
SHP1
Shc
Grb2
PI-3-K
PI(3,4,5)P3
PI(3,4)P2
SHIP-1
SHIP-1
p62Dok
Ras-GAP
Ras
SoS
Syk
PLCγ
Btk
BLNK
InsP3
InsP3R
ER
Ca2+
PI-3-K
PI(3,4,5)P3
SHIP-1
Akt
PTEN
PI(3,4)P2
PI(4,5)P2
PI(4)P
Akt
MEK
ErkMAPK
Pac-1
ErkMAPK
Rb
E2F
Rb
P
E2F
NF-κB?
nucleus
transcription of
S-phase genes
p53
?
mitochondrial
disruption

Figure 6.3 A potential mechanism for the initiation of autoimmunity by the differential expression of FcγRIIb isoforms.

B cell homeostasis is dependent on the balance between the activities of activation and inhibitory receptors. Indeed, loss of suitable inhibitory signalling is frequently associated with the development of inflammatory responses and, in some cases, autoimmunity. Normally, FcγRIIb ligation by immune-complexes on B cells will induce growth-arrest and/or commitment to apoptosis. However, differential expression of the two isoforms of FcγRIIb, FcγRIIb1 and FcγRIIb2, may modulate the ability of immune-complexes to promote B cell inactivation. Normal expression levels of FcγRIIb isoforms will promote down-regulation of B cell function and prevent T cell mediated help. However, down-regulation of FcγRIIb1 expression may permit endocytosis of complexed antigen by FcγRIIb2 and presentation via MHC II to cognate helper T cells, thus breaking normally tolerant B cells. T cell derived CD40-L and IL-4 will promote B cell activation and further suppress any negative regulatory effects by FcγRIIb2. Thus, down-regulation of FcγRIIb1 expression provides a potential mechanism for the initiation of autoimmunity by autoreactive B cells that may lead to inflammation and tissue damage.

Autoreactive
B cell
=
Inactivation

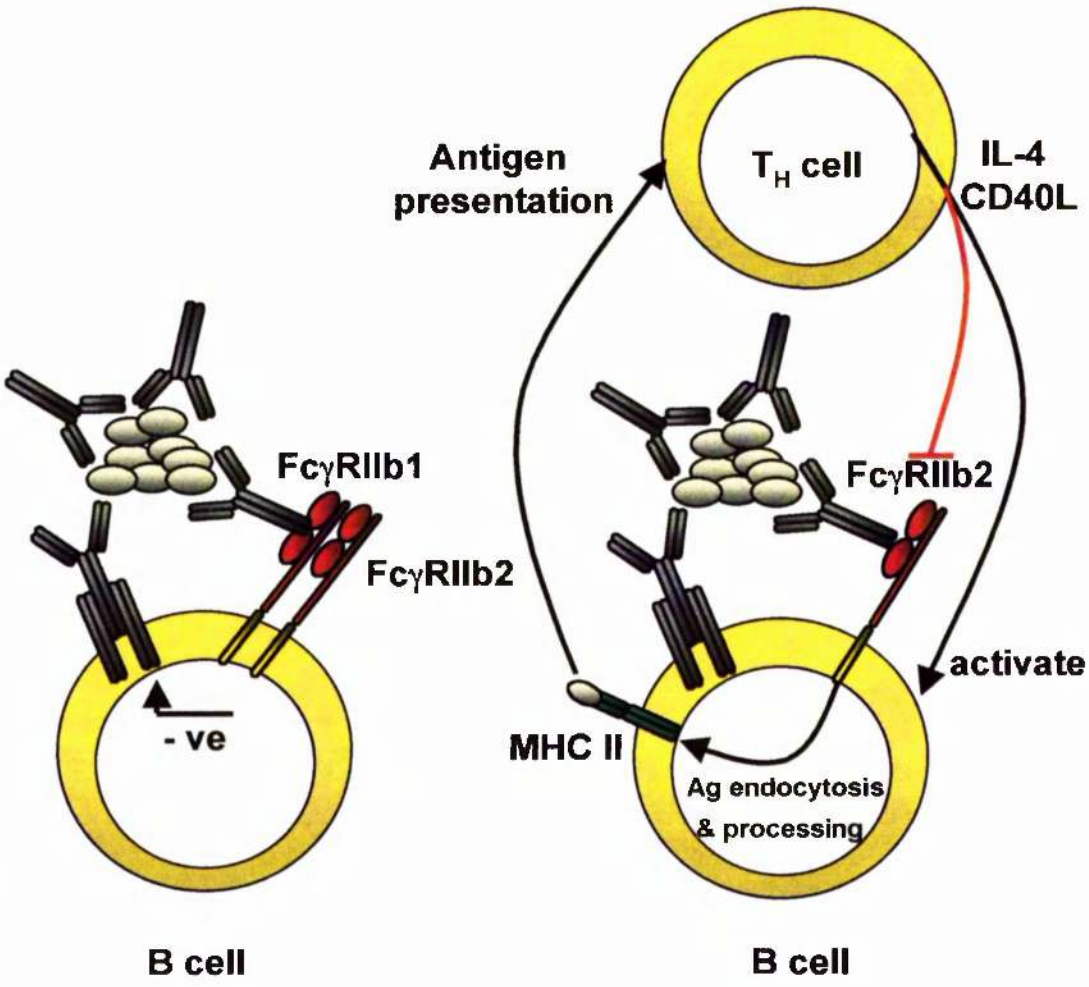
↓

No antigen presentation
NO INFLAMMATION

Autoreactive
B cell
=
Activation

↓

Antigen presentation
INFLAMMATION



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The following abstracts arose from work associated with this thesis.

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