αvβ5 AND RELATED RECEPTORS IN HUMAN B LYMPHOCYTE DEVELOPMENT

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Synopsis

CD23 is a multi-functional protein which exists in membrane-bound and soluble forms. Its functions include acting as the low affinity receptor for IgE and generating pro-inflammatory cytokine release in monocytes.

CD23 has been found to interact with αvβ5 and this interaction greatly enhances growth of the B cell precursor cell line SMS-SB. This interaction may have a role in the development of normal human B cells and in cancer as the integrin is expressed on both precursor and ALL cells but not on normal mature B cells.

One of the aims of this investigation was to expand on the finding that CD23 peptides containing an RKC motif had the same positive growth effect on SMS-SB cells as CD23. Other B cell lines – representative of both precursor and mature stages – were studied to ascertain whether this proliferative effect was dependent upon cell differentiation stage and/or presence of the αvβ5 integrin. It was found that peptides containing the basic RKC motif were mitogenic only for precursor B cells which were expressing αvβ5. Details of these peptides and their varying effects on the different cell lines are in Chapter 4.

Stimulation of SMS-SB cells, presumably via the αvβ5, results in signalling through PI3K and subsequent phosphorylation of Akt. The growth of SMS-SB cells observed following stimulation with peptides containing the RKC motif was abrogated by the PI3K inhibitor LY294002 and western blotting revealed that phosphorylation of Akt was enhanced by stimulation with RKS containing peptides.

Among CD23's receptors is the integrin αvβ3. This integrin can form a signalling complex with CD47. Ligation of CD47 by anti-CD47 antibodies induces apoptosis in some cell lines. To determine whether a pattern exists between response to this stimulation and expression of αvβ3 integrin, cell lines with and without the integrin were tested. It was found that the myeloma cell lines KMS11 and H929 were responsive to this stimulus. Since these cell lines

differ in their expression of αvβ3 (H929 cells express αvβ3 whereas KMS11 do not) it does not appear that any connection between the presence of the integrin and response via CD47 exists and therefore this signalling mechanism would appear to occur independently of the complex formed by CD47 and αvβ3.

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Acknowledgements

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Declaration

The work in this thesis was performed personally be me unless otherwise stated.

Abbreviations

ALL	Acute Lymphocytic Leukaemia
ADCC	Antigen dependent cell cytotoxicity
AP-1	Activating protein-1
APAF-1	Apoptotic protease activating factor-1
APC	Antigen presenting cell
BAD	BCL-XL/BCL-2 associated death protein
BAK	BCL-2 agonist/killer
BAX	BCL-2 associated X protein
BCL-2	B cell leukaemia/lymphoma 2
BCR	B cell receptor
bp	base pair
BH3	BCL-2 homology domain 3 only
BID	B cell lymphoma 2 homology domain 3 only protein interacting BH3 domain agonist
CALLA	Common ALL antigen
CD	Cluster of differentiation
CLL	Chronic lymphocytic leukaemia
CR2	Complement receptor type 2
DC-SIGN	Dendritic cell specific ICAM-grabbing non-integrin
DNA	Deoxyribonucleic acid
EBV	Epstein Barr Virus

ECM	Extraceullular matrix
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
Flt-3	Fms-like tyrosine kinase 3
H	Histone
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
hMSCS	Human marrow stromal cells
I	Insertion
IAP	Integrin associated protein
ICAM	Intercellular adhesion molecule-1
ICE	Interleukin Iβ converting enzyme
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kbp	kilo base pair
kDa	kilo Dalton
LC	Light chain (of Ig)
LD-TGF	Leukocyte derived transforming growth factor
LIMBS	Ligand associated metal binding site
LTBMC	Long term bone marrow cultures
MBP	Mannose binding protein
MIDAS	Metal-ion-dependent-adhesion-site
NMR	Nuclear magnetic resonance

PBMCs	Peripheral blood mononuclear cells
PCD	Programmed cell death
PDGF	Platelet derived growth factor
PI3K	Phosphotidylinositol-3-kinase
PKB	Protein kinase B
PS	Phosphatidyl serine
RA	Rheumatoid arthritis
RAG	Recombinase activating gene
RANTES	Regulated upon activation normal T cell expressed and secreted
SB-AF	SMS-SB autocrine factor
SDF-1	Stromal cell derived factor-1
SIRP-α	Signal recognition particle alpha (transmembrane)
SLE	Systemic lupus erythematosus
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl Transferase
TGF	Transforming growth factor
TNF	Tumour necrosis factor
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
VDJ_H	Variable diversity and joining Ig heavy chain

INTRODUCTION

1.1 The immune system

The vertebrate immune system is a highly evolved, complex, adaptive defence system that exists to protect the host from pathogenic invasion. It is composed of a vast number of cells and molecules that act collectively to ensure that "non-self" organisms are eliminated whilst the "self" is not attacked. The immune response is categorised into two different types: innate and adaptive. The innate immune response is mediated by invariant receptors and is non-specific. This response is mainly mediated by cells of the myeloid lineage - the phagocytic cells, such as monocytes, neutrophils and macrophages in conjunction with the complement cascade of proteins. The innate immune system can be likened to a first line of defence, which can hold off the invaders whilst a specific immune response is mounted. The specific immune response is performed by the B and T lymphocytes and results in a stronger, explicit reaction to a particular invader and generates immunological memory. The cells of the adaptive immune system act via receptors that exhibit remarkable variation, generating a vast repertoire of receptors for antigen recognition.

T cells mediate cellular immunity and can act in either helper or cytotoxic modes to destroy infected cells. T cells generate receptors (T Cell Receptors, TCR) via random recombination of variable receptor gene segments to give rise to cells which each bear a distinct receptor. These receptors can recognise virtually any antigen and when T cells are presented with specific antigen by an antigen presenting cell (APC) they become activated. B cells also carry cell surface receptors with specific binding qualities; these are called immunoglobulin (Ig) receptors and are of the same specificity as the antibody (immunoglobulin) that cell has the ability to secrete following activation. B cells are activated by the binding of specific antigen to the Ig (or B cell receptor (BCR)) and by the receipt of signals from T cells in the form of cell-cell contacts and secreted cytokines. In the presence of all these signals, the B cell can develop into an antibody-secreting plasma cell.

The antibodies produced by plasma cells are soluble immunoglobulins and have the ability to bind to available antigens. Antibodies are generally very important in the primary response to extracellular pathogens such as bacteria and not so important in the response against intracellular invaders such as viruses (although it should be noted that during a secondary

immune response to a virus antibodies can prevent virion entry into cells). Antibodies can neutralize soluble antigens, opsonise particles allowing engulfment and destruction by phagocytes, activate complement and mediate antibody-dependent cell cytotoxicity (ADCC). Besides producing antibodies, B cells can act as antigen presenting cells and develop into memory B cells. The latter are important in immunological memory which comes into play during the rapid secondary immune response seen upon a second encounter with antigen.

This thesis is an investigation into the role of the αvβ5 integrin in normal and neoplastic lymphopoiesis and the following will be discussed in this introduction: B cell development; malignancies of B cells; programmed cell death; the αvβ5 integrin, the CD23 molecule and finally the CD47 molecule.

1.2 B Cell Development in Bone Marrow

B cell development is a multi-step, ordered process during which mature B cells arise from haematopoietic progenitors. Sequential changes in gene expression and external signals originating from microenvironments such as foetal liver and bone marrow direct this process [1]. Early B cells are typified by the absence of CD19, rearrangement of diversity and joining immunoglobulin heavy chain (DJ_H) gene segments and the expression of proteins specific to the B cell lineage such as VpreB and Igα [1]. Support for the existence of early B cells comes from reports showing that DJ_H arrangements [2], cytoplasmic Igα protein [3] and VpreB protein [4] are present in $CD19^-$ lymphoid progenitors.

Using the Hardy classification system [5], pro-B cells are the next stage in B cell development and these cells are characterized by expression of CD10, CD34 and CD19 [6]. In addition, virtually all pro-B cells express terminal deoxynucleotidyl transferase (TdT) [6 7] and variable (V)-DJ_H rearrangements are easily detected [2 8]. A functional VDJ rearrangement is essential for normal pro-B cell differentiation into the pre-B cell compartment. Pro-B cells which do not make successful VDJ rearrangements undergo apoptosis and are thus removed from the proliferative compartment [1].

Pre-B cells are divided into pre-BI and pre-BII: pre-BI cells have lost TdT and CD34 and have acquired cytoplasmic μ heavy chains (μHC) in over 95% of cells and pre-BII cells are re-arranging the light chain locus [6 7]. A crucial checkpoint in B cell maturation is the assembly and surface expression of the pre-BCR. This complex is composed of μHC, VpreB, λ5 and the signal transducing heterodimer Igα/Igβ [9]. Signalling via the pre-BCR induces the pre-B cells to divide [10] and the consequent rearrangement of a κ or λ LC V gene allows cell surface expression of conventional sIgM receptors (BCR) on the immature B cell which exits the bone marrow and journeys to the periphery [11]. Mature B cells in tissues such as the spleen express both sIgM and sIgD receptors and in response to specific antigen and T lymphocytes proliferate and develop into Ig-secreting plasma cells or memory cells [11].

1.2.1 Regulation of precursor B cell development by stromal cells

Stromal cells, haematopoietic cells, extracellular matrix, colony stimulating factors and cytokines contribute to the complex bone marrow microenvironment. These constituents interact to control the proliferation, differentiation and death of B cell precursors (i.e. all B-lineage cells prior to the expression of the BCR) [12]. Stromal cells regulate B lymphopoiesis via cell-cell contacts and the production of cytokines. A system was developed in 1982 by Whitlock and Witte using long term bone marrow cultures (LTBMC) and since then, lines developed from LTBMC have been used to analyse haematopoietic development [13].

1.2.2 Role of cell-cell contacts

Developing B cell precursors have been found in intimate contact with stromal cells in the bone marrow [14] and it has been shown in *in vitro* studies that these direct cell-cell or cell-extracellular matrix (ECM) interactions are required for both murine and human B lymphopoiesis to take place [15,16]. These interactions are responsible for the localisation of precursors in the bone marrow and play an important part in the regulation of progenitor proliferation. Adhesion within the bone marrow has been shown to be developmentally regulated [17] – the most immature B cells displayed the strongest specific adherence to stromal cells and this adherence waned steadily during progression of B lineage differentiation.

The integrin family of adhesion receptors is believed to have a role in early B cell development. The principal interaction is between VCAM-1 (vascular cell adhesion molecule – 1, a member of the Ig superfamily) on bone marrow stromal cells and the integrin VLA-4 ($\alpha4\beta1$) on B cell precursors [18-20]. Growth of precursor cells on stromal cell layers was inhibited by anti-VLA-4 antibodies [21] suggesting a functional role for this interaction in B lymphopoiesis. It has also been found that fibronectin binds VLA-4 at a binding site distinct from that of VCAM-1 indicating a particular role for VLA-4 in the bone marrow. *In vivo* studies using antibodies have identified a role for the β1 integrins in the retention of progenitors in the bone marrow and for the homing of progenitors to the bone marrow after bone marrow transplantation. The β1 integrins, however, are not exclusively expressed on bone marrow cells and hence cannot fully account for the specific interactions. Another receptor(s) must be responsible for the specific interactions of stromal cells and their immediate progeny with components of the bone marrow. Selectins and cartilage link proteins (such as CD44) are also important in the adhesion of B cell progenitors to stromal cells and it is proposed that some of these molecular interactions initiate signals which suppress apoptosis and promote B lymphopoiesis [18].

The influence of stromal cells on the proliferation of precursors is apparently dependent on the presence of cytokines [22]. It has been shown that when precursor cell integrins are engaged by physiological levels of cytokines proliferation is inhibited. When precursors are cultured in pharmacological levels of cytokines integrin engagement triggers entry into the cell cycle [23]. In addition to receiving stromal cell signals, B cell progenitors also possess the ability to induce cytokine release from stromal cells through direct cell contact. IL-6 production by stromal cells was shown to increase 4-fold over three days when cultured in contact with B cells [24] and IL-7 production by stromal cells was shown to require cell-cell contacts with the IL-7 dependent B cell precursors [25].

Although the precise roles of cell-cell and cell-ECM contacts taking place in the bone marrow are not fully understood they are of importance to the development of B cells. In addition, a complete understanding of how B cell precursors affect and are affected by these adhesions has relevance to malignancies arising in the bone marrow. Abnormal adhesion patterns occurring as a result of defective function/expression of adhesion receptors may

participate in the premature appearance of progenitors in the bloodstream or the deregulated proliferation and differentiation seen in leukaemias.

1.2.3 Role of cytokines

IL-7 is a prominent cytokine in B cell development. When it was first identified, IL-7 was described as a 25kDa soluble growth factor with the ability to stimulate the proliferation of B cell precursors *in vitro* in the mouse [26] and it was the first cytokine to be identified and cloned from a stromal cell line [27]. Mice that had been injected with antibodies to either IL-7 or its receptor (IL-7R) were found not to produce B cells – this was the first evidence of the importance of this cytokine [28,29]. Further investigations into IL-7 using mice deficient for IL-7 [30] and IL-7R [31] revealed that signals from IL-7 are required for B cells to progress from the pro-B to the pre-B developmental stage. Injecting normal mice with IL-7 results in an increase in numbers of pre-B cells which then go on to develop into mature $sIgM^+/sIgD^+$ cells [32].

IL-7 has also been implicated in B cell differentiation. The interaction between IL-7 and its receptor has been shown to regulate immunoglobulin (Ig) gene rearrangement. Corcoran showed that if one prevents cell proliferation by abolishing binding of IL7R to phosphotidylinositol-3-kinase (PI3K) via mutation of the α-chain component of the receptor ($Tyr_{449} \rightarrow Phe$) the cells still rearranged and expressed cytoplasmic μ heavy chain (Hμ). The signals which are promoting cell proliferation are therefore distinct from those progressing cell differentiation [33]. The same group went on to analyse mice with IL-7Rα gene knockout and identified impairments in $V_H - (D)J_H$ joining of Ig gene segments. Therefore IL-7Rα ligands may be contributing to the regulation of the primary repertoire of antibody specificities by increasing heavy chain diversity during Ig gene recombination [34].

In addition, it has been shown that the steady decrease in B cell lymphopoiesis in aging mice is due to an impairment in release, rather than production of IL-7 from stromal cells. It was discovered that cell contacts were required between the stromal cells and the B cell precursors to enable proliferation regardless of animal age [25]. Therefore IL-7 release may be influenced by physical contact with B cell precursors, suggesting that these B cells are able to

influence their own futures by signalling the stromal cells via contact. By limiting the amount of IL-7 available to the developing precursor cells, the stromal cells are able to regulate B lymphopoiesis [25].

Although IL-7 has been shown to have a role in the pro to pre B cell transition it does not have any influence over the subsequent maturation to $sIgM^+$ immature B cells [35]. Henderson and colleagues found that late pre-BII cells and immature B cells no longer express IL-7R and therefore no longer respond to IL-7 [36]. They did not, however, define the precise developmental stage at which expression of the receptor ceased.

IL-7 is vital for B cell development in the mouse but it is not as crucial in humans [30] - patients with mutations in the IL-7 receptor α chain have normal numbers of peripheral blood B cells [37]. IL-7 was, however, shown to downregulate RAG-1/RAG-2 (RAG = recombinase activating gene) and TdT (terminal deoxynucleotidyl transferase). These are involved in the rearrangement of Ig chains and their expression can be used to gauge VDJ recombination. Therefore, although IL-7 may not appear to be essential at the single cell level it does have a role in immunoglobulin receptor diversification and therefore contributes to the development of an optimally efficient immune response [38].

Other factors have been found to have effects on B cell proliferation and development. Flt-3 ligand has been reported to boost proliferation of CD19+/CD34+ pro-B cells [39] and the development of CD19+ B lineage cells from CD34+ cord blood haematopoietic stem cells [40]. Stromal cell derived factor-1 (SDF-1) is a chemokine which may play an important role in B cell development. Mice with targeted disruptions in the genes encoding SDF-1 and its receptor CXCR4 have defects in lymphopoiesis [41,42] and it has been concluded that an operational CXCR4 receptor is crucial for the retention of B cell precursors in the bone marrow microenvironment [43].

Not all cytokines produced from stromal cells have positive effects on developing B cells. IL-1α [44,45], IL-3 [44], IL-4 [46] and IFNs α/β [47] have been shown to have an inhibitory effect on B cell precursors. TGF-β is another cytokine which has demonstrated an inhibitory effect on

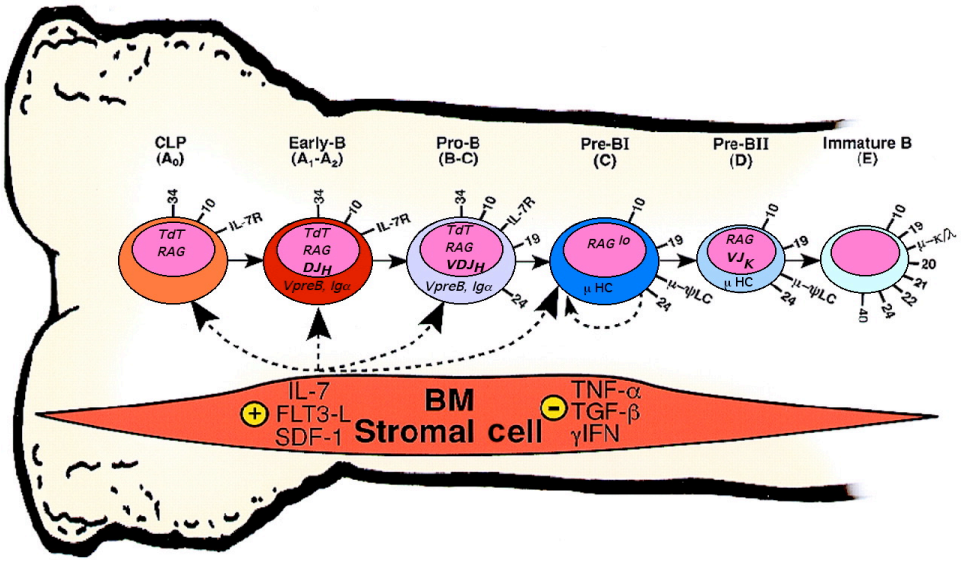
lymphopoiesis. This effect is dose-dependent and has been shown to be partly due to its down-regulation of IL-7 release from stromal cells [48].

Insight into the mechanisms responsible for maintaining the balance between lymphopoiesis and myelopoiesis in the bone marrow environment was gained by experiments performed by Ryan which demonstrated that cytokines inhibitory for lymphopoiesis (IL-1α, IL-4 and TNF-α) simultaneously trigger the release of myeloid growth factors [49].

In summary, many factors - both positive and negative present in the bone marrow milieu in soluble or matrix/membrane bound form – contribute to the tightly regulated advancement of precursor B cells through their sequential stages of development.

Figure 1.1 shows a summary of events during B lymphopoiesis [1].

Figure 1.1 Schematic of Human B Cell Development in the Bone Marrow Six stages are shown with letters in brackets representing an approximation of the counterpart phases in murine B lymphopoiesis using the nomenclature of Hardy and colleagues. Cellular and cell surface protein expression is illustrated pictorially and also shown are the effects of stromal cell cytokines on the developing B cells [1].


CLP
(A0)
Early-B
(A1-A2)
Pro-B
(B-C)
Pre-BI
(C)
Pre-BII
(D)
Immature B
(E)
TdT
RAG
IL-7R
DJH
VpreB, Igα
VDJH
RAG lo
μ HC
RAG
VJK
μ-ψLC
μ-κ/λ
IL-7
FLT3-L
SDF-1
BM
Stromal cell
TNF-α
TGF-β
γIFN

1.3 Apoptosis/programmed cell death

Cell death is fundamental for the development and homeostatic maintenance of multicellular organisms. It is involved in many processes, for example, limb sculpture, cell number adjustment and the riddance of abnormal, misplaced, non-functional or dangerous cells. Loss of control over processes governing cell death contributes to the pathogenesis of diseases such as Alzheimer's disease, which involves increased apoptosis and cancers (for example lymphomas and leukaemias) which involve decreased apoptosis. Cell death is therefore a vital avenue of scientific enquiry.

In 1972 Kerr and colleagues highlighted the significance of "normal cell death" when they identified a distinct set of morphological features that enabled classification of dying cells into one of two categories: necrosis or apoptosis. Necrosis is sometimes referred to as "accidental cell death" because it occurs following acute cellular injury which is characterised by rapid swelling and bursting (lysis) of affected cells. Cell death by apoptosis, in contrast, is typified by regulated autophagy of the cell, evidencing the operation of an active or programmed mechanism of death [50,51]. The word apoptosis is derived from ancient Greek describing the process of leaves falling from the trees in the autumn and was chosen to imply that cell loss is desirable for the survival of the host [50].

Programmed cell death (PCD) and apoptosis are frequently used interchangeably, however, PCD is a functional term describing a cell death that is a normal part of life whereas apoptosis is purely a descriptive term that represents a type of cell death with distinctive morphological features [52].

1.3.1 The morphology of apoptosis

Apoptosis involves the sequential occurrence of a defined sequence of morphological events. Initially, the cell nucleus shrinks and its chromatin becomes condensed into compact masses along the nuclear membrane. The cytoplasm mirrors this process with compaction causing

organelles to become crowded yet maintained in form. The overall cell shrinkage causes the plasma membrane to become "ruffled" – this is known as blebbing [53]. Apoptotic bodies (membrane-bound fragments which retain their integrity) are frequently formed at this point and these are rapidly phagocytosed by cells of the macrophage lineage or neighbouring cells taking on the role of semi-professional phagocytes.

This efficient elimination of apoptotic cells ensures that an immune response is not initiated as cellular components are not released – during necrosis this does occur and is one of the features which distinguishes the two processes. The inflammation that occurs following release of intracellular components may cause collateral damage - destruction of normal cells – and it is therefore crucial that rapid removal of apoptotic cells occurs to prevent this. In the immune system programmed cell death occurs frequently and is vital to normal tissue function so this protection from collateral damage is paramount [53].

Chromatin changes during apoptosis are accompanied by internucleosomal DNA cleavage. At this linker site the DNA is most loosely associated with histone H1 proteins. The cleavage is not sequence-specific and is most likely due to the sensitivity of "open chromatin" to degradation. When first characterised this event was though to be the biochemical hallmark of apoptosis and can be visualised upon gel electrophoresis as multiples of 200 base pairs (bp) oligonucleosome fragments – the "DNA ladder" [51]. Further work has provided evidence that this is a late apoptotic event as larger fragments of either 300 or 50kb are generated prior to the cleavage of oligonucleosomal fragment. These larger fragments are thought to represent rosettes (300kb) or loops (50kb) of chromatin [54] . In addition, apoptosis has been demonstrated to occur in the absence of a DNA ladder, therefore internucleosomal cleavage cannot be a definitive indicator of apoptotic cells [53,55]. Programmed cell death has also been found to occur in the absence of a nucleus which suggests that a cytoplasmic regulator with many targets is a major player in PCD and that nuclear events are not necessary for the process [56].

1.3.2 The role of apoptosis in B cell development

Most B cells that arise from haematopoietic stem cells are not destined to take part in a specific immune response. These cells are removed from the proliferative compartment by apoptosis. Some 75 % of B cells are lost in the bone marrow when precursor B cells which have failed to productively rearrange their immunoglobulin genes at the pro-B to pre-B cell stage are eliminated [57]. Cells with abortive rearrangements die by apoptosis and are engulfed by bone marrow macrophages [58,59].

The next developmental stage sensitive to apoptosis is the immature B cell. These cells characteristically express surface IgM and engagement of the antigen receptor (or B cell receptor – BCR) at this particular stage of development results in clonal deletion, the mechanisms of which appear to cause both anergy (unresponsiveness) and apoptosis [60].

Mature (IgM+/IgD+) B cells are also susceptible to apoptosis. In order to generate antibodies with high affinity for antigen, mature B cells undergo affinity maturation via the processes of somatic hypermutation and antigen-driven selection. Somatic hypermutation of IgV region genes occurs in activated B cells called centroblasts within the dark zones of germinal centres. The descendants of these centroblasts, centrocytes, are then subjected to positive and negative selection processes to ensure that high affinity mutants are retained and low affinity or autoreactive mutants are neglected or deleted by apoptosis [61]. The signals which mediate centrocyte survival are delivered via the antigen receptor and CD40 [62] and also by soluble CD23 and IL-1α [63]. Soluble CD23 is thought to induce differentition into a memory B cell whereas IL-1α promotes plasmacytoid differentiation [63].

1.3.3 The basic apoptotic machinery

All nucleated cells express the protein components needed to execute the death pathway constitutively and therefore intrinsically possess the potential to undergo apoptosis [64]. The drug staurosporine has been used to gather evidence for this by exploiting its broad spectrum inhibitory effect on protein kinases. When used in conjunction with cycloheximide, which

inhibits protein synthesis, staurosporine initiates PCD in all of the cells which can be dissociated from a 13 day mouse embryo [65] and in the cultures of a variety of neonatal and adult rodent organs [66]. Another feature of PCD is that a nucleus is not required in cells which usually possess one. For example, anucleate cytoplasts undergo PCD when treated with staurosporine [56]. This means that all mammalian cells apart from red blood cells, which do not have a nucleus, are capable of undergoing PCD.

The control of PCD and its molecular basis have long been a popular area of scientific research. Many details have been elucidated following the genetic analysis of *Caenorhabditis elegans* which revealed three genes, *ced-3, ced-4 and ced-9* that are crucial to nematode PCD [67]. Loss of function mutations determined that *ced-3*and *ced-4* are both essential for the initiation of programmed cell death [67] whereas *ced-9* acts to prevent cells from undergoing programmed cell death [68]. The order of action of these cell death genes has been determined and is as follows: *ced-9* acts upstream of *ced-4* which acts upstream of *ced-3* [69]. Further studies have revealed finer detail, e.g. the interaction of Ced-4 and Ced-3 which promotes Ced-3 activity and the interaction of Ced-9 and Ced-4 which prevents Ced-4 from binding to, and hence activating, Ced3. These investigations have revealed that Ced-4 plays a central role in the pathway leading to cell death and links the apoptotic regulators to the effectors in a physical manner [70,71].

When mammalian homologues to the nematode genes were identified their significance became far more appreciated. The homologies within apoptotic regulators were found to be: Ced-3 with the caspase family [72], Ced-4 with Apaf-1 [73] and Ced-9 with the bcl-2 family [74].

1.3.4 The caspase family of apoptotic executioners

The name caspase comes from the fact that the proteins are from a family of cysteine proteases which cleave their substrates after aspartate residues [75]. The first caspase to be identified was capase-1, also known as interleukin-1β-converting enzyme (ICE), and as previously stated has homology with the Nematode *ced-3* gene and it was this sequence relationship which led to its recognition [72]. In evolutionary terms caspases are ancient and are common to multicellular organisms. They have been found to take part in cell death and

inflammation and act by cleaving substrates that are particular to the signalling cascades of either of these two processes. Mammalian Ced-3 homologues (caspase-3, caspase-6 and caspase-7) are effectors of many forms of apoptosis in response to various stimuli [76]. The caspases that initiate the secretion of inflammatory mediators are phylogenetically distinct (caspase-1, caspase-4, caspase-5, caspase-11 and caspase-12) and achieve this by proteolysis of the precursors of the inflammatory cytokines IL-1β and IL-18 [77]. The third class of caspases (caspase-2, caspase-8, caspase-9 and caspase-10) are termed initiator caspases as they act upstream of the effector caspases in apoptosis. Despite the separation of caspases into inflammatory and apoptotic, some apoptotic caspases have been found to take part in other cellular processes, including cell cycle progression and, surprisingly, cell survival [78].

1.3.5 Extrinsic and intrinsic pathways

There are two separate pathways of cell death which caspases take part in: the extrinsic pathway and the intrinsic pathway. Extrinsic signals, such as the ligands for the subset of Tumour Necrosis Factor Receptors (TNFRs) that act as death receptors, trigger initiator caspases such as caspase-8. This kind of caspase can directly activate effector caspases like caspase-3, which cause cell death independently of mitochondrial depolarisation [78-80]. These signals are often augmented by pro-apoptotic factors released from mitochondria – this pathway is named the type II extrinsic pathway and involves molecules such as BID. BID stands for B-cell lymphoma-2 (BCL-2) homology domain 3 only (BH3 only) protein interacting BH3-interacting-domain agonist. These types of molecule are cleaved by caspases. The shortened BID is translocated to the outer membrane of the mitochondria where it interacts with the multidomain BCL-2 family members BCL-2-associated X protein (BAX) and/or BCL-2 antagonist/killer (BAK) and initiates their oligomerization. Activation of BAK and BAX is necessary for permeablisation of the outer mitochondrial membrane which enables release of apoptosis-promoting factors housed in the mitochondria [78,81,82].

On the other hand apoptotic stimuli involved in the intrinsic pathway effect the change in mitochondrial permeability either directly or via BH3-containing effector proteins in the

absence of caspases. This can be achieved either by inactivating anti-apoptotic molecules like BCL-2 or activating pro-apoptotic molecules such as BAK and BAX [83].

Mitochondrial proteins released into the cytosol activate the central apoptotic pathway. Cytochrome *c* interacts with apoptotic-protease-activating factor (APAF-1) and caspase-9 to form a complex named the apoptosome [84]. Caspase-9 then drives effector caspase cleavage of apoptotic substrates that leads to the irreversible morphological changes and characteristic cleavage of DNA that typifies apoptosis.

1.3.6 Regulation of BCL-2 family members

Cytokines and other signals contribute to regulation of the BCL-2 family proteins. BAX, for example, is induced in some cells as part of the p53 damage response [85]. Other pro-survival genes are expressed following cytokine stimulation [86] and cytokines also influence cell survival in a post-translational manner. In haematopoietic cells stimulated by (for example) IL-3, BAD is phosphorylated by AKT (also known as PKB - Protein Kinase B) and in this phosphorylated state it is kept sequestered in the cytoplasm by the 14-3-3 protein - preventing it from moving to the mitochondria where it would promote apoptosis [87]. This signal appears to be transduced by phosphoinositide 3-kinase (PI3K) the products of which activate AKT via phosphorylation of threonine 308 and serine 473 [88].

Phosphorylation has the ability to both promote and suppress activity of pro-survival proteins. BCL-X_L activity is thought to be negatively regulated by phosphorylation sites which are located in its flexible loop and BCL-2 is possibly controlled via serine 70 phosphorylation (activation) and several loop sites (suppression) [89].

1.4 SMS-SB cells: a childhood pre-B Acute Lymphoblastic Leukaemia cell line

1.4.1 Acute Lymphoblastic Leukaemia

Acute lymphoblastic leukaemia is thought to originate from various important genetic lesions in blood progenitor cells that are committed to the T-cell or B-cell lineages, including mutations that confer the capacity for unlimited self-renewal and those that lead to precise stage-specific developmental arrest [90,91]. The cells involved in acute lymphoblastic leukaemia have clonal rearrangements in their immunoglobulin or T-cell receptor genes and express antigen receptor molecules and other differentiation-linked cell-surface proteins that largely replicate those of immature lymphoid progenitor cells [90-92]. This malignancy is classified by means of immunotyping, determining the cell lineage and developmental status where the transformation occurred. Cluster of Differentiation (CD) antigens are used a part of this identification method and CD10 (also known as the Common ALL Antigen – CALLA) being the first marker used in the classification of ALL [93]. This antigen is a lineage-independent marker and is not universally expressed in ALL, its absence often being associated with poor prognosis [94].

Chromosomal translocations that activate specific genes are a defining characteristic of leukaemias in general and of acute lymphoblastic leukaemias in particular. About 25% of cases of B-cell precursor acute lymphoblastic leukaemia harbour the TEL-AML1 fusion gene generated by the t(12;21)(13:q22) chromosomal translocation. This is the most common form of acute leukaemia in children [90]. Although the precise molecular role of the fusion protein is unclear the *Tel* gene is an important regulator of haematopoietic cell development and the *Aml1* gene is necessary for definitive embryonic haemopoiesis [95,96]. In adults the most frequent chromosomal translocation is the Philadelphia chromosome, which is a fusion of chromosomes 9 and 22. This fusion results in the joining of the BCR signalling protein to the ABL non-receptor tyrosine kinase and results in constitutive tyrosine kinase activity and interaction with transforming elements such as the RAS signalling pathway [97]. Finally, more than 50% of T-cell acute lymphoblastic leukaemias have mutations that involve the NOTCH1 protein. This protein in its receptor form regulates normal T-cell development and intracellular NOTCH1 can be induced to translocate to the cell nucleus where it activates responder genes including the MYC oncogene [98-100].

1.4.2 The SMS-SB cell line

The SMS-SB cell line was originally characterized by Smith and colleagues in 1981. The cells were derived from a 16 year old African-American girl (SB) in the leukaemic phase of a lymphoblastic lymphoma. The primary lymphoma was atypical in that its development occurred in and around skeletal bone and bone marrow rather than lymphatic tissue and did not express any T-lymphocyte markers which are ordinarily commonplace in lymphoblastic lymphomas [101,102]. This distribution of disease is typical of lymphomas induced in mice by the Abelson Leukaemia Virus [103]. The cultured leukaemic SMS-SB cells are classified as pre-B lymphocytes by virtue of the expression of B-cell markers, the presence of cytoplasmic heavy chains, and the absence of any T-cell specific markers. It is unusual that these cells are referred to as pre-B cells as they do not express the pre-BCR, however, this is how Smith *et al* have classified them in their 1981 paper identifying this cell line[101]. In culture these cells express μ heavy chain on the cell surface enabling their classification as pre-B cells (M. Acharya personal communication). Smith *et* al also state that SMS-SB cells seem to most closely resemble the major population of pre-B cells found in normal bone marrow, and differ from other cultured pre-B ALL cell lines in that they do not express high levels of CD10 (CALLA) or the enzyme TdT [102].

SMS-SB cells do not possess the Epstein-Barr Virus nuclear antigen, therefore this virus did not transform these cells [101]. Analysis of the karyotype of these cells did not reveal any gross chromosomal abnormalities such as the Philadelphia chromosome. This t(9;22) translocation is implicated in acute leukaemia. This transposes the *c-abl* gene (the cellular homologue of the Ableson viral oncogene) to the *bcr* (breakpoint cluster region) gene. The chimeric protein produced has increased tyrosine kinase activity compared to the normal c-ABL protein [104]. *c-abl* analysis in SMS-SB cells revealed no gross rearrangements but discovered 2 additional *abl*-related transcripts not found in normal cells. It was postulated that these abnormal transcripts may have contributed to the commencement of malignancy in these cells perhaps in concert with other activated oncogenes [105]

Advances in molecular diagnostic techniques have enabled the characterization of genetic abnormalities found in ALL that could not have been detected using routine karyotyping [106]. It is therefore quite plausible that SMS-SB cells possess genetic abnormalities that have not been detected thus far.

Further research by Tsai and colleagues has been carried out in an attempt to further classify SMS-SB cells by way of the examination of nuclear proto-oncogenes that are commonly found altered in leukaemias and lymphomas. It was noted that whilst *c-myc, c-myb* and *c-jun* are expressed at normal levels, *c-fos* (and hence the corresponding protein $p55^{c\text{-}fos}$) was found to be over-expressed in SMS-SB cells in comparison with other cell lines. The elevated levels of *c-fos* transcripts were found to be due to up-regulated transcription rather than mutations and this up-regulation could be enhanced by serum. When SMS-SB cells were compared with another ALL cell line, Nalm-6, it was found that SMS-SB cells expressed higher levels of $p55^{c\text{-}fos}$ protein. This protein forms heterodimers with members of the Jun family of proteins to form the transcription factor (AP-1)(Activator Protein 1) [107]. SMS-SB cells were not, however, found to display increased amounts of AP-1 DNA binding activity compared to Nalm-6 cells [108].

1.4.3 SMS-SB cells produce growth promoting factors

Following removal from the patient SB, the leukaemic cells readily adapted to tissue culture without going through a crisis phase. The cells could be grown in serum-free and protein-free media without the addition of exogenous mitogens [101]. This immediate establishment is unusual and pointed to the expression of growth-regulatory factors. Two growth activities were detected in culture supernatants: one which acts as an autocrine factor that is growth-promoting to SMS-SB cells cultured in serum-free medium at low densities; and another named leukaemia-derived-transforming growth factor (LD-TGF), which enhances fibroblast growth but does not affect SMS-SB cells nor other haematopoietic cells [109].

The aforementioned SMS-SB autocrine growth factor (SB-AF) was found by White not to be directly mitogenic for SMS-SB cells, but only able to promote growth under stressful, serum-free conditions in which the cells would normally undergo growth arrest and apoptosis [110]. Subsequent testing of a panel of cytokines with the ability to recreate this specific growth enhancement identified soluble CD23 (sCD23) and platelet-derived growth factor (PDGF). The SMS-SB cells, however, do not express CD23 and the use of neutralising anti-PDGF antibodies ruled out PDGF as being the elusive autocrine factor [110].

1.5 CD23

CD23, also known as the low affinity receptor for IgE (FcRεII) and BLAST-2, exists in two forms: CD23a and CD23b. CD23 is the only Ig receptor not to belong to the immunoglobulin superfamily and can exist as a monomer and a trimer in membrane-bound and soluble forms [111]. The membrane-bound form of CD23 (mbCD23) is cleaved by a membrane-associated metalloprotease to produce soluble CD23 (sCD23) fragments of 37kDa, which are then further cleaved to give fragments of 33kDa, 29kDa, 25kDa and 15kDa. All of the fragments contain the C-type lectin domain of CD23 and varying portions of the stalk region. Cleavage of the mbCD23 producing the 37kDa fragment occurs at close proximity to the cell membrane [112]. These sCD23 fragments not only retain many of the properties of mbCD23, they also process activity akin to cytokines [113-115].

CD23 is usually expressed on mature B cells, activated cells particularly, but in a number of pathological disorders its expression is considerably altered [116-121]. Normal CD23 levels in human serum ranges from 0.5 – 5ng/ml, with children and babies having levels higher than those found in adults [122]. In the malignancy chronic lymphocytic leukaemia (CLL), elevated levels of CD23 may have prognostic value and may correlate with progression of the disease [123-126]. In the inflammatory condition rheumatoid arthritis, CD23 levels are observed where the levels of CD23+ B cells are increased as is the level of sCD23 in the blood and synovial fluid [127-130]. In addition to being a marker for certain pathological conditions, CD23 also displays cytokine-like properties when in its soluble form. sCD23 provides growth signals to cell lines transformed by the Epstein-Barr virus (EBV) [131], prevents apoptosis in germinal centre B cells [63] and induces prothymocyte differentiation [132]. CD23 is clearly a

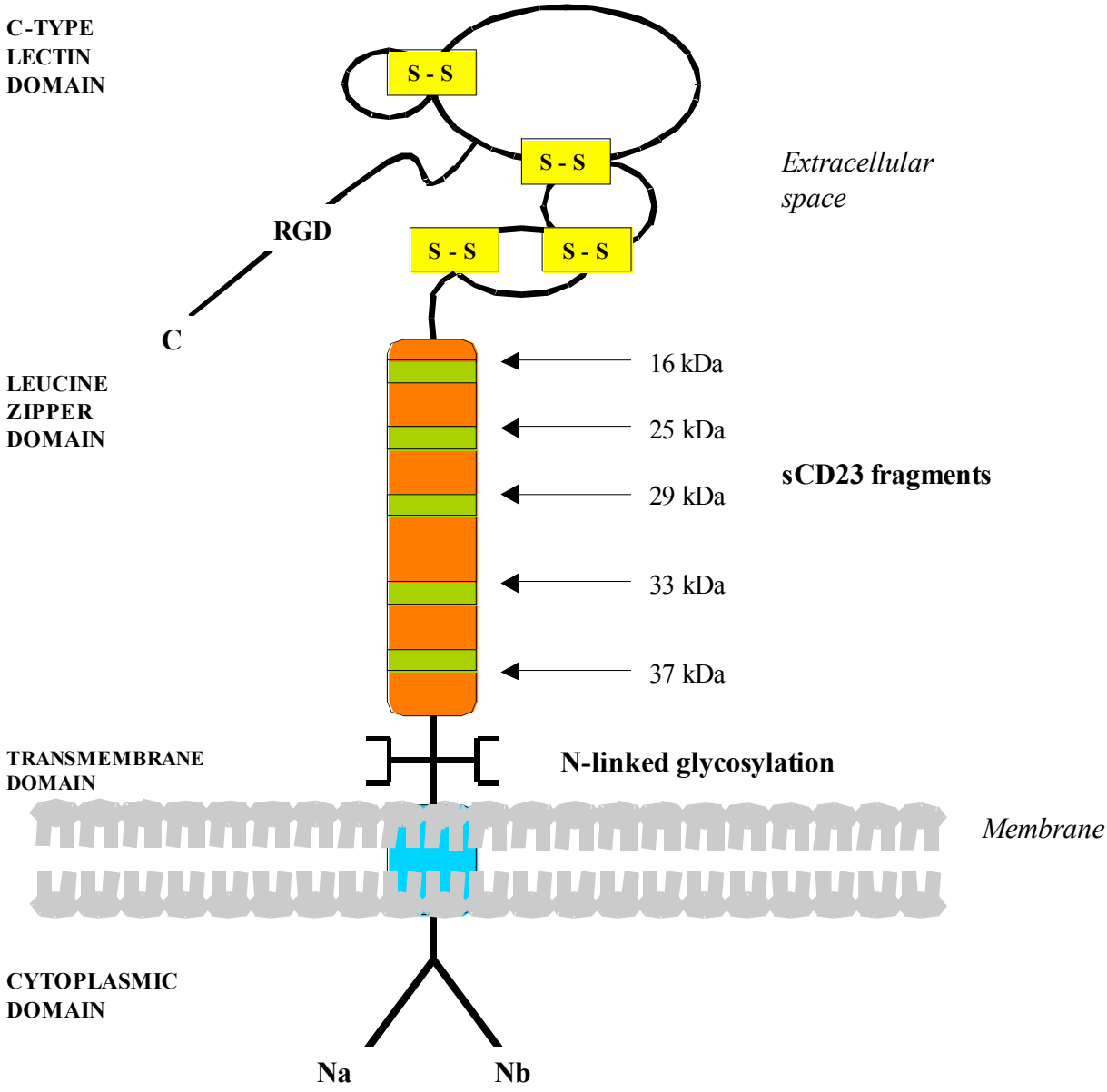
multifunctional protein with many interesting properties associated with cell growth and development.

1.5.1 CD23 Structure

CD23, as previously stated, is the only Ig receptor that does not belong to the immunoglobulin superfamily. It can exist in soluble and membrane-bound forms and its presentation partly mediates its diverse activities. As Figure 2 shows at the structural level CD23 is made up of a number of domains and motifs that are central to its functions [111,133].

Figure 1.2 Schematic representation of CD23

CD23 is comprised of distinct functional groups that define its biological roles. Membrane-bound CD23 is composed of an extracellular C-type lectin head connected to a transmembrane domain by a leucine zipper domain. Cleavage of membrane-bound CD23 occurs in this region to generate a range of soluble forms of CD23. The cytoplasmic domain of CD23 differs in sequence to produce the isoforms CD23a and CD23b.


C-TYPE
LECTIN
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S - S
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S - S
Extracellular
space
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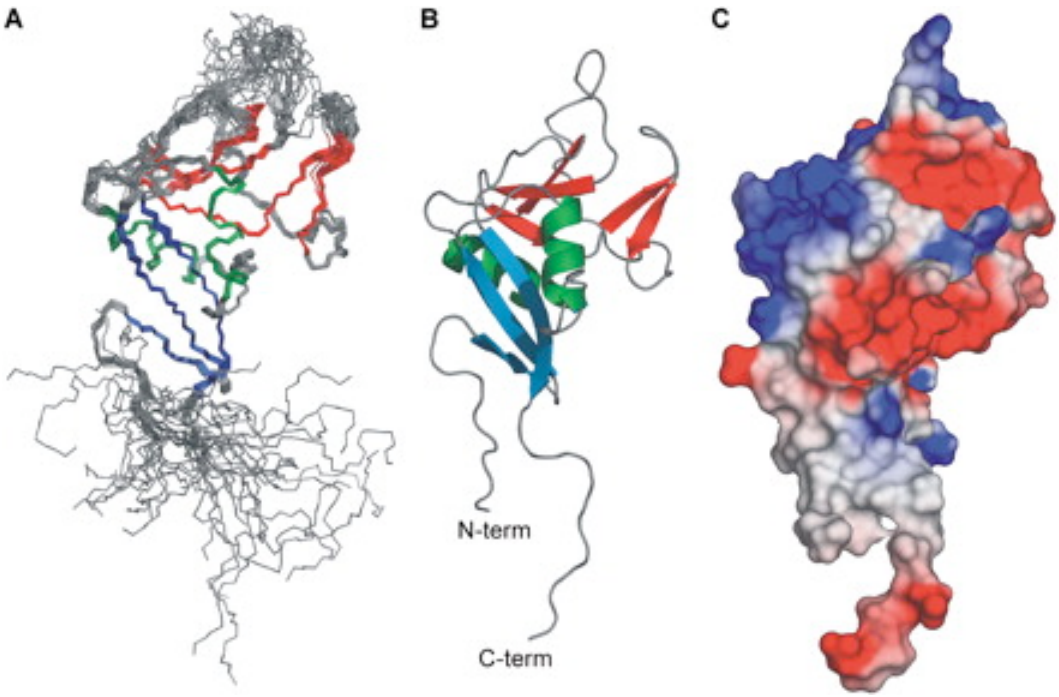
C
LEUCINE
ZIPPER
DOMAIN
16 kDa
25 kDa
29 kDa
33 kDa
37 kDa
sCD23 fragments
TRANSMEMBRANE
DOMAIN
N-linked glycosylation
Membrane
CYTOPLASMIC
DOMAIN
Na
Nb

1.5.3 C-type Lectin Domain

The C terminal extracellular domain of CD23 is similar structurally to the C-type lectin family. C-type lectins require calcium for carbohydrate binding. The sequence of this domain of CD23 is also similar to a family of proteins including the asialoglycloprotein receptor and the selectin adhesion proteins [133].

The 3-D structure of this head domain has recently been determined by NMR spectroscopy [134]. This domain was previously modelled on mannose binding protein (MBP) and has now been shown to have greatest sequence and structural homology with the lectin domains of DC-SIGN. It consists of two α helices and 8 β strands which make up 2 antiparallel β sheets. Seven tryptophans contribute to the formation of a hydrophobic core and 4 disulphide bridges help to maintain tertiary structure. This area of the protein also displays a polarity with regard to its electrostatic nature. The positive and negative residues of the highly charged surface are located on opposite faces of the molecule which may affect the way CD23 interacts with ligands/itself [134]. Figure 3 shows the structure of derCD23 (CD23 cleaved by Der p I, a dust mite protease that cuts the stalk of CD23 close to the lectin domain). The NMR structure does not clearly define the calcium binding region but the crystal structure determined by Wurzburg in 2006 does. This structure has determined that four CD23 residues (E249, T251, N269 and D270) contribute five ligands for the calcium ion and it was also confirmed that calcium binding occurs at the principal binding site but not at an auxiliary binding site as was proposed by the NMR structure [135].

Figure 1.3 The structure of derCD23. (A) View of the backbone of 20 superimposed NMR-derived structures of the head domain of CD23. (B) A ribbon diagram of the head domain of CD23, with secondary structural elements identified. (C) A surface representation of the head domain of CD23 coloured according to opposing charges.


A
B
C
N-term
C-term

1.5.4 Stalk/Leucine Zipper Domain

The stalk domain of CD23 is an alpha helical coiled-coil structure consisting of 7 hydrophobic repeats [136]. This part of CD23 connects the transmembrane domain to the lectin domain and is the site of oligomerisation into CD23 trimers. The leucine zipper region is close to the transmembrane domain and has a motif consisting of 7 amino acids beginning with either a Leu or Ile residue which is repeated 5 times in human CD23 [133]. The stalk is the site of proteolytic cleavage of CD23 into sCD23 fragments and therefore it contributes to the regulation of the functions of CD23 via production of CD23 molecules with particular functions [137].

1.5.5 N terminal cytoplasmic sequence

The N terminus of CD23 is cytoplasmic. CD23 is found is two forms, namely CD23a and CD23b which differ in sequence at this region. The a form contains the sequence "Met-Glu-Glu-Gly-Gln-**Tyr-Ser-Glu-Ile-Glu**" (MEEGQ**YSEIE**) at the N terminus, whereas the b form has the sequence "Met-Asn-Pro-Pro-**Ser-Gln-Glu-Ile-Glu**" (MNPP**SQEIE**) [138]. The residues in bold represent the consensus N terminal pentameric sequence described by Vega and Strominger as being common to receptors capable of being internalised via coated pits [139]. Both forms are derived from the same gene and formed by alternative splicing. Different promoters drive expression of the distinct first exons which are then spliced to give forms CD23a and CD23b. CD23a is expressed constitutively on certain cell types (for example B cells and follicular dendritic cells [140,141]) whereas CD23b is induced by IL-4 and expressed on a wide range of cell types (T-cells, eosinophils, monocytes, platelets and Langerhan's cells [141,142]). The two isoforms display different functions. The a isoform has a role in endocytosis (Tyr based motif) and the b isoform is involved in phagocytosis (Asn-Pro motif). In terms of its structure and expression pattern CD23a is equivalent to murine CD23 [138,143]. Murine CD23 is equivalent to the human CD23a isoform and has a Tyr reside in a similar position. This implies murine CD23 can be endocytosed and draws a parallel with the ability of murine CD23 to focus and present antigens to T cells in an IgE-dependent manner. Murine CD23, however, does not contain the entire pentameric sequence from CD23a [133].

1.5.6 Inverse RGD Sequence (DGR)

Located near the C terminus of human CD23 is a reverse RGD sequence (DGR). This sequence is not present in murine CD23. The RGD sequence is a well-characterised recognition motif for integrin binding and is found in a wide range of proteins that mediate cell adhesion [144]. Although a function for the DGR sequence has not yet been defined it has been suggested that it interacts with a sequence at the root of the N linked sugar chain. This sequence has 8 out of 12 amino acid homology (in reverse) with an "RGD-binding inhibitory peptide" (the gpIIIa chain of platelet-integrin gpIIb/IIIa). This peptide prevents the binding of platelet-integrin and fibrinogen that contains the RGD sequence [145]. Further studies are required to further clarify the functions of these two reverse sequences.

1.5.7 CD23 Functions

CD23 participates in both protein-protein and protein-carbohydrate interactions. To date, CD23 has been found to interact with IgE [146], CD21 [147], and leucocyte integrins ($\alpha X\beta 2$ and $\alpha M\beta 2$) [148] and the vitronectin receptors $\alpha v\beta 3$ and $\alpha v\beta 5$ [114]. The interaction of mbCD23 and sCD23 with these ligands results in specific cellular events and CD23 has been implicated in numerous processes such as antigen presentation, cellular adhesion, growth and differentiation of B and T cells, rescue from apoptosis, liberation of cytotoxic mediators and control of IgE synthesis [111].

1.5.8 CD23 Interaction with IgE

IgE is one of the classes of human antibodies and is produced exclusively in mammals. IgE is well known for its efficacy in combating parasitic infections and is thus found at high levels in the skin, lungs and gut (sites of parasitic invasion). IgE overexpression following exposure to common environmental antigens gives rise to allergies. It accounts for a very low percentage of the total antibody in human serum but the actions of IgE are considerably augmented by its receptor interactions. Immediate hypersensitivity reactions are caused by the interaction of IgE with its high affinity receptor (FcεRI) [122].

CD23 is the low affinity receptor for IgE and binds to IgE alone with an affinity of Ka ~ $10^7 M^{-1}$. It binds IgE-antigen complexes at $10^{-9} M^{-1}$. The high affinity receptor for IgE binds with an affinity of $10^{-9} M^{-1}$ [133]. Mutation of the glycosylation site Asn371 does not affect binding hence the interaction between IgE and CD23 is via protein-protein interactions and not protein-carbohydrate despite the binding site being located in the lectin (carbohydrate-binding) domain [149]. The interaction between CD23 and IgE is important to the regulation of IgE levels. The membrane bound and soluble forms of CD23 have distinct roles in the control of IgE expression [122].

When CD23 and IgE bind on the surface of B cells synthesis of IgE is reduced and a fall in serum levels of IgE follows [111] [150]. CD23 has to be in the membrane-bound trimeric format to facilitate this activity [151]. Release of sCD23 from the membrane is inhibited by IgE, which in turn represses synthesis of IgE further. CD23-/- mice tend to have higher levels of IgE following stimulation with antigen, whereas mice overexpressing CD23 produce notably lower amounts of IgE following the same antigen treatment [152]. The ligation of mbCD23 by IgE is thought to stabilise the stalk region thereby preventing proteolytic cleavage. Support for this lies in the fact that sCD23 production is reduced by monoclonal antibodies which act to stabilise the stalk region, whilst the opposite is true of monoclonal antibodies which act to destabilise the stalk region [153]. mbCD23 expressed on B lymphocytes (trimeric form) also enhances the presentation of antigen complexes to T cells. Antigen presentation requires internalisation of the antigen, its cytoplasmic processing and subsequent external presentation. This fits with the role in endocytosis ascribed to CD23a. IgE-dependent presentation of antigens to T cells [154] also involves mbCD23 and homotypic adhesion between B cells [155] and mbCD23 on follicular dendritic cells prevents apoptosis in germinal centre B cells [63].

All sCD23 fragments can bind to IgE but only fragments larger than 28kDa promote IgE synthesis. CD23 is found in solution in monomeric and trimeric form and the site of oligomerisation is located in the stalk region. As such, only fragments over 25kDa which possess this part of the stalk region can form trimers [122]. The effect on IgE production is thought to be due to the ability of CD23 to cross-link CD21 and IgE at the cell surface [156].

Soluble CD23 also promotes the proliferation and differentiation of plasma cells, T cells and myeloid precursors, presumably through its cytokine activity [157] [131].

The interplay between IgE and CD23 is also significant in IgE-mediated cytotoxicity, IgE-dependent antigen focussing, promotion of B-cell growth and prevention of germinal centre B cells, maturation of early thymocytes and proliferation of myeloid precursors [111,158].

1.5.9 CD23 Interaction with CD21

CD21 (the receptor for Epstein-Barr virus - EBV), also known as the Complement Receptor type 2 (CR2), is a 145kDa, type I transmembrane glycoprotein. It is important to both human B cell proliferation and activation [155]. Like CD23, CD21 has both membrane bound (mbCD21) and soluble forms (sCD21) that retain biological activity. Expression of CD21 occurs on B cells, thymocytes, a portion of T cells and follicular dendritic cells. CD21 expression is developmental stage-dependent with greatest expression on mature B cells and a subset of immature thymocytes [147]. EBV infection of T and B cells results in an increase of CD21 expression [159]. CD21 and CD23 interact via protein-protein interactions and N-linked sugar chains on CD21 and this takes place at the CD23 lectin head domain [147]. Figure 4 shows a representation of a CD23 trimer.

In humans, the interaction between mbCD21 and mbCD23 contributes to the homotypic adhesion of B cells [155]. It has been demonstrated that sCD21 acts as a functional ligand for CD23-expressing monocytes. In such monocytes (IL-4-induced) this interaction leads to an increase in the production of IL-6 and TNF-α. Also, CD23 interacting with sCD21 leads to monocyte differentiation via a process involving cyclic nucleotide metabolism and stimulation of the nitric oxide pathway [160]. The interaction also produces an upregulation of CD40 and HLA-DR. This increase may reinforce the contact with T cells to facilitate antigen presentation or amplify cooperation with CD40 ligand expressing T cells which would further boost cytokine release [161].

IgE synthesis can be both positively and negatively regulated by CD23. Positive regulation of IgE synthesis occurs following co-ligation of membrane IgE and CD21 (on a human B cell

committed to IgE synthesis) by soluble CD23 that has been released from membrane-bound CD23. CD23 trimers are unstable in the B cell membrane and can be cleaved by ADAM10 (a disintegrin and metalloproteinase 10). CD23 trimers are stabilized by binding of IgE and co-ligation of membrane CD23 and membrane IgE by IgE-antigen complexes negatively regulates IgE synthesis. Concentration of IgE is central to this model. When IgE concentration is low CD23 is not protected from cleavage and therefore synthesis of IgE is increased. At high IgE concentrations CD23 is protected from cleavage and positive signalling for IgE synthesis is prevented. In addition the co-ligation of CD23 and IgE by antigen-IgE complexes results in negative signalling for IgE synthesis [134].

Figure 1.4 A (Ribbon) and B (cartoon) forms of the CD23 trimer. A – red and blue coloured areas correspond to opposing electrostatic charges. B – cartoon. The calcium binding site is shown in yellow, the IgE interaction region in green, the oligomerisation sites red and blue and the cyan area representing the CD21 binding site [134].

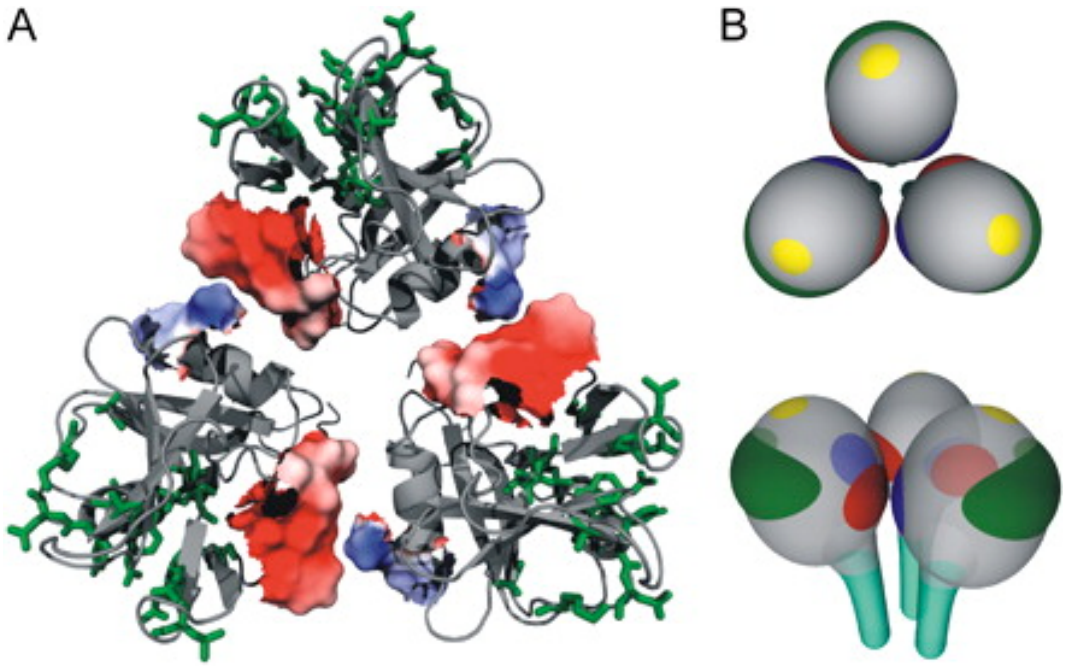
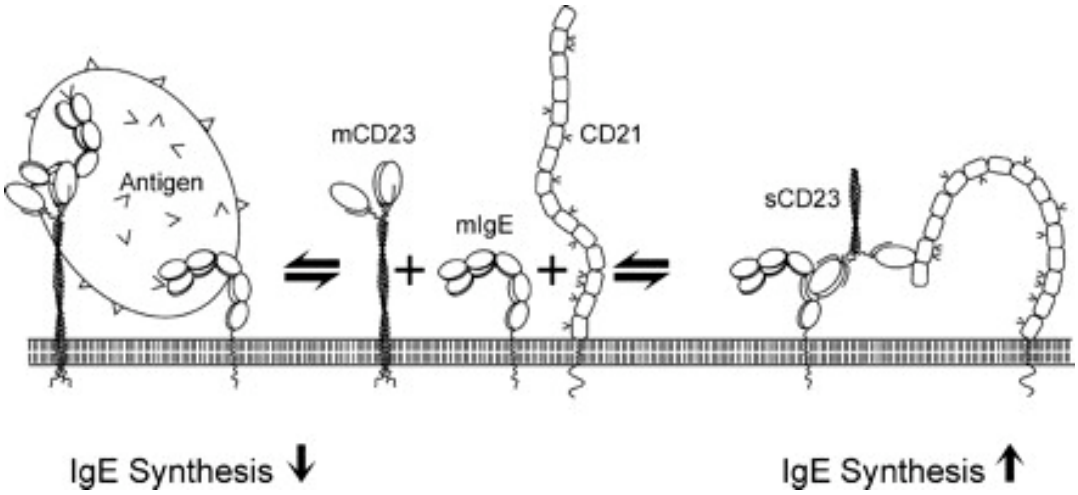

A
B

Figure 1.5 Diagram depicting competition between membrane (mb)CD21 and mbCD23 for IgE and its effect on IgE production [134]. Synthesis of IgE is **inhibited** by the crosslinking of mbCD23 and mbIgE by IgE-bound antigen and **promoted** by crosslinking of mbCD21 and mbIgE by soluble trimeric CD23.


Antigen
mCD23
mIgE
CD21
sCD23
IgE Synthesis
IgE Synthesis

1.5.10 Clinical relevance of CD23

The involvement of CD23 in B cell development is highlighted by its atypical expression and regulation in a malignancy arising from the clonal expansion of mature B cells: chronic lymphocytic leukaemia (CLL). It has been found *in vitro* that B cells from CLL patients produced 8-50 times more CD23 than normal B cells [162]. The excessive CD23 production in CLL patients is due to both the enlarged pool of CD23+ B cells and over-expression of CD23 on B-CLL cells. CD23 is not constitutively expressed on B-CLL cells and is upregulated by IL-4 [163]. It has also been found that these abnormal CD23 levels are prognostic indicators in CLL – high soluble CD23 levels (sCD23) correlates with poor prognosis [164].

Increased levels of sCD23 are present in the synovial fluid of patients with rheumatoid arthritis (RA) classified as of erosive status. This may have predictive value in the monitoring of joint destruction [127]. RA patients present with an increase in CD23 expression on B cells and an increase in CD23 produced by peripheral blood mononuclear cells (PBMCs) [65]. A model for human RA has been established using collagen-induced arthritis in mice. Anti-CD23 antibodies have been found to reduce the destruction of bone and cartilage as well as lessening the clinical severity of the disease [165].

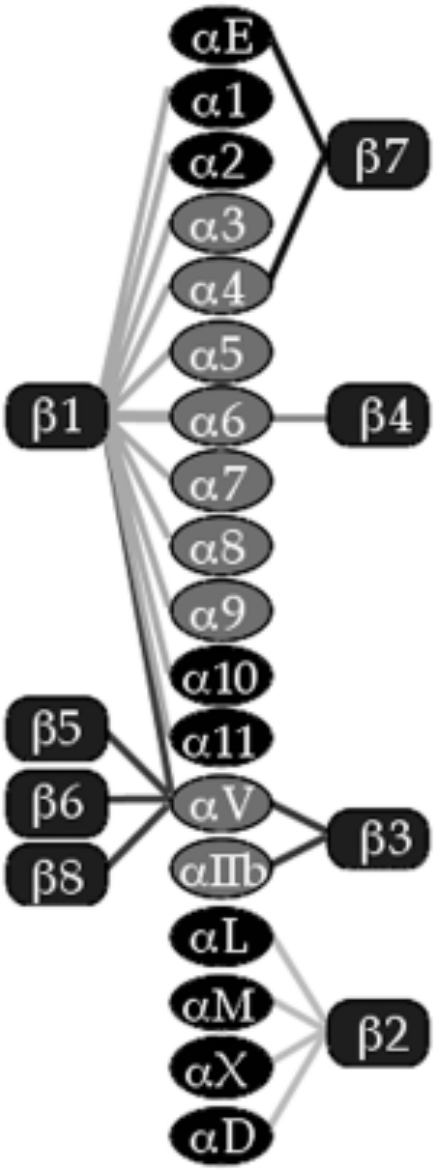
Patients with primary Sjogren's syndrome and systemic lupus erythematosus (SLE) also exhibit elevated serum levels of CD23. Treatment with glucocorticoids significantly lowers the CD23 levels in comparison with patients not receiving this treatment. It appears that B cell hyperactivity in these diseases is associated with the elevation of serum sCD23 [166].

1.6 Integrins

CD23 has been shown to have multiple effects in soluble and membrane bound forms. Induction of pro-inflammatory cytokines by sCD23 is mediated by the interactions of CD23 with members of the integrin family of cell surface adhesion molecules.

Integrins are a family of cell surface receptors that attach cells to and mediate signals from the extracellular matrix (ECM). They provide the cell with the means to communicate with its environment. These signals regulate the activities of cytoplasmic kinases, growth factor receptors and ion channels and control the organisation of the actin cytoskeleton [167]. Cell adhesion is a fundamental event that is critical to a range of biological processes including embryonic development and tissue morphogenesis as well as pathological processes such as tumour cell invasion and metastasis, thrombosis and inflammation [168-171].

Figure 1.6 The integrin family. The α subunits shown darkened contain the A domain of von Willebrand factor (also known as the I domain) [172].


αE
α1
α2
α3
α4
α5
α6
α7
α8
α9
α10
α11
αV
αIIb
αL
αM
αX
αD
β1
β5
β6
β8
β7
β4
β3
β2

Integrins are heterodimeric transmembrane adhesion receptors made up of 2 subunits - α and β, and each αβ grouping has its own binding specificity and signalling characteristics [168,171,173]. Figure 6 shows an illustration of the families. The name integrin refers to their function of integrating the cell ECM to the cytoskeleton. The 18 α and 8 β subunits that have been identified are known to form 24 distinct heterodimers. The combination of the integrin pairing determines the ligand specificity of the integrin. Many integrins have binding specificities for the same ligands and it is the amalgamation of the integrin expression/activation pattern and the accessibility of ligand that specifies the interactions *in vivo* [174]. Although this overlapping of integrin binding specificities exists, the loss of almost every integrin subunit results in some form of biological defect in knockout mice [175].

1.6.1 Integrin Structure

Integrins are formed by two non-covalently associated type I glycoprotein α and β subunits. The cytoplasmic domains are short except for in the case of the β4 subunit which is specialized to connect to the keratin cytoskeleton [176]. The globular headpiece binds to ligand and two stalk-like regions connect this to the cytoplasmic domains.Twelve extracellular domains exist in integrins that do not contain an I domain [177].

1.6.2 α subunit

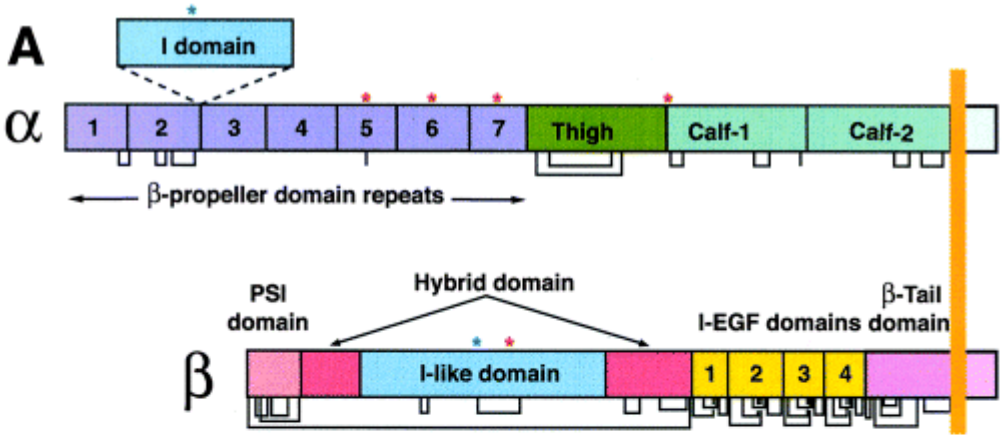
The N terminal domain contains 7 segments which form a β-propeller structure [177-179] and mutagenesis studies show that ligand binding sites are located in this structure [180]. Half of all α integrin subunits contain a domain of approximately 200 amino acid known as the I (insertion) domain or a von Willebrand factor A domain. These I domains are the major sites for ligand binding in integrins which possess them [181]. The I domain forms a Rossmann fold and contains a divalent cation co-ordinating metal-ion-dependent-adhesion-site (MIDAS) which binds to negatively charged residues in ligands [182].

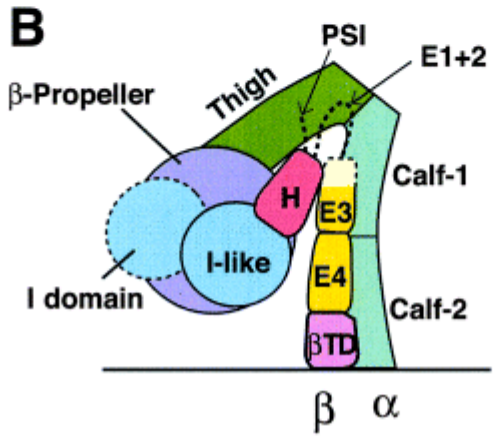
The domains C terminal to the β propeller account for approximately 500 residues of the extracellular portion and much of this corresponds to the stalk region and the crystal structure revels that there are three β-sandwich domains designated the thigh, calf-1 and calf-2 [177]. Figure 7 shows a visual representation of integrin domain arrangements in a primary form and structural illustration.

1.6.3 β subunit

The N terminal region has been termed PSI (plexins, semaphorins and integrins) due to its structural likeness to plexins and semaphorins [183]. An I-like domain is present in the β subunit and this region appears to directly bind ligand in integrins that do not have an I domain [184]. The hybrid domain is a β-sandwich domain that is connected to the I-like domain by two covalent associations. Four EGF-like domains provide rigidity and with the cysteine-rich tail domain complete the extracellular β subunit.

Figure 1.7. Integrin structure. A shows integrin make up within the primary integrin structure. Asterisks show Mg^{2+} (blue) and Ca^{2+} (red) binding sites and lines below the stick diagrams show disulphide bonds. **B** shows a representation of the 3 dimensional arrangement of domains derived from the crystal structure of αvβ3 [184].


A
I domain
α
1
2
3
4
5
6
7
Thigh
Calf-1
Calf-2
β-propeller domain repeats
PSI domain
Hybrid domain
β-Tail
I-EGF domains domain
β
I-like domain
1
2
3
4


B
PSI
E1+2
β-Propeller
Thigh
Calf-1
H
E3
I-like
E4
I domain
Calf-2
βTD
β
α

1.6.4 Integrin activation and signalling

Integrins provide a physical link between the cytoskeleton and the ECM and activate intracellular signalling pathways. They are capable of transmitting signals from the ECM into the cell ("outside-in signalling") and from inside the cell to the exterior milieu ("inside-out signalling"). Stable integrin complexes need to form in order to allow mechanical signals to be sent between cells and the ECM [185]. Signal transmission via integrins requires long-range conformational changes and co-operation between integrin domains. Many integrins are not constitutively active but require activation before ligand binding and signalling can occur. The current model for activation of integrins is called the "switchblade model" and was defined following the definition of the structure of the αvβ3 integrin [186]. The inactive form of the αvβ3 integrin has a bent or genuflexed conformation (the site of such bending being the genu region) and activation results in a straightening and separation of the legs [187]. Binding of extracellular ligand – outside-in signalling – enhances separation of the cytoplasmic domains of the integrin and therefore enables their interaction with the cytoskeleton and signal transduction molecules. Reciprocally, separation of the legs by activators inside the cell could activate the head to facilitate ligand binding. This is inside-out signalling [185].

Integrins have a role in adhesion to ECM ligands and also trigger a large variety of signalling events that modulate processes such as proliferation, cell survival/apoptosis, shape, polarity, gene expression and differentiation. Many cellular responses to soluble growth factors such as EGF, PDGF, thrombin etc are dependent upon the cell's adherence to substrate via integrins [188]. Prevention of apoptosis via PI3K and Akt and progression of the cell cycle through ERK and cyclin D1 have been established to be integrin-mediated [185].

1.6.5 Ligand recognition by integrins

The classical recognition sequence for most integrins is the RGD (Arg-Gly-Asp) motif [189-191]. ECM Proteins containing RGD motifs include fibronectin, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, osteonectin, laminin and collagens [192].

Ligand binding is cation-dependent and involves amino acid residues from both α and β subunits. The Asp of the RGD sequence is co-ordinated by a divalent cation co-ordinated by the MIDAS on the α subunit. In I-domain containing integrins the MIDAS is found at the I domain and in integrins without I domains there is a corresponding MIDAS located on the β subunit. The β-propeller structure from the α subunit can sometimes donate residues to bind the ligand. The β subunit possesses two sites which have metal ion-coordinating capability – the MIDAS and the LIMBS (ligand-associated metal binding site) that can contribute to ligand binding [179].

Other recognition sequences include NGR which binds to α5β1 with low affinity [193]. This is exploited by the Adeno-Associated Virus Type 2 which uses this integrin as a co-receptor for cell entry [194]. The sequence LDV binds to α4β1 and α4β7 integrins and is found in a splice variant of fibronectin. The α4β1 integrin can also recognise two other sequences in that region of fibronectin IDA(PS) and REDV [195]. The integrin-binding motifs all share one amino acid, the aspartic acid, or the closely related glutamic acid residue. Asp may be important because of its potential to contribute to divalent cation binding [196]. Disintegrins are snake venom proteins which can interact with integrins and can prevent blood clotting through high-affinity binding to the αIIbβ3 integrin in platelets [197]. Disintegrins contain an RGD sequence and in one instance this sequence is KGD. Homologous to the integrins are the mammalian ADAMs which have disintegrin domains that lack RGD. At least one ADAM, fertilin, can act as a ligand for α6β1 and this is via a TDE motif which is analogous to the disintegrin RGD [198].

1.6.6 Alternate binding sites

In addition to the RGD recognition site of integrins other ligand binding sites have been identified. The binding of the integrin ligand tumstatin to αvβ3 was found by Maeshima and colleagues to be independent of RGD and that tumstatin contained two RGD-independent binding sites for αvβ3 as binding of tumstatin-derived peptides was not inhibited by cyclic RGD peptides [199].

The αvβ5 integrin interacts with the HIV Tat protein via the Tat basic domain not the Tat RGD domain. This finding was based on experiments involving the binding of Tat peptides to the integrin and their ability to support cell attachment. Given that HIV Tat protein contains an RGD sequence it is perhaps surprising that recognition does not occur in an RGD-dependent manner. It is possible that the RGD sequence is not presented in a suitable context to allow binding to occur. In addition to HIV Tat interacting with αvβ5 in an RGD-dependent manner, the viral protein also interacts via a divalent cation-independent mechanism. The physiological implications of this interaction are yet to be elucidated [200].

1.6.7 CD23/Integrin interactions

1.6.7a Beta 2 (CD18) or Leukocyte Integrins

The expression of β2 integrins is confined to leukocytes. These are heterodimeric proteins composed of a partnership between the β2 integrin and one of αD, αL, αM or αX. The αMβ2 (Mac-1, CD11b/CD18 or CR3) and αXβ2 (CD11c/CD18 or p150/95) integrins are adhesion molecules that participate in many cell-cell and cell-ECM interactions and have been reported to bind to several ligands including CD54, fibrinogen, factor X, lipopolysaccharide (LPS), zymosan and concanavalin A [201,202]. An absence of the β2 integrin results in defects in immune processes including the macrophage oxidative burst, phagocytosis and proliferation of lymphocytes and individuals afflicted with this deficiency present with recurrent, often life-threatening bacterial and fungal infections [202-205]. Homozygous CD18 null mice display chronic dermatitis, increased numbers of neutrophils, increased immunoglobulin levels, lymphadenopathy, splenomegaly and a defect in T cell proliferation [206]. The αM and αL β2 integrins are involved in the functions of myeloid and lymphoid cells respectively [203] and the αDβ2 integrin is expressed on eosinophils [207].

CD23 interacts with the α chains of the leukocyte integrins αMβ2 (CD11b/CD18) and αXβ2 (CD11c/CD18) [148]. Liposomes coated with CD23 were found to bind to monocytes and CD23 was found to interact with these integrins on macrophages [148]. CD23 appears to

recognise an epitope on CD11b and c with similarity to factor X as this ligand inhibited the CD23 interaction. Fibrinogen did not decrease the interaction therefore it is not likely that binding occurs via the RGD motif. The interaction is likely to be cation dependent as Ca^{2+} chelation by EDTA decreased binding of CD23 to monocytes and addition of Ca^{2+} led to a dose-dependent increase in monocytes binding. Tunicamycin (glycosylation disrupting agent) decreases the interaction between CD11b/c and CD23 and therefore CD23 may be acting like a C type lectin recognizing sugars on the integrins to facilitate binding [148].

sCD23 was shown to be capable of inducing proinflammatory cytokine synthesis in monocytes via the CD11b and CD11b integrins. This increased production of IL-1β, IL-6 and TNF-α occurred in response to CD23 ligation of CD11b/c in a nitric oxide synthase (NOS) dependent manner [208]. The signalling pathways of this mode of cytokine production is not yet well defined.

1.6.7b CD23 interaction with alpha v (αv) integrins

The αv family of integrins (vitronectin receptor family) include the αv subunit paired with either β1, β3, β5, β6 or β8. All of these group members bind to the RGD sequence found in substrates including vitronectin, fibronectin, osteonectin, fibrinogen, von Willebrand factor and thrombospondin. Apart from αvβ6 all the integrins bind vitronectin.

Some cell types including embryonic fibroblasts [209], smooth muscle cells, [210], neural crest cells [211] and melanoma cells [212] express up to three αv heterodimers which could potentially act as vitronectin receptors. This apparent redundancy could reflect the same ligand binding to different receptors to facilitate the transmission of separate signals to invoke various cell behaviours. An example of this is seen in the Chinese hamster ovary cell line which expressed the α5β1 fibronectin receptor and does not undergo apoptosis when plated on fibronectin following withdrawal of serum. In the same cells αvβ1 is also expressed where is also acts as a receptor for fibrinogen but does not have the same effect of protection from apoptosis [213]. In addition, co-operation between integrins has been observed where αvβ5-expressing FG carcinoma cells which bind to but do not migrate on vitronectin were able to

migrate on vitronectin but variants of this cell type which expressed αvβ3 were capable of migration on vitronectin. Further, addition of epidermal growth factor (EGF) to an αvβ3-deficient FG carcinoma cells allowed vitronectin migration via an αvβ5-mediated signalling event through PKC (protein kinase C). This suggests that ligation of the αvβ3 integrin produced a signal not generated through αvβ5 that could be replicated by activation of PKC [214]. In the differentiation of oligodendroglial cells the relative levels of αvβ1 and αvβ5 alter, perhaps indicating a role for particular integrins at defined windows in cell development [200]. These integrins are therefore capable of acting alone or in conjunction with other integrins or environmental cues to modify cellular behaviours/functions [212].

Out of all the vitronectin receptors, αvβ3 and αvβ5 are the best described in terms of function. αvβ5 appears to mediate spreading only on vitronectin [211,214] whereas αvβ3 can mediate migration via a range of substrates including fibronectin, vitronectin, fibrinogen, laminin, osteopontin and collagen [210,215,216]. In addition the cytoplasmic tail of β5 does not appear to be able to convey growth promoting signals following ligation but β3 has been implicated in providing positive growth signals to melanoma cells [217-219]. This evidence makes αvβ3 a prime candidate for a major role in tumour progression and metastasis [212].

CD23 interacts with the vitronectin receptor αvβ3. αvβ3 is associated with CD47 (also called Integrin Associated Protein – IAP), a 50kDa 5-transmembrane protein to form the αvβ3 signalling complex. This complex regulates leukocyte activation and mediates the phagocytosis of aging apoptotic leukocytes. This function resolves inflammation by removing these leukocytes before they disgorge their potentially harmful contents [220]. The highest expression levels of αvβ3 are found on osteoclasts, specialised macrophage variant cells that degrade mineralised tissue and are responsible for the modelling and remodelling of bone [221]. Osteoclasts express both αvβ5 and αvβ3 integrins and it has been demonstrated by Inoue et al that this expression is developmentally regulated. The sole integrin expressed on these cells at a precursor stage is αvβ5 and this integrin is replaced by αvβ3 as differentiation progresses [222].

Studies of $\beta3^{-/-}$, $\beta5^{-/-}$ and $\beta3^{-/-}/\beta5^{-/-}$ mice reveal abnormalities in angiognesis but not lymphopoiesis [223] which suggests $\alpha v\beta5$ does not have a role in murine lymphocyte development. The CD23-$\alpha v\beta5$ interaction may well be exclusive to humans – although murine CD23 does act as a receptor for IgE it is devoid of cytokine activities and apparently does not bind integrins. Likewise, the functions of human and murine IL-7 in lymphopoiesis are dissimilar [1,224] and murine stromal cells have been found to be inferior to human stromal cells in sustaining human B lymphopoiesis *in vitro* [225].

1.7 Multiple Myeloma

Multiple myeloma accounts for 10% of malignant blood diseases. It is defined by the clonal proliferation of malignant plasma cells in the bone marrow compartment, secretion of monoclonal antibodies, and inhibition of normal antibody synthesis and of normal differentiation. The malignant plasma cells found in the bone marrow originate from lymph nodes and then migrate across the endothelium of bone marrow sinuses to the bone marrow environment where they can interact with bone marrow stromal cells [226]. This interaction with stromal cells is crucial to the homing and survival of these cells in the bone marrow. It is also found that osteoclast activity is increased in areas close to the multiple myeloma cells and patients often exhibit skeletal destruction [227].

This migration is though to be mediated via the action of the chemokine SDF-1 (stromal cell-derived factor 1) and its receptor CXCR4. Stromal cells constitutively secrete SDF-1 at high levels and this chemokine is a chemotactic factor for many cells including haematopoietic progenitor cells [228]. CXCR4 has a role in B cell migration and proliferation and is expressed by endothelium and myeloma cells but not by marrow stromal cells [229]. It is thought that SDF-1 regulates the migration of myeloma cells by transiently upregulating VLA-4 ($\alpha4\beta1$)/VCAM-1 inducing cell adhesion to the endothelium and contributing to the trafficking of multiple myeloma cells [230]. Other chemokine receptors expressed by multiple myeloma cells are CXCR3, CCR1, CCR5 and CCR6. These receptors are ligands for MIP-1α, MIP-1β, CXC and RANTES. These chemokines, along with the "homing" chemokine SDF-1, have a role to play in the processes of tumour growth and bone destruction in multiple myeloma [231].

1.8 CD47

Multiple myeloma cells have been found to undergo apoptosis when exposed to an antibody directed against CD47 [232]. A bivalent single chain antibody fragment directed against CD47 has been put forward as a potential anti-tumour treatment in conjunction with conventional chemotherapy due to its induction of apoptosis via a caspase-independent manner. The model used by Kikuchi et al (grafting of CD47-expressing human KPMM2 cells into SCID (Severe Combined Immunodeficiency) mice) showed that this molecule can be used with chemotherapy as the cells do not possess Fc and therefore do not rely on induction of apoptosis with assistance from other immune cells. During chemotherapy these cells would be in short supply and therefore success of treatment would be greatly reduced [232].

Ligation of CD47 also promoted caspase-independent apoptosis in cells such as T cells and B-CLL [233,234]. The cell death induced by CD47 in B-CLL was characterised by cell shrinkage, exposure of phosphatidylserine (PS), and mitochondrial matrix swelling in the absence of nuclear degradation [233]. It was then further elucidated that there is loss of mitochondrial membrane potential and generation of reactive oxygen species (ROS) but this does not result in release of cytochrome c or apoptosis inducing factor (AIF) from the mitochondria [235]. In addition, the CD47 ligand thrombospondin and anti-CD47 antibodies caused apoptosis-like cell death in breast tumour cells [236], monocytes and dendritic cells [237] and certain fibroblasts [238]. Mateo et al found that blood cells susceptible to CD47-induced cell death included U937 cells (monocytes), Jurkat cells (T cells) and RPMI8866 (lymphoblastoid cells) [234]. Further, on immune cells, ligation of CD47 by soluble mAbs resulted in inhibition of cytokine release from dendritic cells [239] and, when immobilized, CD47 mAb costimulated T-cell receptor (TCR) activated T cells [240]. How CD47 acts appears to be influenced by the way it is engaged, the surface molecules it interacts with and the cell type it is expressed upon [241,242].

CD47 (also known as IAP – integrin associated protein) is a member of the Ig superfamily and runs on SDS-PAGE at 45-55 kDa. This broad migration is due to heavy glycosylation of the extracellular IgV (V for variable) domain. CD47 is a transmembrane type I glycoprotein (C terminus is cytoplasmic and N terminus is extracellular) [243] which spans the membrane 5

times and is ubiquitously expressed on haematopoietic cells and non-haematopoietic cells [244]. It acts as a receptor for thrombospondin [245] and for SIRPα (transmembrane signal recognition particle) [246]. Through its interaction with β1, β2 and β3 integrins it modulates processes such as cell motility, leukocyte adhesion, phagocytosis and platelet activation via heterotrimeric G protein signalling [243]. The complex formed between CD47 and the integrin has been described as an "ad hoc" seven-transmembrane receptor composed of two membrane-bound domains from the heterodimeric integrin and the five membrane spanning CD47 domains [247]. As previously mentioned, CD47 forms a signalling complex through an association with the αvβ3 integrin and on monocytes this complex binds to CD23 and results in cytokine synthesis [114]. The apoptotic effects of CD47 ligation seen in B-CLL cells are not thought to be mediated by the αvβ3/CD47 complex however, this is though to be distinct from integrin binding as ligation of αvβ3, αvβ5 or CD23 with their respective immobilised antibodies did not induce apoptosis [233]. In B-CLL cells treated with anti-CD47 the resultant apoptosis could not be reversed by addition of survival factors such as IL-4 or IFN-γ. These are "rescuing" cytokines that do not function this way in this context [233].

1.9 Work leading up to the project

The Cushley laboratory has recently discovered an interaction between CD23 and αvβ5. The interaction between sCD23 and αvβ5 is implicated in the inhibition of apoptosis of pre-B cells and is independent of the classical RGD recognition site on the integrin as RGDS peptide did not inhibit the interaction. The region of CD23 which binds to the integrin contains the motif RKC (Arg-Lys-Cys). Peptides derived from CD23 containing this motif were able to bind cells expressing αvβ3 and αvβ5 and to αvβ3 and αvβ5 alone. These new data suggest an alternative recognition site on CD23 and a further role for this multi-functional protein.

A library of overlapping nonapeptides derived from CD23 was produced and several of these peptides (#9-12) were found to bind to the pre-B cell line SMS-SB. This cell line does not express any of the known CD23 receptors and affinity isolation identified the αvβ5 integrin as the receptor on these cells. αvβ5 is expression is limited to B cell precursors and

universally expressed on acute lymphocytic leukaemia (ALL) cells. B-CLL (B-chronic lymphocytic leukaemia) cells do not express αvβ5 (figures 8 and 9). In addition to this identification of a new CD23 receptor it was found that binding of CD23 to the integrin was via a novel motif, the RKC motif. Classical integrin recognition is via the RGD motif and the RKC motif was discovered following collection of evidence that RGD was not involved – RGDS peptide did not inhibit the interaction and the active site on CD23 did not contain the RGD sequence. This motif is located at a site distinct from those of the IgE binding site, the CD21 binding site and the oligomerisation site (figure 10).

Soluble CD23 has been shown to influence growth of SMS-SB cells in a dose-dependent manner (figure 11). It was found the CD23 peptides containing the RKC motif exhibited biological activity. When SMS-SB cells are seeded at low densities (5 X 10^4 cells/ml or less) they will undergo apoptosis. As previously stated, the SMS-SB cells are adapted to culture in protein free hybridoma medium by virtue of the production of an unidentified autocrine factor. It has been determined that this autocrine factor is not CD23 [110]. Seeding of these cells at such low densities removes the autocrine factor and places the cells under stress and it is under these conditions that "rescue from apoptosis" treatments can be assessed. Use of peptides containing the RKC motif leads to significant increase in proliferation and this mimics the effect of full length sCD23.

1.10 Research Aims

One of the aims of this thesis was to examine the interaction between stromal cells and SMS-SB cells in terms of CD23 and αvβ5. It has been found previously that hMSCs (human marrow stromal cells) support the growth of SMS-SB cells in protein-free medium and it was intended to test the importance of the interplay between CD23 from stromal cells and αvβ5 expressed on the B cell precursors.

Another aim of this thesis was to examine the effects of the CD23-derived peptides on pre-B cell lines other than SMS-SB and also multiple myeloma cell lines. These cells were to be assessed for their expression of CD23 receptors and response to peptides in terms of binding and proliferation in order that information could be gathered to determine whether response to CD23-derived peptides was dependent upon the CD23 receptor(s) expressed or the developmental stage of the cell line being examined.

The final aim of this thesis was to investigate the effects of anti-CD47 antibodies on a range of cell types at varying stages of development. It was hoped to further expand upon work already in the literature which has found that some cell types undergo apoptosis upon ligation of CD47 and others are resistant to such apoptosis. The cell types in question were to be assayed for their expression of CD47 and its signalling partner, the CD23 receptor, αvβ3. It was hoped that such information could enable the identification of a pattern between induction of apoptosis/resistance to apoptosis and presence/absence of αvβ3 or whether the response depends upon developmental stage.

Figure 1.8. **αvβ5 expression.** Graph produced from FACS data showing expression of αvβ5 integrin on various leukaemia cells. It can be seen that only B-CLL cells do not express this integrin.

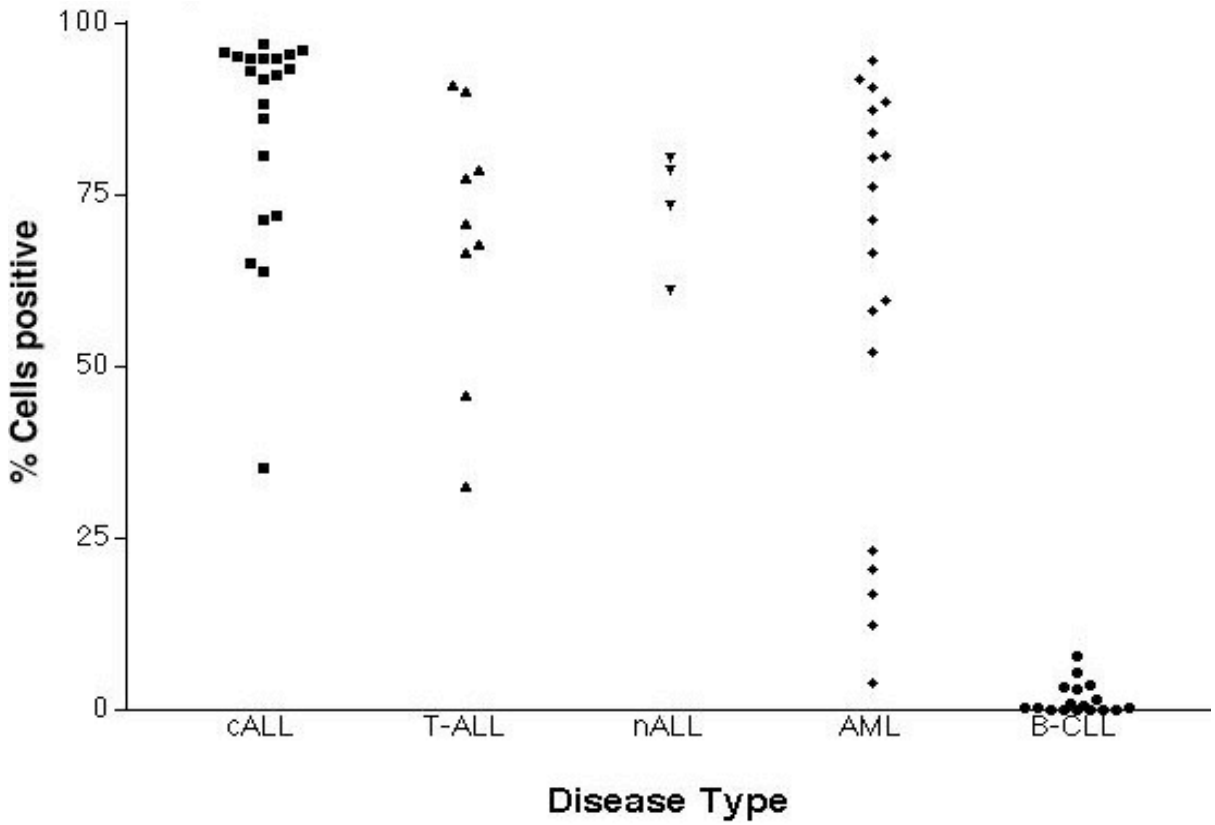
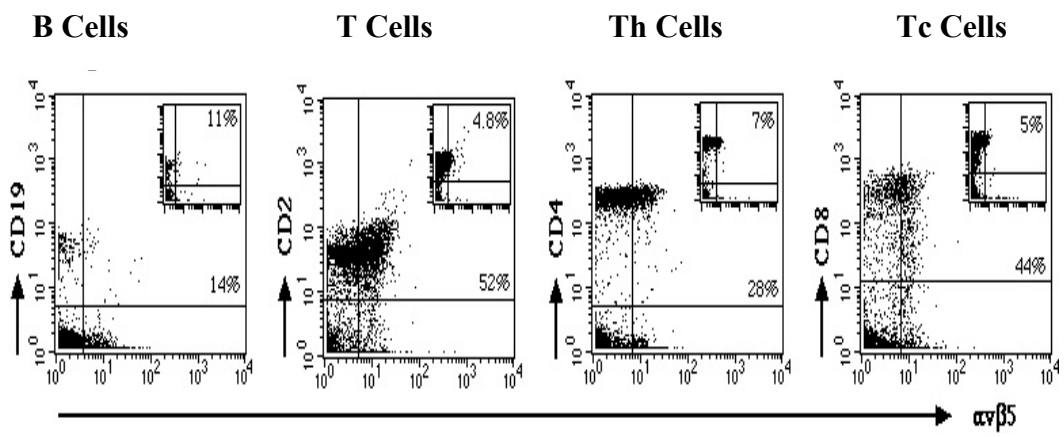

100
75
50
25
0
% Cells positive
cALL
T-ALL
nALL
AML
B-CLL
Disease Type

Figure 1.9. αvβ5 expression. FACS staining showing expression of αvβ5 on non-leukaemic peripheral blood and bone marrow. B cell precursors express αvβ5 but mature B cells do not.

Peripheral Blood Cells



Precursor B Cells

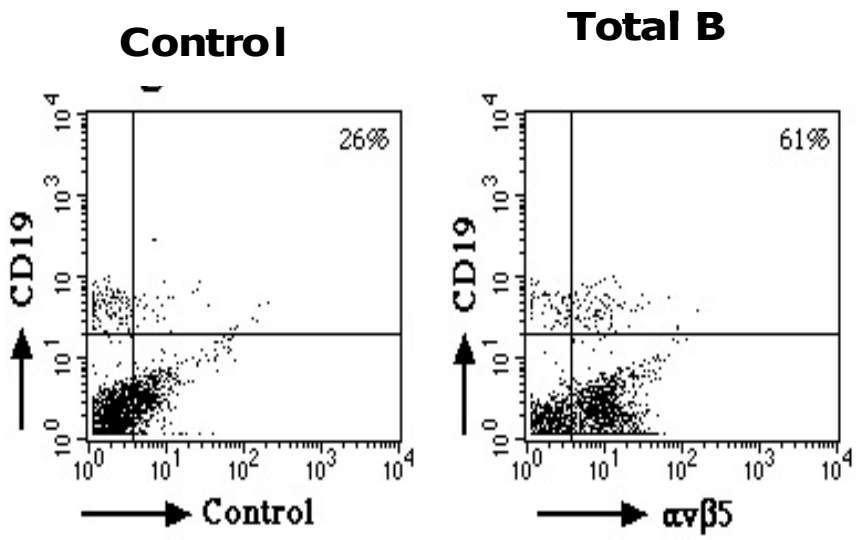


Figure 1.10. CD23 and its binding sites [248]. Cartoon of full membrane-bound CD23 trimer (a) and ribbon diagram of the head domain showing binding sites of IgE, CD21 and αvβ5 integrin (b). The αvβ5 binding site is the RKC motif.

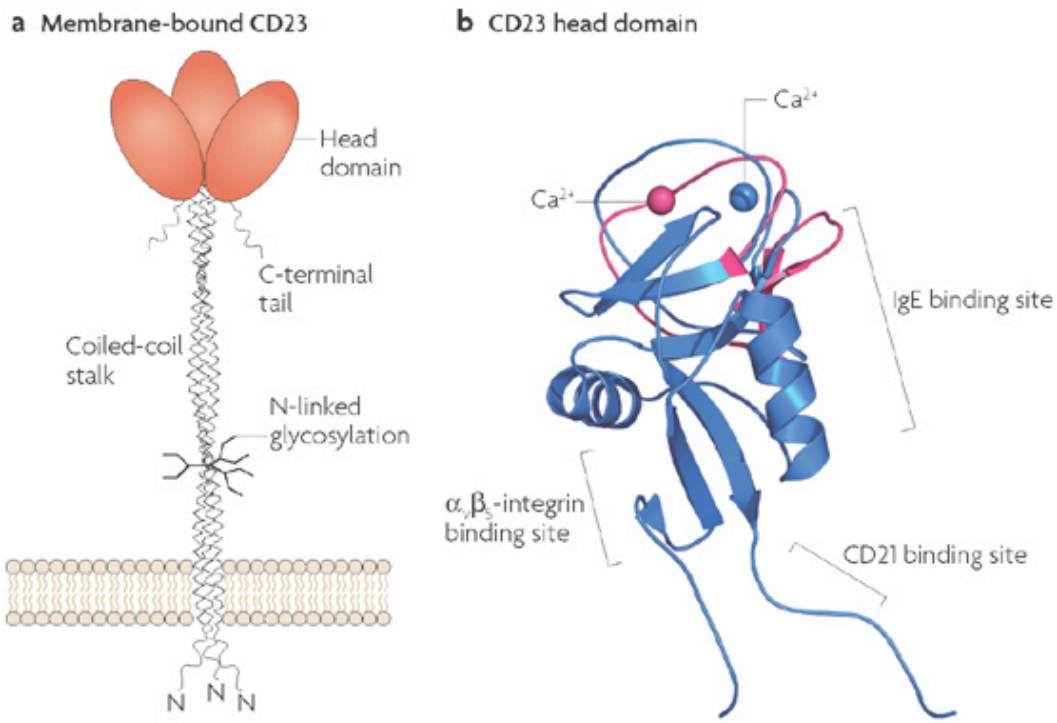


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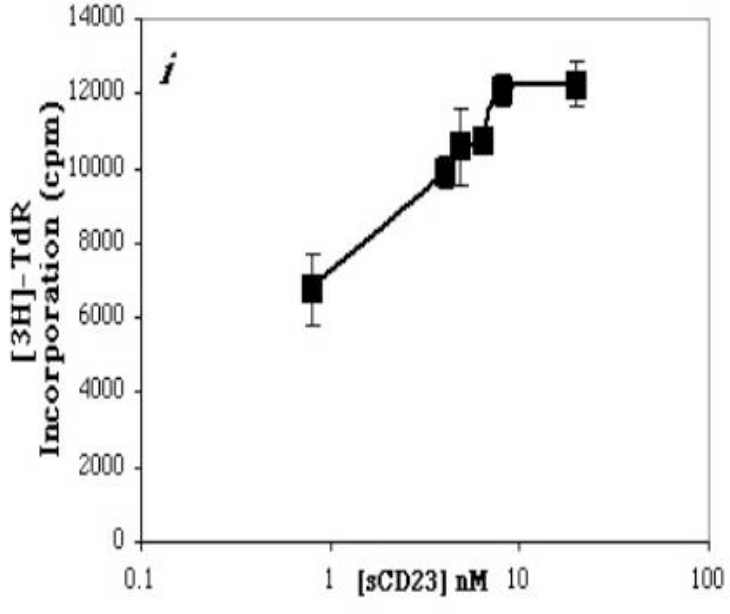
Figure 1.11. **Effect of sCD23 on SMS-SB cell proliferation.** Data from a tritiated thymidine incorporation proliferation assay. It can clearly be seen that proliferation increases with increasing concentrations of sCD23. At 10nM the proliferation rate reaches a plateau and proliferation no longer depends on raising sCD23 concentration.


i
14000
12000
10000
8000
6000
4000
2000
0
[3H]-TdR
Incorporation (cpm)
0.1
1
[sCD23] nM
10
100

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Unless otherwise stated all chemicals were purchased from Sigma, Poole, Dorset.

2.1.1 General chemicals and materials

Bovine Serum Albumin DMSO (Dimethyl Sulphoxide) Propidium Iodide	Sigma, Poole, Dorset
Supersignal West Pico western blotting detection system	Perbio Science UK Ltd, Cramlington, UK
Protein multicoloured standards	Invitrogen, paisley UK
Annexin V – FITC	PharMingen International
NOVEX 4-12% polyacrylamide gels and gel tanks including blotting module and 20X MES Running buffer	Invitrogen, Paisley, UK
TRIZOL reagent	Sigma, Poole, Dorset
RNase free plasticware	Thistle Scientific, Glasgow UK

2.1.2 Cell culture materials and reagents

G418	Calbiochem-Novabiochem, Nottingham, UK
Tissue culture flasks Disposable cell scrapers 96 well plates 60mm cell culture dishes	Corning Costar, Birmingham, UK
RPMI-1640 medium Dulbecco's Modified Eagle's Medium (DMEM) Trypsin Trypan blue L-glutamine Penicillin/streptomycin	Sigma, Poole, Dorset, UK
Haemocytometer	Philip Harris Scientific, Lanarkshire, UK
Protein-Free Hybridoma Medium II (PFHM II)	Gibco, Invitrogen, Paisley UK

DOTAP liposomal transfection reagent (1,2-Dioleoyl-3-Trimethylammonium-Propane (Chloride Salt)	Boehringer Mannheim Ltd, Sussex, UK
Foteal Calf Serum (FCS)	TCS Cellworks Ltd, Buckingham UK
Cryovials	Corning Costar, Birmingham, UK
25ml pipettes 10ml pipettes Cell scrapers 96 well plates (flat bottom) 75cm flasks 25cm flasks	Corning Inc., Costar, BirminghamUK

2.1.3 Antibodies

Mouse anti-human CD23 Clone BU38 (Inhibits IgE binding)	Alexis Corporation UK, Nottingham, UK
Mouse anti-human CD23 Clone ML233 (binding site unknown)	
Mouse anti-human Akt (PKB) (cat #2966)	New England Biolabs, Hitchin, Herts UK
Rabbit anti-human phospho-Akt (PKB) (Ser473 cats #4058 and 9271)	New England Biolabs, Hitchin, Herts UK
Rabbit anti-human Akt (PKB) (cat #9272)	New England Biolabs, Hitchin, Herts, UK
Mouse anti-human αvβ5 Clone P1F6 (precise binding site unknown but inhibits binding of HT29 cells, carcinoma cells and myeloma cells to vitronectin.)	Upstate (Now Millipore),Chandlers Ford, Hampshire UK
Mouse anti-human αv Clone AMF7	Chemicon (Now Millipore), Chandlers Ford, Hampshire UK
Mouse anti-human αvβ5 Clone 15F11 (precise binding site unknown but does not block ligand binding or adhesion)	Chemicon (Now Millipore), Chandlers Ford, Hampshire UK
Mouse anti-human αvβ3 Clone 23C6 (precise binding site unknown but inhibits binding in adhesion assays)	Chemicon (Now Millipore), Chandlers Ford, Hampshire UK
Mouse isotype control IgG1	Sigma, Poole, Dorset, UK
Mouse isotype control IgG2a	Sigma, Poole, Dorset, UK
Goat anti-mouse immunoglobulins PE conjugate	Dako, Ely, Cambridgeshire, UK
anti-rabbit HRP conjugate Cat#A6154	Sigma, Poole, Dorset, UK

2.1.4 Cell Lines

BLIN-1	Pre-B cell stage of development, abnormality in chromosome 9p.
NALM-6	Human pre-B cell line established from ALL (acute lymphocytic leukaemia) patient. T(5;12) (translocation of chromosomes 5 and 12)
SMS-SB	Pre-B cell line established from ALL patient. c-fos overexpressed.
697	Pre-B ALL cell line t(1;19)
BAF03	Mouse pro-B cell line. Dependent upon IL-3.
RPMI8866	EBV transformed lymphoblastoid cell line from CML patient.
hMSC-TERT	Bone marrow stromal cells immortalised with the catalytic subunit of telomerase [249].
KMS11	Multiple myeloma cell line. t(4;14)
H929	Multiple myeloma cell line (t4;14) FGFR (fibroblast growth factor receptor) negative
HEK293	Human embryonic kidney cells.

2.1.5 Plasmids

CD23a	Lab stocks
CD23b	Lab stocks

2.1.6 CD23 Peptides - Synthesized by Mimotopes. CD23 sequence (M^{151}-S^{321}). RKC sequence shown in bold. Peptide sequences indicated by black lines.

MELQVSSGFVCNTCPEKWINFQ**RKC**YYFGKGTKQWVHARYACDDMEGQLVSIHSP
EEQDFLTKHASHTGSWIGLRNLDLKGEFIWVDGSHVDYSNWAPGEPTSRSQGEDCV
MMRGSGRWNDAFCDRKLGAWVCDRLATCTPPASEGSAESMGPDSRPDPDGRLPTP
SAPLHS

Peptide 9 sequence	KWINFQRKC
Long Peptide sequence	KWINFQRKCYYFGKG
Peptide 8 sequence	PEKWINFQR
Peptide 58 sequence	GSGRWNDAF

2.2 Methods

2.2.1 Culture of Human B Lymphocytes

SMS-SB cells were routinely maintained in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS) (heated at 57°C for 50 minutes), 2mM L-glutamine and 50 units/ml penicillin and 50μg/ml streptomycin. These cells were also cultured in Protein-Free Hybridoma Medium II (PFHM II). It should be noted that when adapting these cells to PFHM II this must be done by passaging every 2 days by adding 10ml of cells growing in complete RPMI to 10ml of PFHM II. After 7 of such passages the cells are fully adapted to PFHM. If these cells are taken from complete media culture, centrifuged and resuspended in PFHM II immediately then the cells will become strongly adherent to plastic and their morphology will resemble apoptotic cells.

All other B cells were cultured in supplemented RPMI-1640 medium only. Cells were sub-cultured every 2-3 days depending on experimental demands. Cells were cultured in 75cm^2 flasks at 37°C in a humidified 6% CO_2 incubator and manipulated aseptically in a Laminar flow hood.

2.2.2 Culture of non-B cell lines

hMSC-TERT cells were routinely maintained in Dullbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FCS, 2mM L-glutamine and 50 units/ml penicillin and 50μg/ml streptomycin. Cells were sub-cultured at a 1 in 10 split every week using trypsin (0.25 w/v) in PBS. HEK293 cells were detached from the flask once per week and subcultured at a split of 1 in 10. All cells were cultured in 75cm^2 flasks under the same conditions as the B cells. Transfected HEK cells were revived from liquid nitrogen stocks and maintained in the presence of 70μg/ml G418.

2.2.3 Frozen Cell Stocks

Frozen stocks of each cell line routinely used in culture were stored long-term in liquid nitrogen. 10^7 logarithmically growing cells were centrifuged and resuspended in 1ml of freezing medium (90% (v/v) heat inactivated FCS and 10% (v/v) dimethyl sulphoxide (DMSO) and quickly transferred to cryovials. The vials were kept at –70°C for 3-4 days before being conveyed to liquid nitrogen. Cells being taken from liquid nitrogen were allowed to thaw and then washed in 10ml of appropriate medium to eliminate any remaining DMSO. The cells were then resuspended in medium and placed into culture flasks where they were allowed to recover prior to any experimental use or manipulation.

2.2.4 Flow Cytometry

Flow cytometry was performed using a Becton Dickinson FACScan Flow Cytometer fitted with an argon laser. Samples were prepared in 5ml FACS tubes.

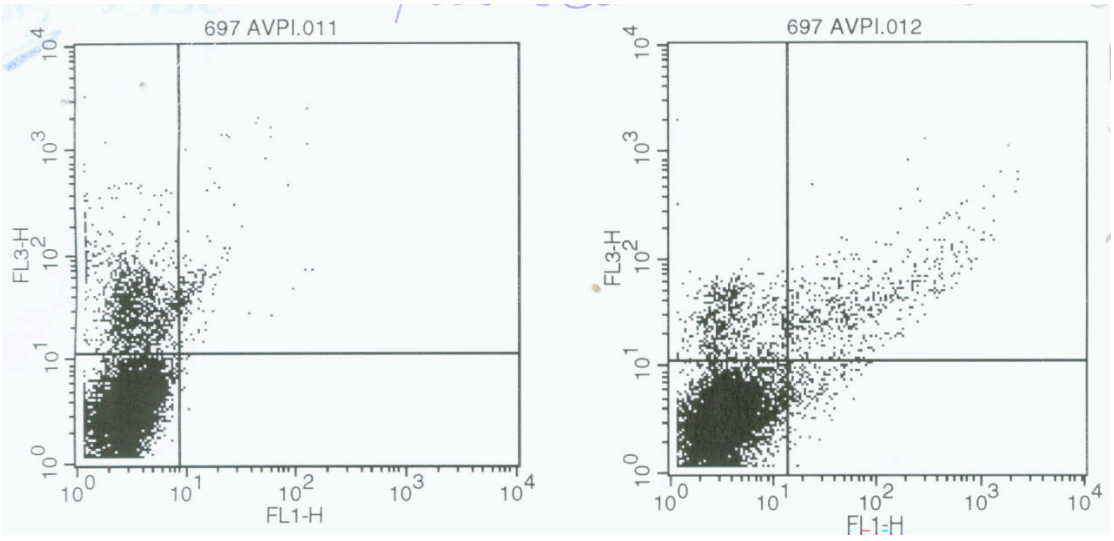
2.2.5 Cell Phenotyping

5µl of antibody (at appropriate concentration and either unlabelled or flurophore conjugated) was added to the cells and incubated in the dark for 30-60 minutes. The samples were washed twice in cold PBS and, if necessary (i.e. for unlabelled primary antibodies), 5µl of secondary flurophore-conjugated antibody was added and the samples incubated for a further 30-60 minutes. Cells were then washed a further two times in cold PBS and resuspended in 300µl of PBS for analysis on the FACScan. Cytometer channel FL1 (525nm) was used to detect FITC fluorescence against cell count and FL2 (575nm) was used to detect PE fluorescence. FL3 (>630nm) was used to detect Cy5.

2.2.6 Annexin V/Propidium Iodide Staining

Ninety-six well flat-bottomed plates were coated with 10µg/ml antibody in 100µl 0.1M $NaHCO_3^-$ pH9 and left overnight at 4-8°C to immobilise. The following day the plates were washed and blocked with culture medium. 4 X 10^6/ml cells in 100µl were added to the wells and the soluble antibodies/treatments were also added and incubated at 37°C for 16 hours. The cells were transferred to FACS tubes, washed twice in ice-cold PBS and resuspended in binding buffer to a concentration of 2 X 10^6/ml in 200µl. 100µl of this was taken into a separate FACS tube and added to this was 0.5µg/ml Annexin V-FITC and 0.5µg/ml propidium iodide. The samples were then incubated at room temperature in the dark for 15 minutes, resuspended in 400µl of binding buffer and analysed on the FACScan within the hour. For Annexin V-FITC staining cytometer channel FL1 (525nm) was used and FL3 (>630nm) was used for detection of propidium iodide.

All cells were assayed by flow cytometry for FL1/FL3 (no gating of populations/elimination of debris).
Quadrants were applied as follows on the flow cytometric data from the above assay:-



Single stained for propidium iodide Single stained for annexin V

2.27 Proliferation Assay

This assay was carried out in 96-well plate format. Treatments were carried out in triplicate and are as detailed in separate figures. Cells were added at densities of 2,000 – 10,000 per

well in a volume of 80µl bringing the total volume to 100µl. The plates were incubated for 3 days at 37°C in a humidified incubator. Cells were then pulsed with 0.3µCi/well ^{3}H-thymidine for 16-18 hours, following standard radioactivity safety procedures, then harvested to a filtermat, dried and counted in scintillation counter. Mean counts per minute (CPM) were calculated for triplicates and graphs constructed using Microsoft Excel. Statistical analysis was performed by way of a Student's T test and asterisks were applied where p values were <0.5 when comparing like for like.

2.2.8 Transfection Assay

All transfections were carried out using the DOTAP transfection reagent (Boehringer Mannheim Ltd). Cells were sub-cultured into 60mm culture dishes and grown overnight to 60-70% confluence. The cells were then transfected with 5µg of plasmid DNA plus 70µl DOTAP transfection reagent in accordance with the manufacturer's instructions. Fresh medium was supplied 24 hours post-transfection. Selection in 500µg/ml G418 sulphate for plasmids containing the neomycin resistance gene began 48 hours later. Cells which had been successfully transfected survived the antibiotic selection and were then further sub-cultured.

2.2.9 Fluorescent Microscopy

20mm diameter coverslips were placed into wells of 6-well plates and seeded onto these was approximately 10^{5} cells. The cells were incubated for 2-3 days and then the coverslips were rinsed three times in ice-cold PBS. Primary antibody was then added to the cells and incubated at room temperature for 30 minutes. The coverslips were then rinsed again three times and secondary antibody added. After a further 30 minute incubation at room temperature the coverslips were rinsed three times and then examined using a fluorescent microscope.

2.2.10 Cell Lysate Preparation

Cells were washed twice in ice-cold PBS and resuspended in RIPA buffer (see Appendix) to a concentration of 5 X 10^7/ml. The cells were then left on ice for 30 minutes and then the samples were centrifuged at 13,600g for 15 minutes. The supernatant was collected and then centrifuged for a further 15 minutes. The supernatant was then aliquotted for freezing at -20°C. When preparing lysates from time-course experiments, the samples were placed into RIPA buffer without detergents, centrifuged and the pellets resuspended in RIPA buffer for 30 minutes etc as above.

2.2.11 Immunoprecipitation

1μg of antibody was added to 100μl of cell lysate and gently mixed. The cells were then left on ice for 30 minutes before addition of 30μl of protein G-sepharose beads in RIPA buffer without detergents. The samples were incubated overnight at 4-8°C on a rotary shaker. The next day the beads were pelleted by centrifugation in a microfuge at 3000g and the supernatant discarded. The beads were washed five times with RIPA buffer and then suspended in 20μl of 2X protein loading dye (see Appendix), vortexed and boiled for 5 minutes. The supernatant was then either frozen or used in SDS-PAGE.

2.2.12 SDS-PAGE Gel Electrophoresis, Western Blotting and ECL detection

2.2.14a SDS-PAGE

Reducing sample buffer (see Appendix) was used in a 1:1 ratio with sample before loading onto the gel. NOVEX pre-cast gels were used in conjunction with NuPAGE® MES SDS running buffer (Invitrogen). The gel was electrophoresed at 200V for approximately 45 minutes in this running buffer.

2.2.12b Western Blotting

The protein was transferred from the gel to nitrocellulose in transfer buffer at 30V for one hour and then blocked for one hour in PBS/10% marvel (w/v). The blot was then washed 5 times over one hour in PBS/0.1% Tween 20 (v/v). Primary antibody was diluted to the

optimum concentration determined in PBS/0.1% Tween (v/v)/5%BSA (w/v) and incubated overnight with shaking. The blot was then washed 5 times over one hour in PBS/0.1% Tween 20 (v/v). Secondary antibody was then added and the blot incubated for one hour with shaking and washed a further 5 times.

2.2.12c ECL Detection

Pierce Supersignal West kit was used according to the manufacturers instructions. Solutions 1 and 2 were mixed in equal proportions and added to the blot in a dish and the blot submerged for one minute. Excess liquid was removed from the blot and the blot was placed inside a development cassette and exposed to Kodak film in the darkroom for between 10 seconds and 1 hour, depending on the strength of signal, to detect antibody binding.

2.2.13 RNA Isolation

RNA was isolated from cells using TRIZOL reagent according to the manufacturer's instructions using Rnase-free pipette tips and plasticware. Briefly, the pelleted cells ($2.4X10^7$) were washed once with 1XPBS then lysed with 0.2ml TRIZOL per 10^6 cells by pipetting the cells. Chloroform was then added (0.1ml per 1ml of cell homogenate) and the samples were shaken vigorously for 15 seconds and put on ice for 5 minutes. The samples were then centrifuged at 12,000 g for 15 minutes at 4°C. Following centrifugation the homogenate forms two phases; a lower phenol-chloroform phase and an upper aqueous phase where the RNA is found. This phase was carefully removed and transferred into a fresh tube and added to this was an equal volume of isopropanol and this was left on ice for 15 minutes to allow the RNA to precipitate. The sample was then centrifuged as before and the resultant pellet was washed with 75% ethanol, air dried and resuspended in 30μl of RNase- free water. Samples were then either analysed or stored at -80°C prior to analysis. Before use, sample concentrations were determined by spectrophotometer readings at A_{260} and A_{280}. Samples had A260/280 ratios greater than 1.9 indicative of DNA and protein-free preparations.

2.2.14 PCR (polymerase chain reaction)

The following oligonucleotides were used as PCR primers for detection of CD23 mRNA:

Forward:
5' CGT GTA CGG TGG GAG G 3'
Reverse:
5' CTT CGT TCC TCT CGT TCA ATT C 3'

and for GAPDH as control:

Forward:
5' TCC ACC ACC CTG TTG CTG 3'
Reverse:
5' ACC ACA GTC CAT GCC ATC AC 3'

PCR reactions were performed using a Promega RT-PCR kit. Reactions were carried out in 0.5ml microcentrifuge tubes and were performed in a Techne Genius PCR machine. After PCR, the samples were analysed on a 1% (w/v) agarose gel.

Reaction mixture:

mRNA	1ug
upstream primer	$50mol^{-1}$
downstream primer	$50mol^{-1}$
DNTP mix	1μl
AMV/Tfl 5X buffer	10μl
DNA Pol Tfl	2μl
AMV Reverse Transcriptase	1μl

dH20 to make up volume to 50µl

Cycle parameters:

94°C 5 minutes	1 cycle	
94°C 30 seconds		(denaturation)
62°C 30 seconds	30 cycles	(annealing)
72°C 1minute		(extension)
72°C 1 minute		
72°C 10 minutes	1 cycle	

4°C HOLD

CHAPTER 3

RESULTS

ANALYSIS OF HUMAN BONE MARROW STROMAL CELLS AND SMS-SB CELLS

3.1 Introduction

Human Marrow Stromal Cells (hMSCs) are found in the bone marrow and contribute to the microenvironment which supports early B cell development from pluripotent stem cells [1]. Stromal cells express cytokines such as IL-7 [26], Flt-3 ligand [250] and SDF-1 [43] which have a positive effect on the progression of B cell development and TNF-α, TGF-β and IFNγ which have a negative effect on B cell development. In addition, stromal cells are believed to express CD23 [249], which has a supportive effect on the growth and proliferation of precursor B cells. Some precursor B cells have been found to express the integrin αvβ5 which acts as a receptor for CD23 and it is proposed that in precursor cells which express αvβ5 as the sole CD23 receptor the interaction between the two proteins has a supportive effect on growth, whether this effect is due to enhanced proliferation or suppressed apoptosis.

3.1.1 hMSCs and SMS-SBs

It is possible to plate out approximately 5,000 hMSCs per well in 96-well plate format and to use this layer of cells as a "feeder layer" upon which to grow SMS-SB cells. SMS-SB cells are pre-B cell-like and, as discussed earlier, overexpress the oncogene *c-fos* and express cytoplasmic μ heavy chains but not light chains [101]. These cells have been found to survive under culture conditions of growth in protein-free hybridoma medium and do not require additional protein growth factor supplements. The reason for this unusual characteristic is that the cells produce an autocrine factor which, when expressed at an adequate level, sustains the cells in protein-free medium. In order to perform experiments with these cells it is necessary to remove the influence of this autocrine factor either by centrifugation and medium replacement, or by seeding the cells at extremely low densities. SMS-SB cells treated in this manner will certainly die unless rescued by specific experimental treatments.

It has been discovered that "seeding" very low densities of SMS-SB cells onto the "feeder layer" of hMSCs described above allows growth of the SMS-SB cells, resulting in wells full of SMS-SB cells after approximately two weeks. This has been observed to be the case when cells are seeded as low as 5 cells per well. When the cells are at this very low density the time taken for the SMS-SBs to reach confluence is longer than, for example, cells seeded at

100 cells/well. From this it is clear that the stromal cells are providing the SMS-SB cells with all the signals they require for growth and proliferation and in addition it has been observed that the stromal cells enjoy better viability in the presence of the SMS-SB cells (stromal cells plated in protein-free medium alone have been observed to lose adherence and do not survive). The cells are displaying symbiotic behaviour and appear to benefit hugely from the presence of each other – especially in the case of the SMS-SB cells, which face rapid cell death in the absence of survival signals. To determine whether this benefit to the SMS-SB cells was due to secreted factors conditioned medium from stromal cells was used to culture the SMS-SB cells but it was found that growth was not supported (data not shown).

3.2 Results

3.2.1 SMS-SB Cells

As previously stated, the SMS-SB cells do not express any CD23 receptors other than the integrin αvβ5. This means that any effect CD23 has on these cells has to occur via this receptor.

Figure 3.1 shows the results of flow cytometry performed with SMS-SB cells. It can be seen that the SMS-SB cells stain positive for αvβ5 (antibody P1F6) and negative for αvβ3 (antibody 23C6). If the SMS-SB cells are interacting with the stromal cells in a growth-affecting manner it has to be via the only CD23 receptor expressed on these cells, i.e. the integrin αvβ5. Other CD23 receptors - β2 integrins (found on monocytes), CD21 and αvβ3 are not found on SMS-SB cells [110].

3.2.2 hMSCs and SMS-SBs

Figure 3.2 shows a photograph of hMSCs and SMS-SB cells. At some points (shown by arrows) it appears that the SMS-SB cells are growing in physical contact with the stromal cells. This leads to the hypothesis that a physical interaction between membrane bound cell surface molecules (perhaps CD23 and its receptor the integrin αvβ5) is sustaining SMS-SB cell growth. It is possible, of course, that simple random placement of the cells in this

particular field gives this impression. As previously stated, Fourcade and colleagues identified long term bone marrow culture derived stromal cells which expressed CD23 mRNAs at high levels and secreted soluble CD23 in their supernatants [249]. The hypothesis that hMSCs express CD23 was evaluated.

3.2.3 Detection of CD23 protein in hMSCs

Flow cytometry was performed on hMSCs obtained in solution via their removal from the bottom of the tissue culture flask using a cell scraper and subsequent resuspension. The cells were incubated with an anti(α)-CD23 antibody as the primary antibody and an RPE-conjugated secondary antibody. As a positive control RPMI-8866 cells were also stained for CD23 – this cell line produces a high level of surface CD23 and the cells can be seen microscopically as large clumps as a result of the interaction between the CD21 and CD23 surface molecules [155]. From the results of the flow cytometry it could not be demonstrated that the stromal cells have surface CD23. Comparison with the high levels of CD23 produced by the RPMI8866 cells indicates that the stromal cells are not producing CD23 within the detection limits of flow cytometry (Figure 3.3).

Western blotting analysis of stromal cell lysates also failed to detect CD23 protein. Figure 3.4 shows immunoblots of recombinant sCD23 and stromal cell lysates and it can be seen that only the control sCD23 can be identified.

Some growth factors are not secreted by bone marrow cells until they are induced to do so by precursor cells; for example, IL-7. Stromal cells were incubated in a flask with SMS-SB cells for one week, the SMS-SB cells washed off and lysates prepared from the stromal cells left behind in the flask. The rationale behind this experiment was that if the stromal cells do not express CD23 without contact from precursor cells, this might induce the hMSCs to produce CD23. However, again, western blotting failed to detect any CD23 protein from these lysates (data not shown). hMSC cell culture supernatants were assayed for CD23 protein using a CD23 ELISA. No CD23 could be detected in these supernatants (Figure 3.5).

3.2.3 Detection of CD23 mRNA in hMSCs

The level of CD23 transcripts was assessed by RT-PCR analysis of hMSC RNA. Primers were selected to encompass the coding sequences of exons X and Y, thereby avoiding spurious products arising from contaminating DNA and also including both CD23a and CD23b transcripts as potential amplification targets.

Figure 3.6 shows a scanned picture of a DNA gel. The DNA fragments separated in this gel were produced by RT-PCR from stromal cell RNA which was obtained using Trizol reagent. The predicted PCR fragment size produced using CD23 left and right primers is 256 and this gel shows that a fragment of this size has been produced by the PCR and confirms that the hMSCs contain CD23 mRNA. From the intensity of the DNA fragment it is clear that, from this sample, the cells are not producing a high level of CD23 RNA compared to GAPDH. A positive control from RPMI8866 cDNA is not included due to failure of the PCR but would have been extremely revealing in terms of mRNA levels as it is known that these cells express a very high amount of CD23.

3.2.4 Transfections

Attempts were then made to transfect the hMSCs with a CD23a plasmid. This would hopefully have boosted the CD23 levels to a detectable level and investigations could have been made into the effect of this on the SMS-SB cells seeded thereon. The plasmid used carried a drug resistance gene for kanamycin resistance to enable selection of cells which survive culture in kanamycin and are therefore successfully expressing the CD23a plasmid.

The cells were transfected using DOTAP as the transfection reagent and this appeared to have been successful as cell growth occurred over the next few days through 3 passages. However, further culture of the transfected cells failed and the cells lost adherence and viability. This occurred approximately 10 days post-transfection. The cells were not assayed for CD23 expression.

This was repeated numerous times, varying the antibiotic concentration from 10μg/ml to 50μg/ml but the cells never survived longer than 10 days post-transfection. The cells were

only transiently expressing the CD23 plasmid and therefore were not going to prove appropriate for use in the long-term culture system used. Further attempts to transfect these stromal cells with CD23 were abandoned.

The only evidence that the stromal cells produce CD23 came from the RT-PCR performed using CD23 primers on hMSC mRNA. It was not possible to confirm whether these cells expressed either membrane bound or soluble CD23, only message was able to be detected and not protein. If these cells are expressing CD23 it may be at very low levels beneath the detection limits of the techniques attempted.

3.2.5 Disruption of CD23/αvβ5 Interaction

Using the assay described above involving stromal cells and low densities of SMS-SB cells, various different clones of anti-CD23 antibodies were added to the stromal cells prior to the seeding of SMS-SB cells. This was carried out in an attempt to prevent the integrin from physically contacting the CD23, if present, on the surface of the hMSCs. Figure 3.7 shows photographs of cells subjected to these treatments. Examination of these fields reveals that the antibodies do not have any effect on the growth of the SMS-SB cells. The SMS-SB cells still have a viable appearance, are still able to adhere to the stromal cells and have not decreased in number. The photograph showing the cells treated with the BU38 clone does have some novel features, however, when compared with the ML233 clone-treated cells. The SMS-SB cells are found in "beads on a string" along the stromal cells rather than in individual sites. The cells appear to be dividing in very close contact with the hMSCs. In the absence of any effect on the growth of SMS-SB cells following these treatments, this approach was not continued any further. If CD23 is expressed on these hMSCs, then its interaction with αvβ5 on the SMS-SB cells is not required for the growth of these precursor B cells. Alternatively, it is possible that the antibodies used did not bind at the interaction site and the CD23/αvβ5 contact was not prevented (it is known that BU38 and MHM6 both block IgE binding and the IgE binding site is distinct from the αvβ5 binding site). The data of figure 3.7 show that anti-CD23 Mabs had no effect on adhesion of SMS-SB cells to hMSC monolayers. This may be explained either by a failure of the anti-CD23 Mabs to bind at or near the site on CD23 recognised by the integrin, or that a second adhesion interaction,

particularly the VLA-4/VCAM-1 interaction, has a dominant role that swamps any effect of the CD23-αvβ5 interaction. A further possibility is that the stromal cell line does not express CD23 either at all or not at sufficient levels to allow it to have a meaningful effect on SMS-SB adhesion or survival.

3.2.6 Conclusions

The data for CD23 expression by hMSC cells are inconclusive but suggest that the line does not express CD23. The only results which support the assertion that the hMSCs have CD23 are those from the RT-PCR. FACS, microscopy and western blotting did not detect CD23. It is possible that the levels of CD23 expressed by the hMSCs were below that of the detection limits in these assays, or perhaps the cells do not produce CD23 until driven to do so by other cells/matrix factors in the bone marrow stroma.

SMS-SB cells express only αvβ5 as a CD23 receptor. This means that any effect on the SMS-SB cells from CD23 has to occur via this integrin.

The stromal cells support the growth of the SMS-SB cells at extremely low densities (as low as 5 cells per well on top of 5,000 stromal cells). This growth cannot be prevented using antibodies against CD23. Perhaps the interaction is simply not the sole factor in the growth sustenance, or, perhaps the antibodies are not directed against the epitopes specifically involved in the interaction.

Physical separation of these two cell types using, for example, the Corning transwell system, would allow for diffusion of soluble factors from stromal cells to SMS-SBs and vice versa. This would allow for determination of the importance of secreted factors versus physical interactions between the cells.

Figure 3.1 Integrin expression on SMS-SB cells:

A. staining for αvβ5 and B. staining for αvβ3. Green solid represents the experimental antibody staining and the pink line represents isotype control antibody staining. These data are representative of experiments performed five times.

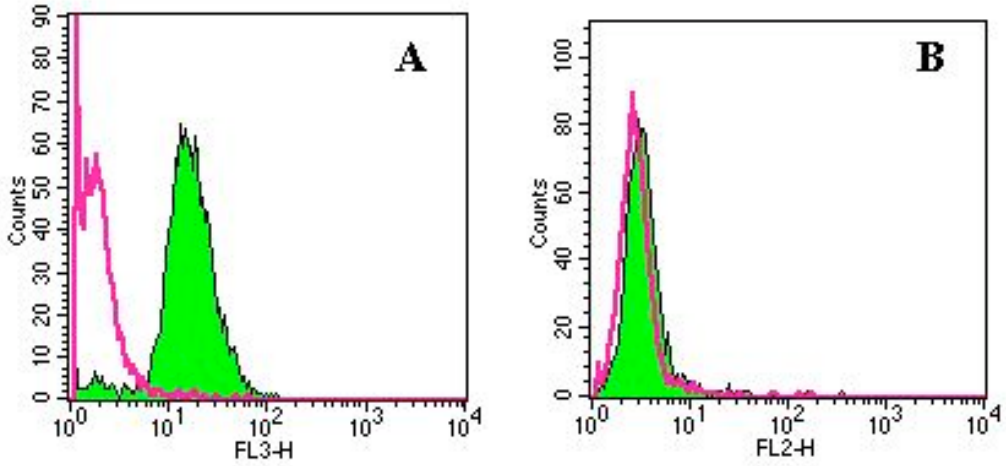

A
Counts
FL3-H
B
Counts
FL2-H

Figure 3.2 SMS-SB cells adhere to and grow upon human stromal cells.

The stromal cells are the adherent cells and the SMS-SB cells are the rounded cells. Arrows show SMS-SB cells growing directly alongside hMSC cells. This photograph shows a field of a well in a 96-well plate. Stromal cells were plated at 5,000 cells per well and SMS-SBs at 25 cells per well. Pictures were taken one week after addition of SMS-SB cells. This result is typical of experiments performed in excess of 20 times.

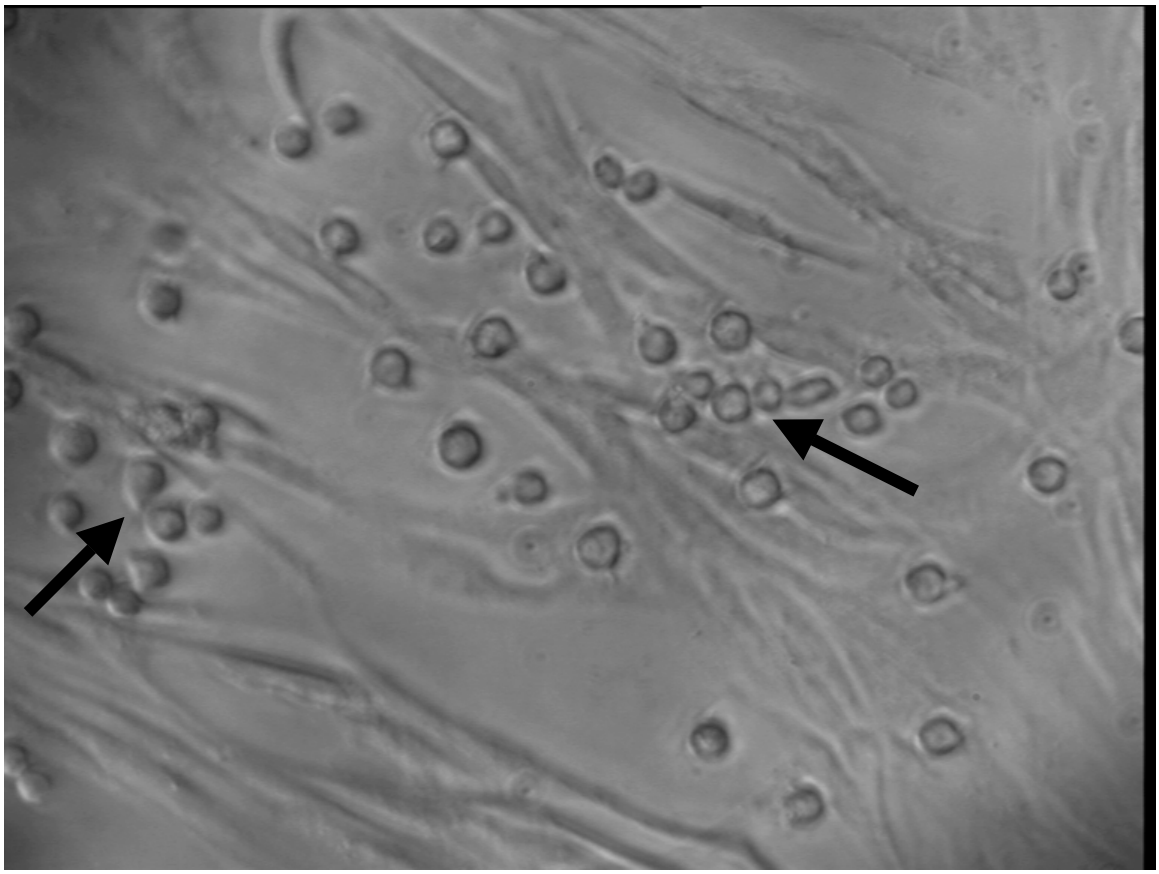
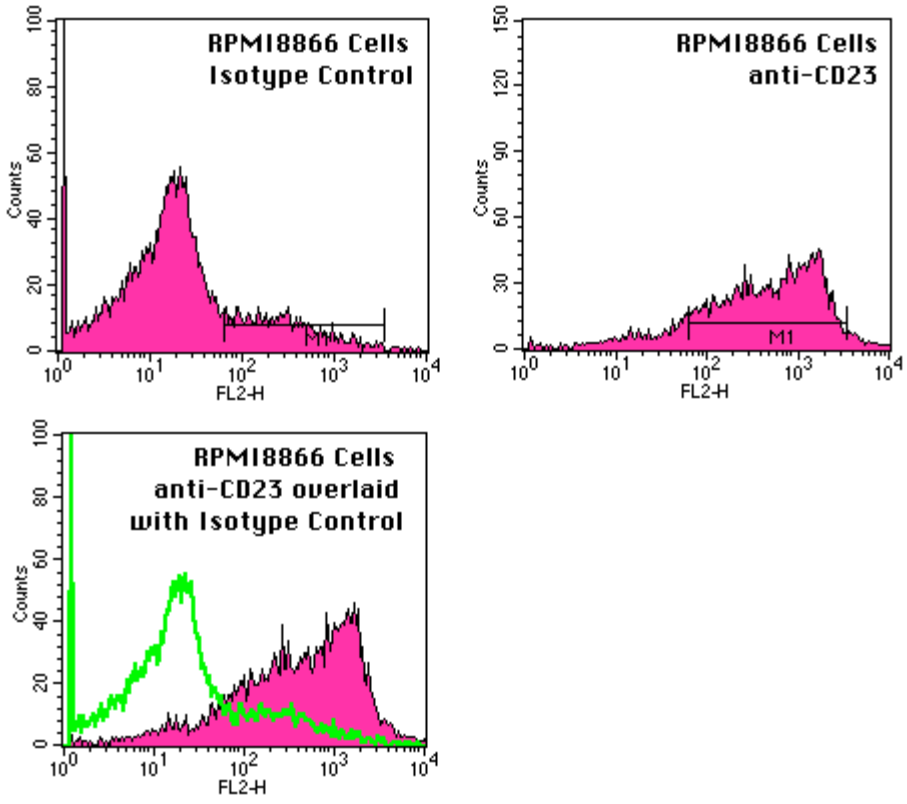


Figure 3.3 A CD23 Expression on RPMI8866 cells.

(i) shows the isotype control antibody staining of the RPMI8866 cells and (ii) shows the staining achieved using α-CD23. (iii) shows an overlay of the isotype control (green line) and the CD23 staining (pink solid). This experiment was performed five times and the data shown are representative data.

Figure 3.3 A

(i) **(ii)**



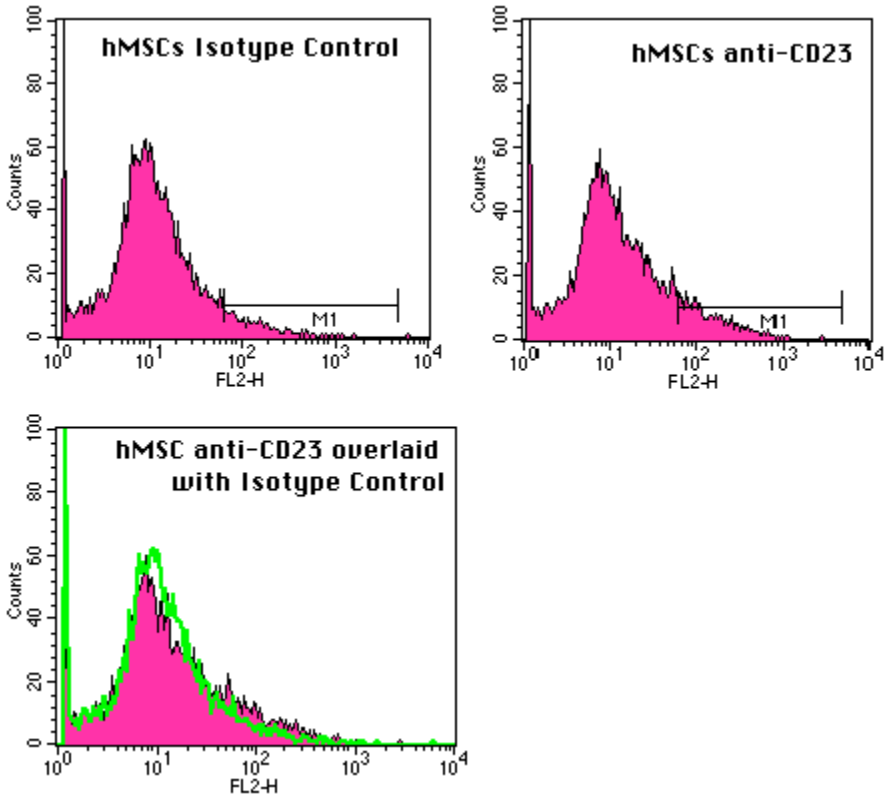
(iii)

Figure 3.3 B CD23 expression on hMSCs

(i) shows the isotype control antibody staining of the hMSC cells and (ii) shows the staining achieved using α-CD23 (iii) shows an overlay of the isotype control (green line) and the CD23 staining (pink solid). This experiment was repeated five times and the data displayed are representative.

Figure 3.3 B

(i) **(ii)**

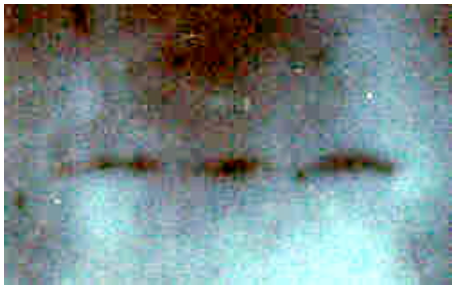


(iii)

Figure 3.4 CD23 Immunoblots.

A. recombinant sCD23 and B. hMSC lysates. In A 3 lanes of the same sample were analysed and in B 3 lanes were also analysed. The result seen in A is representative of two experiments and in B the result is representative of 8 experiments.

A



CD23 (45 kDa)

B

CD23 (45 kDa)

Figure 3.5 CD23 ELISA.

Standard curve graph for CD23 ELISA. Inset box shows values for hMSC supernantants (s/n) (G. Borland, unpublished results). This is data from an experiment performed once.

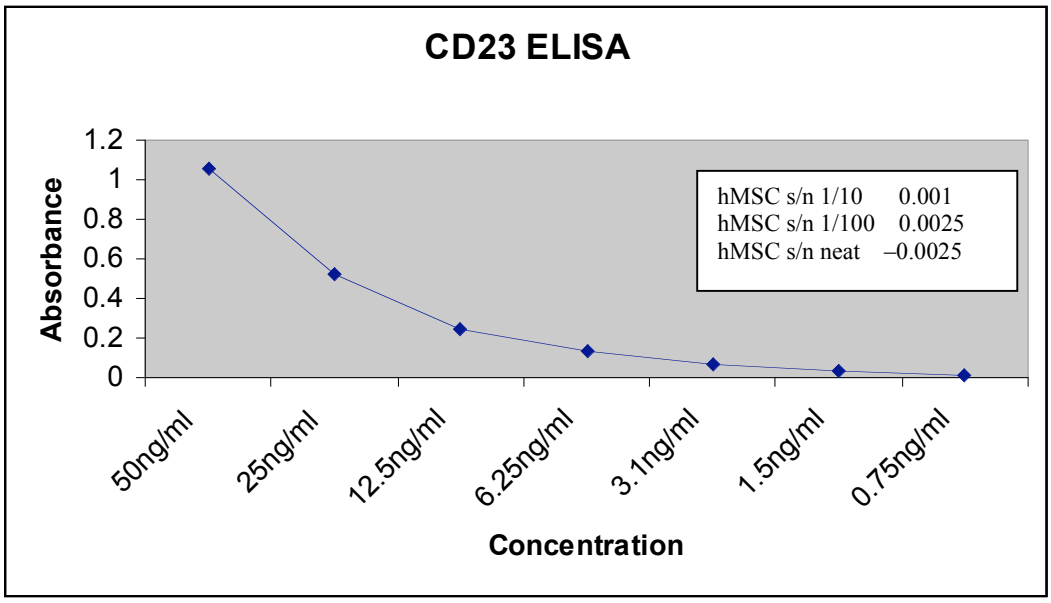

CD23 ELISA
Absorbance
1.2
1
0.8
0.6
0.4
0.2
0
hMSC s/n 1/10 0.001
hMSC s/n 1/100 0.0025
hMSC s/n neat –0.0025
50ng/ml
25ng/ml
12.5ng/ml
6.25ng/ml
3.1ng/ml
1.5ng/ml
0.75ng/ml
Concentration

Figure 3.6 Stromal cells produce CD23 mRNA.

Photograph shows Lane 1 - fragment produced following RT-PCR using primers for housekeeping gene GAPDH; Lane 2 – DNA fragment produced following RT-PCR with CD23 primers (predicted size 256 bp) and Lane 3 – no fragment (no primers used in this RT-PCR reaction). Lane 4 – DNA ladder showing high intensity fragment of 500bp. This experiment was repeated twice with the same result both times.

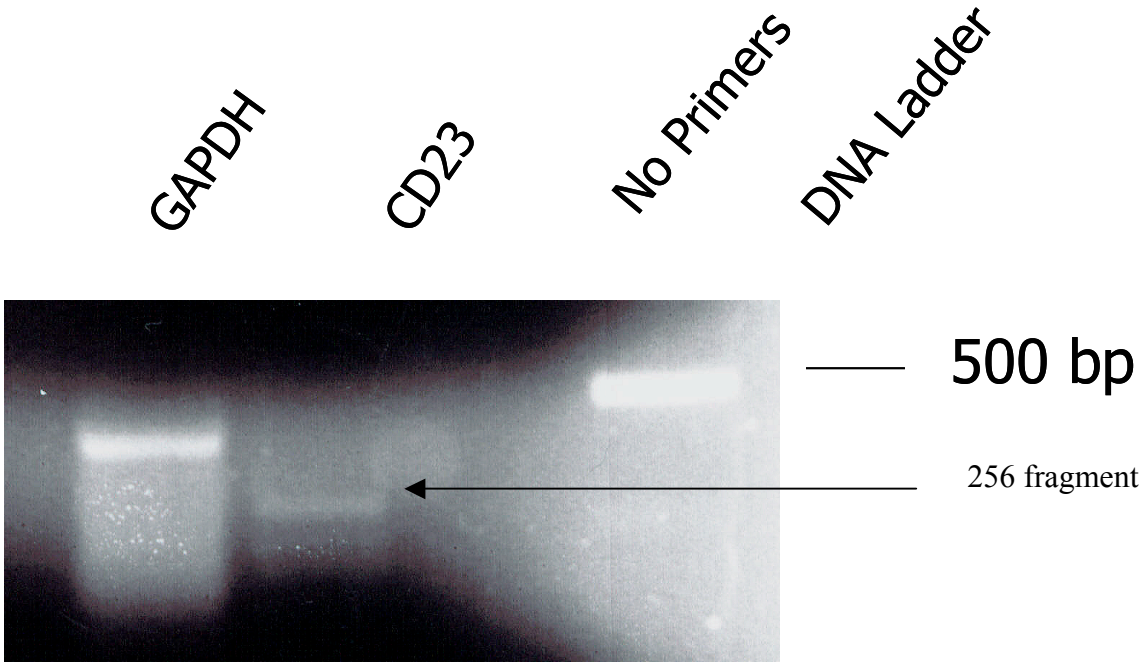
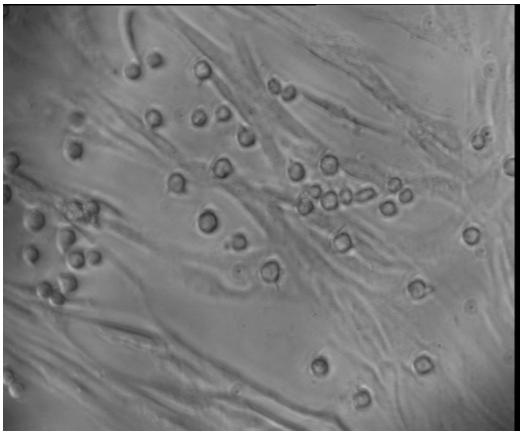
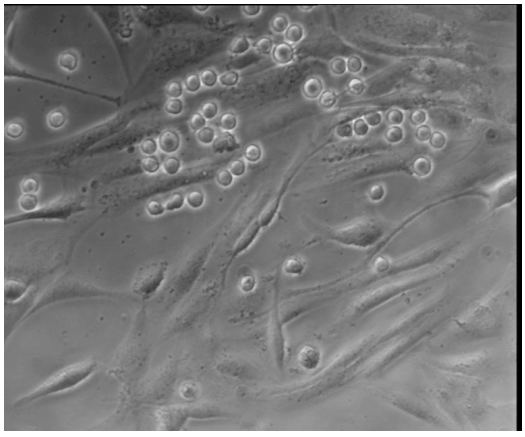

GAPDH
CD23
No Primers
DNA Ladder
500 bp
256 fragment

Figure 3.7 Interaction between hMSCs and SMS-SBs.

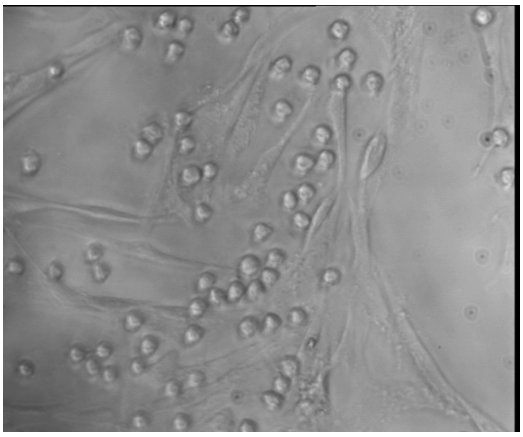
A shows cells without treatment, B shows cells treated with clone BU38, C shows cells treated with an isotype control antibody and D shows cells treated with clone ML233. This experiment was performed 5 times and that pictures shown are representative of these.



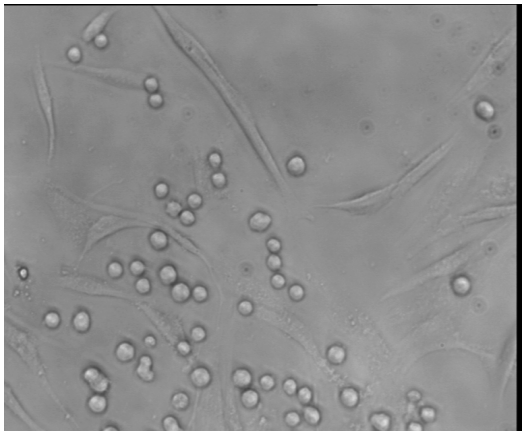
A. No Treatment



B. Anti-CD23 (Clone BU38)



C. Isotype Control



D. Anti-CD23 (Clone ML233)

CHAPTER 4

GROWTH OF B CELL PRECURSORS AND PLASMA CELLS

4.1 Introduction

The cells which are used as the model cell line in this thesis are SMS-SB cells. These cells produce an autocrine factor which sustains the growth of SMS-SB cells once above a certain culture density (this density is 10,000 cells/well). If the SMS-SB cells are plated out at low densities, the effect of the autocrine factor is negated and the cells will not survive unless they are rescued. Proliferation assays span 72 hours and during this time cells become apoptotic and are lost in the absence of protective treatments. Previous work has demonstrated that sCD23 will inhibit apoptosis in these cells and that the AMF7 antibody, which recognises the αv integrin subunit, also has a positive effect on the growth of these cells. Here the effects of CD23 peptides are assessed, on SMS-SB cells and on other precursor B cell lines and on myeloma lines. The presence of CD23 receptors on these cell lines is also measured and binding of CD23 peptides determined. It was also intended to initiate investigations into the mechanisms at work following peptide stimulation.

4.1.1 CD23 Peptides

In this thesis use of CD23-derived peptides is described. A library of 83 overlapping nonapeptides was synthesised and the effects of these peptides investigated. Biotinylated peptides were used to identify which peptides could bind to cells using streptavidin conjugated to a fluorophore to allow detection by flow cytometry. Some of these peptides have been found to have the ability to bind to SMS-SB cells and noted to have effects on cells similar to those seen following treatment with the intact sCD23 molecule. These peptides all contain a tripeptide motif of Arg-Lys-Cys (RKC) derived from the CD23 molecule, and are #9-#12 in the library.

Peptides 9 – 12 were investigated for their ability to bind to cells which express the CD23 receptors αvβ5 and αvβ3. Although all 4 peptides have been found to bind to cells bearing these receptors only two have been found to have an obvious agonistic effect. These are peptides 9 and 12, and peptide 9 has been used exclusively for further investigation due to its more consistent effects. In addition, a peptide was synthesised which spans the entire area of

sequence between peptides 9 – 12 and this has been found to bind to cells and also to have a positive effect on growth. This peptide has been termed the "long peptide". The work in this chapter concentrates on the effects of two peptides on various cell lines: 9 and long.

4.2 Results

4.2.1 Growth of Precursor and Mature B Cells

Proliferation assays were carried out using incorporation of tritiated thymidine into DNA as the method of detection. As previously stated SMS-SB cells cannot grow at low cell densities unless rescued, so these cells were plated out in 96-well plates at between 2,000 and 7,000 cells per well. Treated and untreated cells were incubated for 72 hours then pulsed with tritiated thymidine for 16-24 hours. Untreated cells do not survive over the 4-day experiment window. Treatments were performed in triplicate and averages and standard deviations calculated for the results. P values were determined and statistical significance is indicated by the presence of asterisks. Individual figures specify treatments and cell densities. The finding that SMS-SB cells at this density are predisposed towards apoptosis creates an intrinsic difficulty when performing these assays. When preparing the cell suspensions for the experiments the longer they remain without stimulation the more likely it is that they will no longer be able to be rescued. This resulted in experiments being rendered useless due to the cells being non-viable prior to treatments even being applied. In addition it was observed that, at these low densities, the SMS-SB cells would adhere very strongly to the plastic of the plates. This was not such an issue for this particular assay as when harvesting the plates the cells are aspirated to a filter by the power of a vacuum pump but this property of the cells could be problematic in other instances.

Figure 4.1 shows the results of a typical proliferation assay performed using SMS-SB cells. The cells were plated out at a concentration of 3,000 cells per well. A stimulatory effect is obvious in the cells treated with peptide 9 and the long peptide (both at 5μg/ml) and the AMF7 antibody (also at 5μg/ml). The AMF7 antibody recognises the αv subunit of the integrin heterodimer. The P1F6 antibody reacts with an epitope formed by the entire heterodimer. The cells treated with P1F6 antibody, the untreated cells, the cells treated with

isotype control antibodies and control peptide-treated cells do not survive being placed at low density. Figure 4.2 demonstrates that the effects of #9 and long peptide (LP) on SMS-SB cells growth are dose-dependent. Peptide 58 has no effect.

As previously discussed, the SMS-SB cells do not express any other of the known receptors for CD23 and, therefore, it must be the αvβ5 integrin that is being ligated by the growth-promoting peptides and antibody and presumably transmitting some form of survival/anti-apoptotic signal. The CD23-derived peptides which have been shown to bind to the integrin have in common a sequence of three amino acids, arginine-lysine-cysteine (RKC). Much of this work has been in the form of binding assays using the BIACORE platform and has shown that the peptides bind the integrin with varying affinities. SMS-SB cells have been tested using flow cytometry for peptide binding and it has been found that peptides 9, 11 and 12 bind to SMS-SB cells. As previously mentioned these peptides possess a biotin tag and therefore can be detected using a streptavidin secondary reagent. Figure 4.3 shows the data obtained from this flow cytometry analysis. The peptides binding to the cells to the greatest extent are 9, 11 and 12. Peptides 8, 13 and 58 do not bind appreciably to the cells.

Other B cell precursor cell lines were used in proliferation assays to determine their responses to peptides. The cell lines used express either one or both of the integrins αvβ3 and αvβ5 and it was investigated whether the integrin expression profile of the cells correlated with their growth response to the CD23 peptides 9 and the long peptide. One such cell line is the 697 line which, like SMS-SB cells, expresses the αvβ5 integrin as the sole CD23 receptor. Figure 4.4 shows flow cytometry data to confirm that 697 cells only express the αvβ5 integrin and are negative for αvβ3. On the basis of this similarity to the SMS-SB cells it was predicted that they may respond in a comparable manner when subjected to growth assays. 697 cells were also analysed for peptide binding and Figure 4.5 shows the results of this. Like the SMS-SB cells peptides 9-12 bind to the 697 cells with peptides 11 and 12 binding to the greatest extent. It should be noted that the peptide binding profiles are quite distinct with regard to these two cell types despite comparable expression of αvβ5. 697 cells express a very small amount of αvβ3, which does not necessarily account for the differences in peptide binding observed. The possibility that the peptides are binding to other αv integrins on these

cells must be considered. Further analysis of the 697 cells was not undertaken in this thesis but should be considered for future work.

Figure 4.6 illustrates the data for a proliferation assay using 697 cells and shows that these cells do respond similarly to SMS-SB cells. Peptide 9 and LP both have a positive growth effect on the cells at a concentration of 5μg/ml. LP has a much more pronounced effect than peptide 9 which is the converse of what is seen with the SMS-SB cells which respond most robustly to peptide 9. It should be noted that the long peptide is not available in biotinylated form and so is not included in the binding analysis. The SMS-SB cells and the 697 cells express αvβ5 as the sole CD23 receptor in common and 697 cells appear to express slightly more αvβ5 than SMS-SB cells (comparing Figures 3.5 and 4.4). Perhaps variation in integrin conformation or activation could account for this disparity, e.g. the integrins on the 697 cells have a conformation more suited to binding or activation by the long peptide. It is also possible that these peptides are also able to signal through other αv integrins present on the cells, following on from the peptide binding results. Support for this hypothesis can be gained from the fact that the AMF7 antibody (which only binds αv) has a positive effect on growth, but this hypothesis is not supported by the finding that peptide 9 binds to the integrin β chain [251].

Two additional precursor B cell lines, NALM-6 and BLIN-1 cells were assayed for their integrin profiles using flow cytometry. Figure 4.7 shows that these precursor B cell lines express both αvβ3 and αvβ5 integrins. This is where the NALM-6 and BLIN-1 cells differ from the SMS-SB cells and 697 cells. NALM-6 and BLIN-1 cells are representative of a more advanced developmental stage of B cells and perhaps it could be proposed that expression of the integrins is stage-dependent, i.e. at earlier phases in development the αvβ5 integrin is expressed and this expression wanes as differentiation progresses and progressive expression of the αvβ3 integrin occurs concomitantly with αvβ5 integrin loss. Osteoclasts have been shown to follow this pattern of integrin expression so perhaps a similar process is relevant in B lymphopoiesis [222].

Peptide binding was then carried out on the NALM-6 and BLIN-1 cells and the results are shown in Figure 4.8 and 4.9. These cells display higher peptide binding than SMS-SB cells and 697 cells as would be predicted given that the cells are expressing more than one receptor for CD23. Proliferation assays were then carried out on these cells to determine how they would respond to the growth-promoting peptides given their possession of more than one receptor.

Figure 4.10 demonstrates that NALM-6 cells show a broadly similar pattern of response to peptide 9 and LP to that observed in SMS-SB cells. Thus both CD23-derived, RKC-containing peptides promote thymidine incorporation in a dose-dependent manner in NALM-6 cells, while peptide 58 is without effect on the cells. Figure 4.11 illustrates the equivalent data set for BLIN-1 cells and these cells also respond in a specific, dose-dependent manner to stimulation with peptide 9 or LP. As stated previously, the NALM-6 and BLIN-1 cell lines are thought to be representative of a stage in B cell development that is more advanced than that of the SMS-SB cells and 697 cells. A further growth assay was carried out using the IL-3-dependent mouse pro-B cell lineBAF03.

Figure 4.12 shows a graph of the response of the mouse pro-B cells to peptide 9 in the presence and absence of IL-3. A combination of IL-3 and peptide 9 causes a striking increase in growth – approximately 24-fold, compared with an approximately 14-fold increase upon provision of IL-3 alone.

The myeloma cell lines KMS11 and H929 were also subjected to proliferation assays using CD23-derived peptides and the results contrasted with those from the B cell precursor lines. Neither of these cell lines responded to the peptide treatments and the counts were so low in the case of KMS11 cells that it is likely these cells were no longer viable at the time of harvesting. The H929 cells appeared to have better viability at these low densities, but there was no growth enhancement achieved with the peptide treatments. Figure 4.13 shows a flow cytometric analysis of these two cell lines to establish which of the CD23 receptor integrins they expressed. The KMS11 cells express $\alpha v\beta 5$ integrin but not $\alpha v\beta 3$ whereas H929 cells express $\alpha v\beta 3$ but not $\alpha v\beta 5$. The presence of $\alpha v\beta 5$ on the KMS11 cells does not render these cells responsive to the CD23 peptides. From the results (Figures 4.14 and 4.15) it can be

deduced that the positive growth response to CD23 peptides occurs in the B cell precursor lines but not in the mature B cell lines. Thus, in H929 cells, peptide 9 causes an enhancement in proliferation compared to untreated controls, but this is no greater than that detected with peptide 13 which lacks the RKC motif and does not bind cells. The enhancement of proliferation, which has been observed and documented in this thesis, is confined to cells that express the αvβ5 receptor and are at an early stage in B cell differentiation. The lack of precursor B cell lines expressing only αvβ3 as a CD23 receptor precluded further testing of the CD23 peptides to attempt to characterise growth responses in cells with such a profile. Further testing of CD23 peptides in the cell line RPMI8866 (a plasma cell line) revealed that these cells do not undergo proliferation in response to the peptides. These cells express αvβ3 but not αvβ5 (i.e. same profile as H929 cells) as shown in Figure 4.17 and the proliferation data is shown in Figure 4.16. Peptide binding for these cells is shown in Figure 4.18.

4.2.2 Action of RKC-containing peptides

Previous studies had indicated that the pro-survival effect of recombinant soluble CD23 on the growth of SMS-SB cells could be explained by sustained expression of the protein Bcl-2. Intracellular staining using of SMS-SB cells following treatment with recombinant soluble CD23 using flow cytometry showed maintenance of Bcl-2 levels [110]. As this protein is an anti-apoptotic member of the Bcl-2 family, it was a reasonable assumption that phosphatidylinositol 3-kinase (PI3K) signalling could be implicated because integrin signalling via PI3K through Akt (also known as protein kinase B (PKB)) and the Bcl-2 family member Bad inhibits mitochondrial induction of apoptosis. This form of apoptosis is known as negative induction whereas positive induction involves ligand binding e.g. the FAS/FAS ligand system. Phosphorylation of Akt at serine 473 by PI3K leads to phosphorylation of Bad at serine 112 and when bad is phosphorylated it is sequestered in the cytosol by the 14-3-3 protein[252]. When Bad is dephosphorylated it interacts with other Bcl-2 family members and is though to contribute to the overall balance between pro- and anti-apoptotic Bcl-2 family proteins. These proteins influence the mitochondrial induction of apoptosis via changes in mitochondrial membrane permeability which is dependent upon the balance of homo/heterodimers of pro and anti-apoptotic Bcl-2 family proteins. The

sequestration of Bad in the cytosol and the increased expression of Bcl-2 would contribute to suppression of apoptosis.

Western blotting was performed on immunoprecipitates (IPs) obtained from lysates prepared from SMS-SB cells treated with peptide 9 over a short time-course. These lysates were then incubated overnight with an anti-Akt antibody and then immune complexes were collected using protein G-sepharose beads. These IPs were then subjected to SDS-PAGE, transferred to nitrocellulose, blocked and probed using anti-phospho Akt (serine 473). Figure 4.19 shows the result obtained from this immunoblot and it can be seen that treatment with peptide 9 results in phosphorylation of Akt at serine 473. At time zero there is less phorphorylated protein present than at times 10 minutes, 30 minutes, 60 minutes and 90 minutes. The loading control immunoblot shows that constant levels of total Akt protein were present. This indicates that when SMS-SB cells are stimulated with peptide 9 sustained phosphorylation of Akt-1 occurs as a result of signalling via the αvβ5 integrin. A clear elevation of Akt phosphorylation is evident at 10-90 minutes, but this begins to reduce after 90 minutes.

Following on from this proliferation experiments were carried out using the specific PI3K inhibitor LY294002. Cells treated with this inhibitor failed to undergo proliferation in the presence of peptide 9 (see Figure 4.20), which reinforces the hypothesis that growth induced by CD23-derived peptides containing the RKC motif occurs via a signalling mechanism involving PI3K and Akt.

4.3 Conclusions

Studies performed with various cell lines have revealed that those which correspond to early stages of B cell differentiation undergo proliferation when treated with the CD23-derived RKC-containing peptides whereas those derived from later stages of differentiation do not.

The SMS-SB cell line and the 697 cell line express only αvβ5 as a CD23 receptor and therefore it is assumed that this integrin is the one these peptides are signalling through in these cells. Other precursor B cell lines, which respond in the same way to the peptides,

express αvβ3 in addition to αvβ5, hence the peptides could conceivably be signalling through both of these CD23 receptors to enhance growth and division. Further experimentation with these cell lines would have been important – using siRNA, for example, to abrogate expression of each integrin separately could have revealed information regarding which integrin is crucial to this growth mechanism. Such studies would be a logical progression to the work in this thesis. In addition work to test the hypothesis that other αv integrins are present on B cell precursors and can be bound by and affected by CD23-derived peptides would also be important.

In SMS-SB cells it was found that peptide 9 enhances phosphorylation of Akt at residue ser473. This residue is a target of PI3K and therefore the growth mechanism induced by RKC operates via these proteins. These experiments should be followed up with further studies to elucidate the remainder of the signalling pathway. Use of inhibitors and antibodies relevant to various Akt cascades would be of particular importance. The MAPK (Mitogen Activated Protein Kinase) pathway would be one such candidate for examination.

Figure 4.1 Effect of CD23 peptides on SMS-SB cells.

This proliferation assay graph shows the incorporation of tritiated thymidine into the DNA (cpm = counts per minute). The cells (3,000 per well) were treated with peptides 9, long and 13 and also with the antibodies P1F6 and AMF7 and isotype control antibody (all at 5μg/ml). It should be noted that the P1F6 antibody recognises the entire αvβ5 integrin heterodimer whilst AMF7 recognises only the αv portion of the integrin.

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 against peptide 13. The data shown are representative of experiment repeated approximately 10 times. It should be noted that technical difficulties necessitated the repetition of this experiment and that these difficulties related to excessive cell death. When positive results were achieved the graph shown in this figure is representative of n=4.

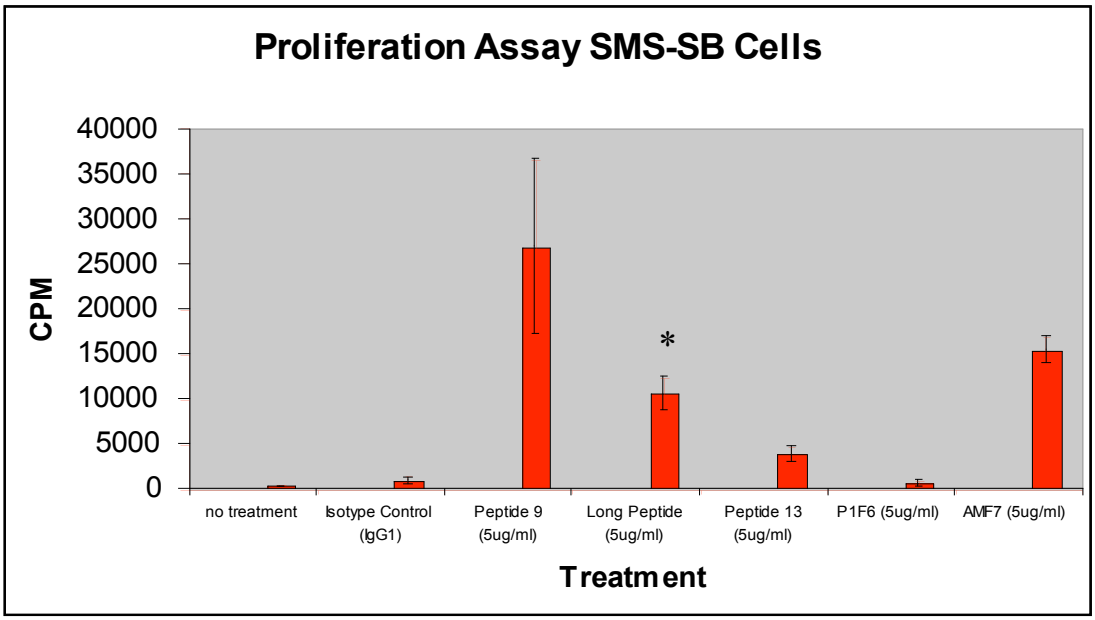

Proliferation Assay SMS-SB Cells
CPM
40000
35000
30000
25000
20000
15000
10000
5000
0
*
no treatment
Isotype Control (IgG1)
Peptide 9 (5ug/ml)
Long Peptide (5ug/ml)
Peptide 13 (5ug/ml)
P1F6 (5ug/ml)
AMF7 (5ug/ml)
Treatment

Figure 4.2 Effect of peptides on SMS-SB cells.

Proliferation data from assay carried out on SMS-SB cells plated at 3,000/well. This experiment was designed to ascertain whether the cells responded to variation in peptide dosage.

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 against corresponding concentrations of peptide 58 (which does not bind to SMS-SB cells) and peptides 9 and LP.

The data shown are representative of experiment repeated approximately 10 times. It should be noted that technical difficulties necessitated the repetition of this experiment and that these difficulties related to excessive cell death. When positive results were achieved the graph shown in this figure is representative of n=4.

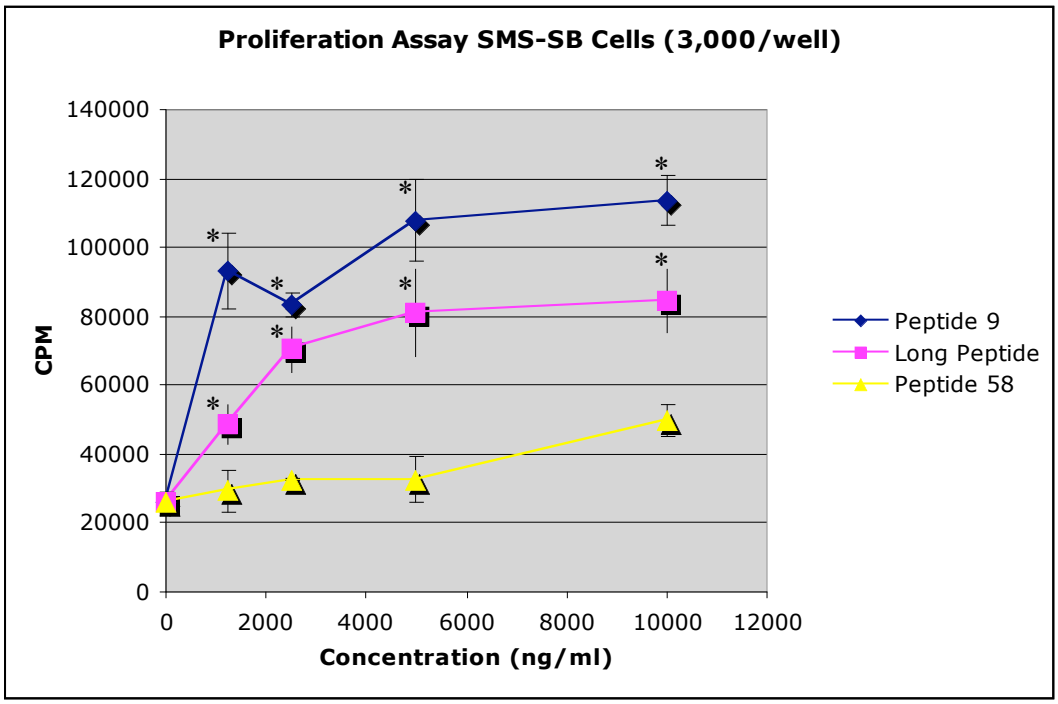

Proliferation Assay SMS-SB Cells (3,000/well)
CPM
140000
120000
100000
80000
60000
40000
20000
0
0
2000
4000
6000
8000
10000
12000
Concentration (ng/ml)
Peptide 9
Long Peptide
Peptide 58

Figure 4.3 Peptide binding to SMS-SB cells.

This assay uses biotinylated peptides and a streptavidin-Cy 5 conjugated secondary capture antibody. The panels show data for peptides 8, 9 10, 11, 12, 13 and 58 (A-G respectively) with peptide 8 being used as the negative control marker.

The data shown are representative of and experiment performed 3 times.

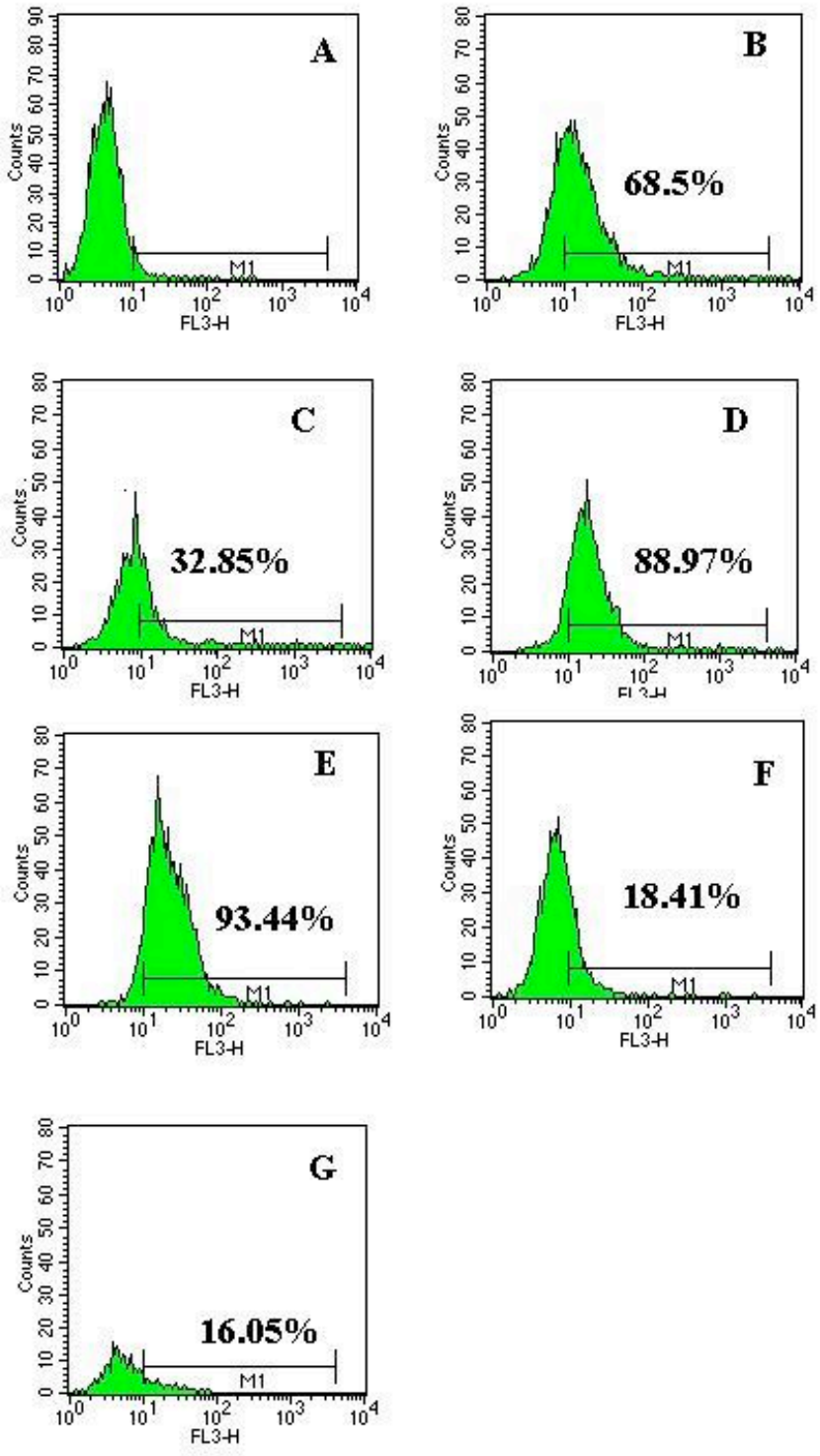

A
B
68.5%
C
32.85%
D
88.97%
E
93.44%
F
18.41%
G
16.05%
Counts
FL3-H
M1

Figure 4.4 Integrin expression on 697 cells.

A. αvβ5 and B. αvβ3 integrins in 697 cells. Green solid shows integrin staining and pink line shows isotype control staining.

The data shown are consistent with 3 experimental repeats.

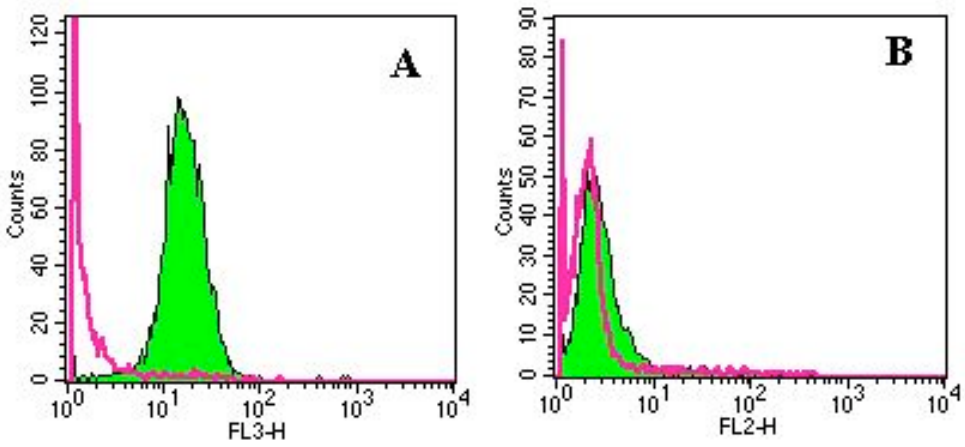

A
Counts
FL3-H
B
Counts
FL2-H

Figure 4.5 Peptide binding to 697 cells.

As before, this assay uses biotinylated peptides and a streptavidin-Cy 5 conjugated secondary capture antibody. The panels show data for peptides 8, 9, 10, 11, 12, 13 and 58 (A-G respectively) with peptide 8 being used as the negative control marker.

The data shown are consistent with 3 experimental repeats.

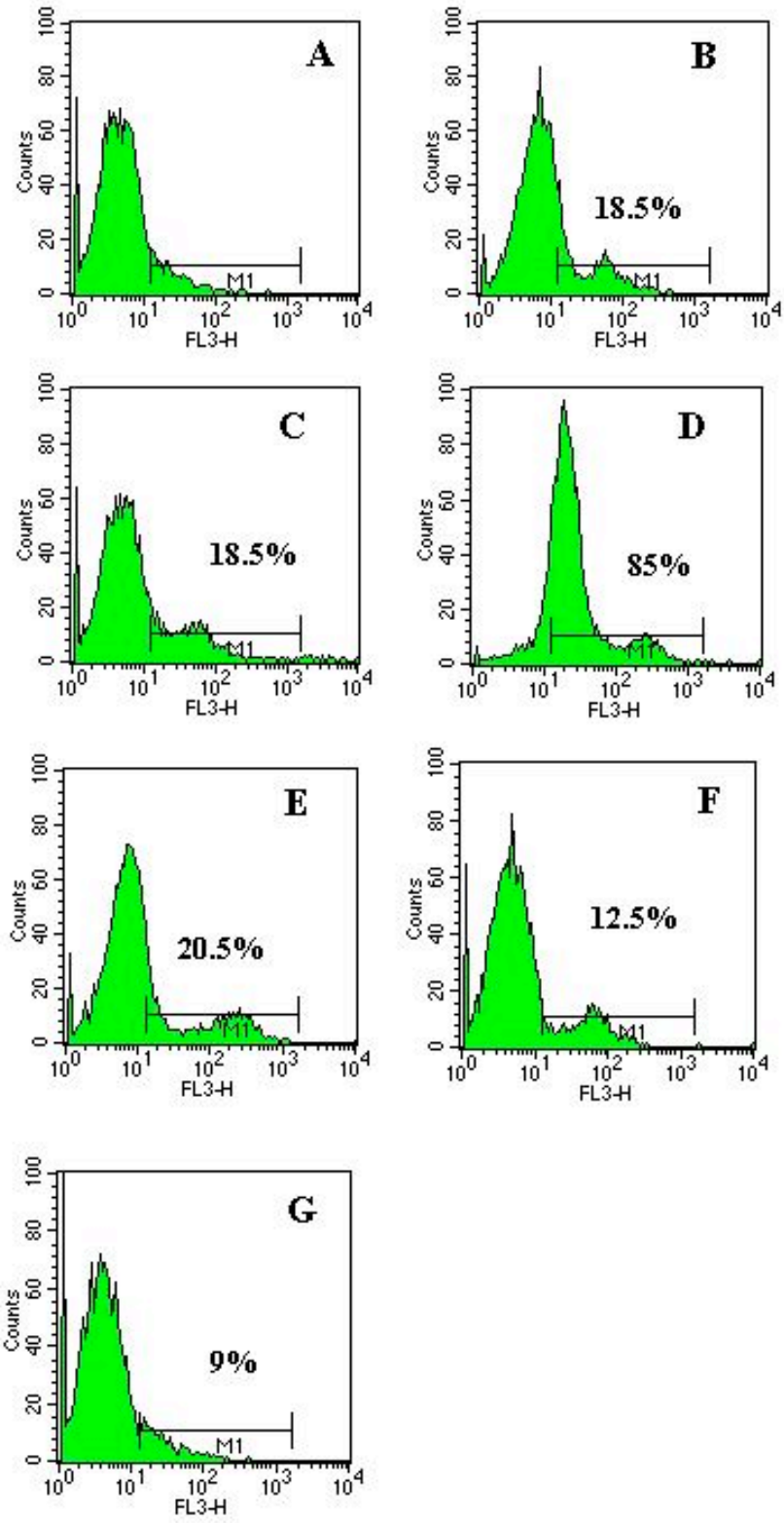

A
B
18.5%
C
18.5%
D
85%
E
20.5%
F
12.5%
G
9%
Counts
FL3-H
M1

Figure 4.6 Effect of CD23 peptides on growth of 697 cells.

Proliferation assay graph showing the response of cells to peptides 9, long and 8 (all at 5μg/ml). Peptide 8 does not bind to 697 cells and is used here as a negative control.

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 against peptide 8 (non-binding peptide).

The data shown are representative of experiment repeated approximately 6 times. It should be noted that technical difficulties necessitated the repetition of this experiment and that these difficulties related to excessive cell death. When positive results were achieved the graph shown in this figure is representative of n=3.

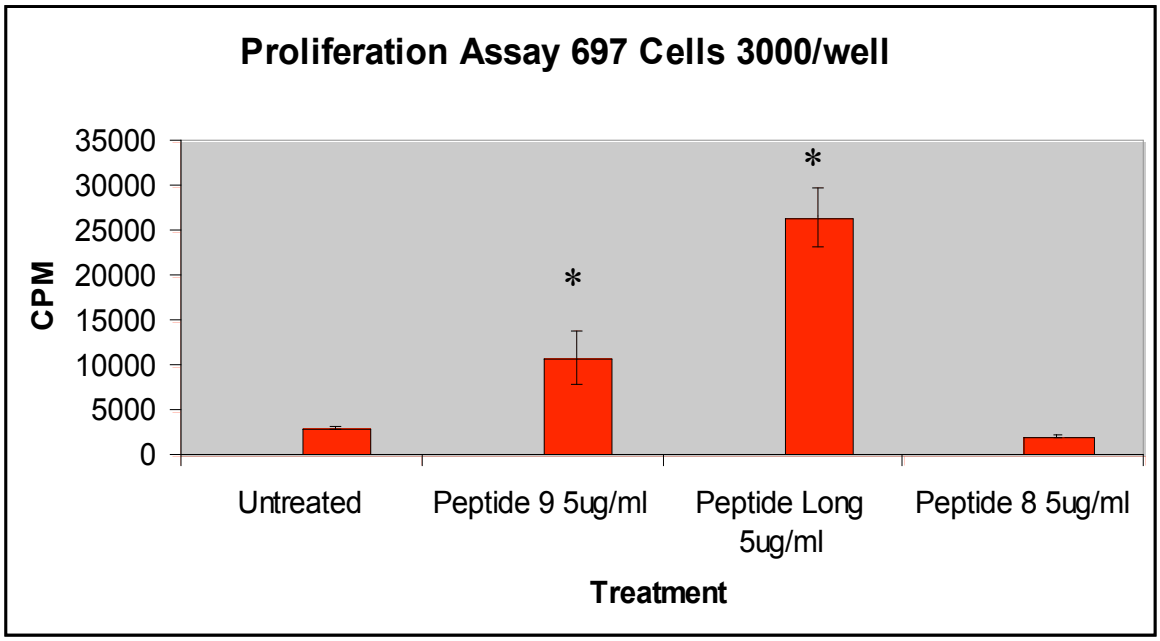
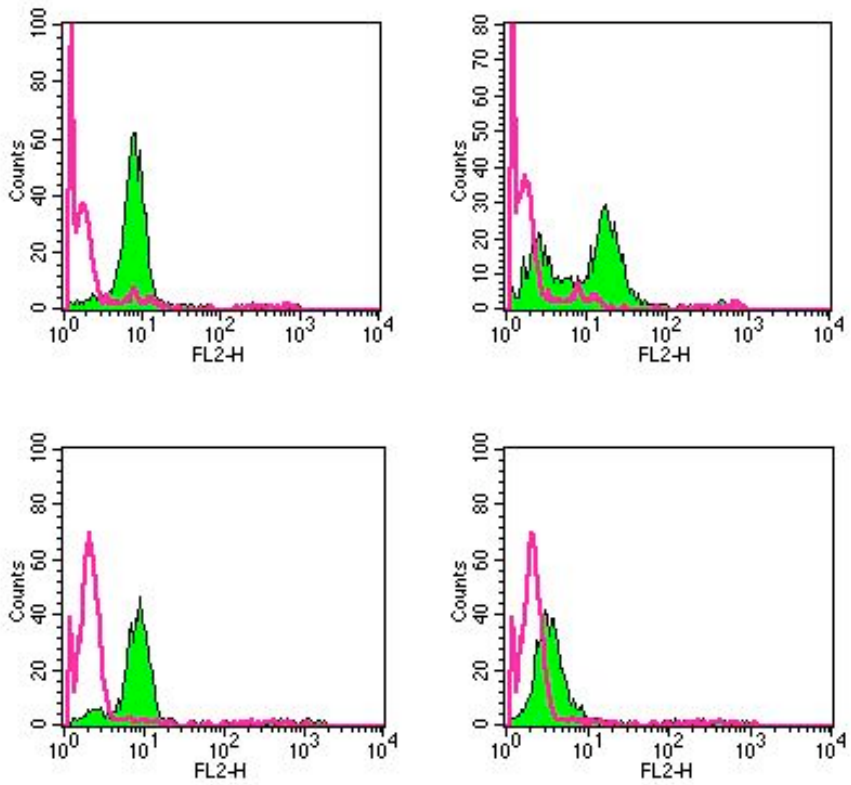

Proliferation Assay 697 Cells 3000/well
CPM
35000
30000
25000
20000
15000
10000
5000
0
*
*
Untreated
Peptide 9 5ug/ml
Peptide Long 5ug/ml
Peptide 8 5ug/ml
Treatment

Figure 4.7 Integrin expression on NALM-6 and BLIN-1 cells.

Panel A is αvβ5 staining of NALM-6 cells and panel B is αvβ3 staining of NALM-6 cells. Panel C is αvβ5 staining of BLIN-1 cells and panel D is αvβ3 staining of BLIN-1 cells. Green solid represents specific antibody staining and pink line represents isotype control.

This experiment was repeated 3 times and these data are consistent with these results.

A **B**

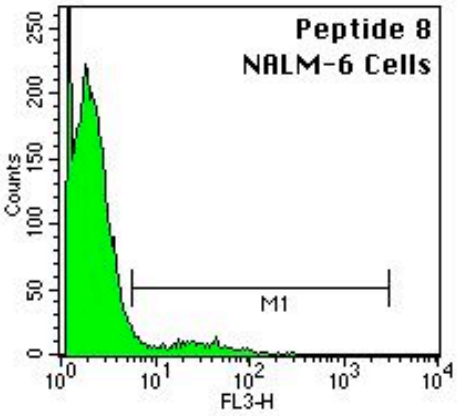


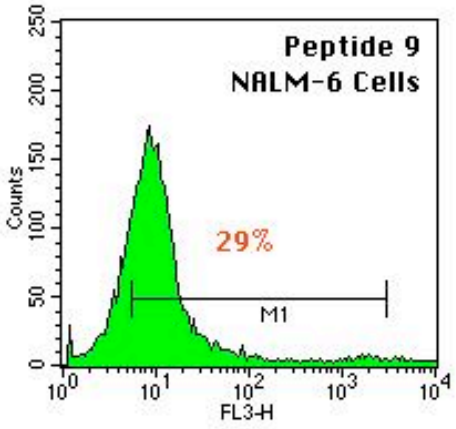
C **D**

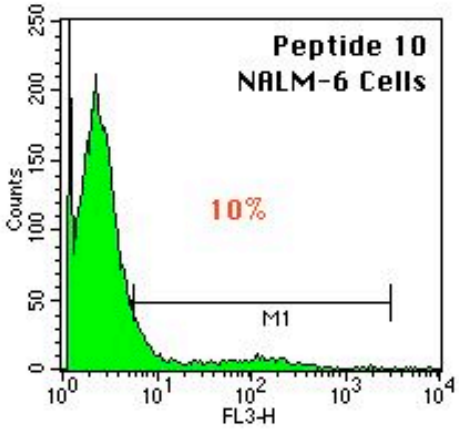
Figure 4.8 Peptide staining of NALM-6 cells.

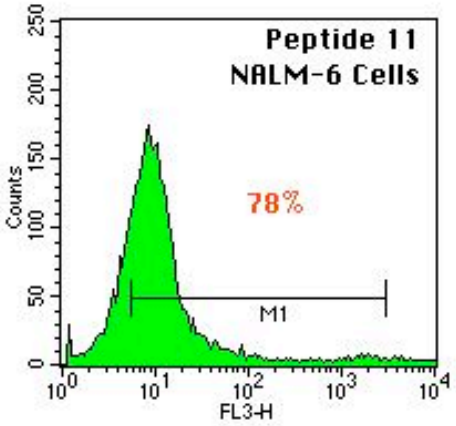
As before, this assay uses biotinylated peptides and a streptavidin-Cy 5 conjugated secondary capture antibody. The labelled panels show data of peptides 8, 9, 10, 11, 12, and 58 with peptide 8 being used as the negative control marker.

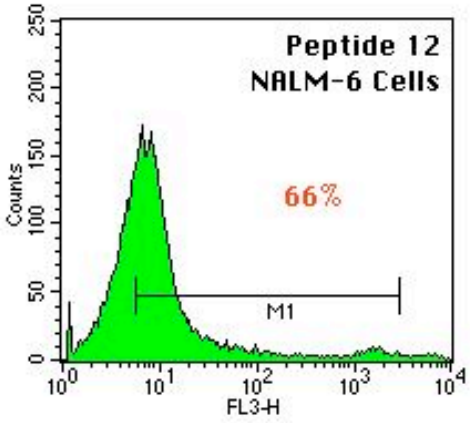
This experiment was repeated 3 times and these data are representative of these experiments.

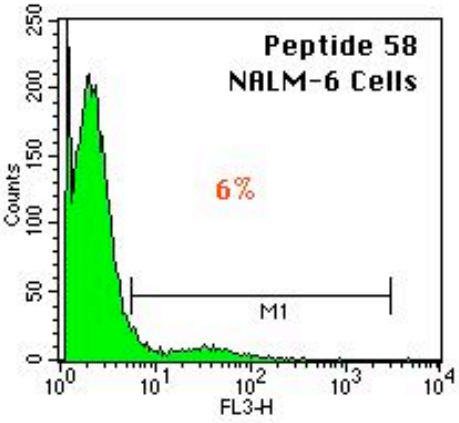

Peptide 8
NALM-6 Cells
Counts
FL3-H
M1


Peptide 9
NALM-6 Cells
29%
Counts
FL3-H
M1


Peptide 10
NALM-6 Cells
10%
Counts
FL3-H
M1


Peptide 11
NALM-6 Cells
78%
Counts
FL3-H
M1

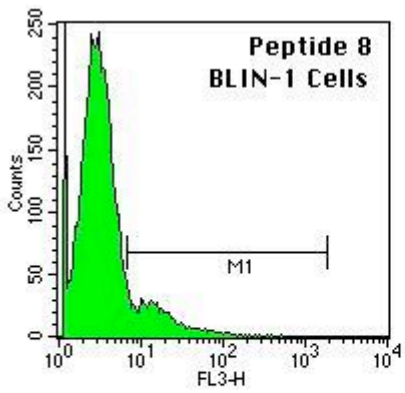

Peptide 12
NALM-6 Cells
66%
Counts
FL3-H
M1

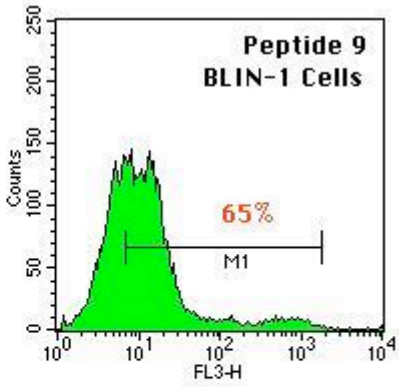

Peptide 58
NALM-6 Cells
6%
Counts
FL3-H
M1

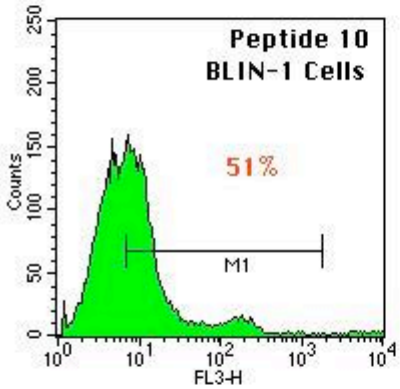
4.9 Peptide staining of BLIN-1 cells.

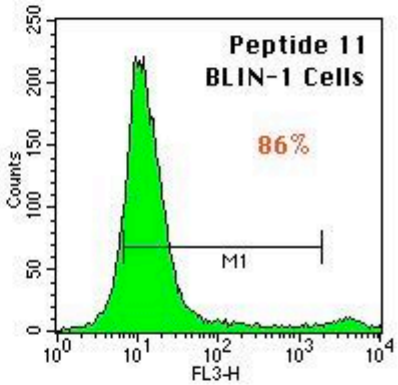
As before, this assay uses biotinylated peptides and a streptavidin-Cy 5 conjugated secondary capture antibody. The panels show data of peptides 8, 9, 10, 11, 12, and 58 with peptide 8 being used as the negative control marker. Peptides 9, 11 and 12 bind most strongly to the BLIN-1 cells.

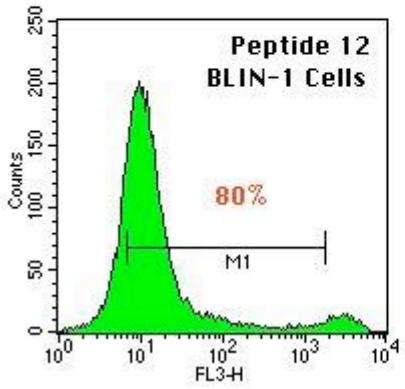
These data are consistent with experiments repeated 3 times.

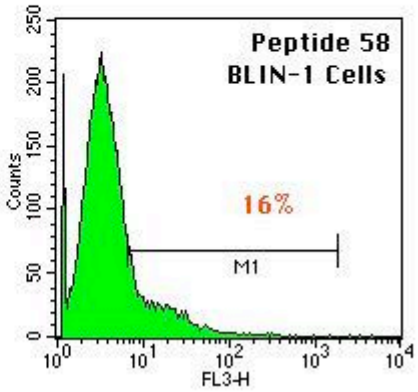

Peptide 8
BLIN-1 Cells
Counts
M1
FL3-H


Peptide 9
BLIN-1 Cells
65%
Counts
M1
FL3-H


Peptide 10
BLIN-1 Cells
51%
Counts
M1
FL3-H


Peptide 11
BLIN-1 Cells
86%
Counts
M1
FL3-H


Peptide 12
BLIN-1 Cells
80%
Counts
M1
FL3-H

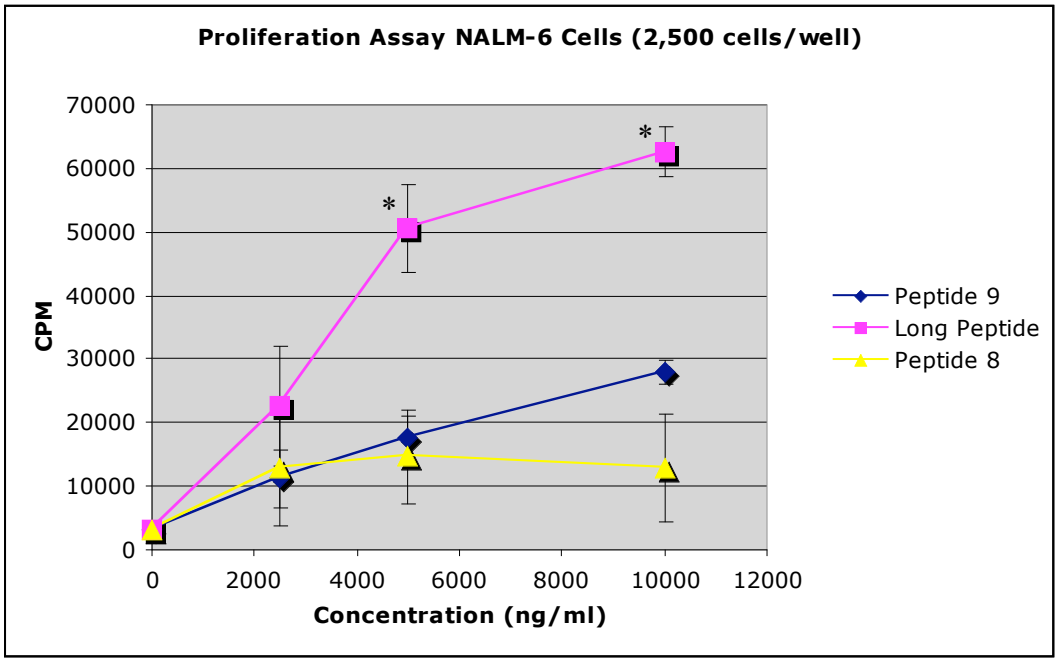

Peptide 58
BLIN-1 Cells
16%
Counts
M1
FL3-H

4.10 Effect of CD23 peptides on NALM-6 cells.

Proliferation assay graph showing effect of peptides 9, long and 8 on NALM-6 cells.

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 between corresponding concentrations of non-binding peptide 8 and peptides 9 and LP.

These data are consistent with experiments repeated to n=2.

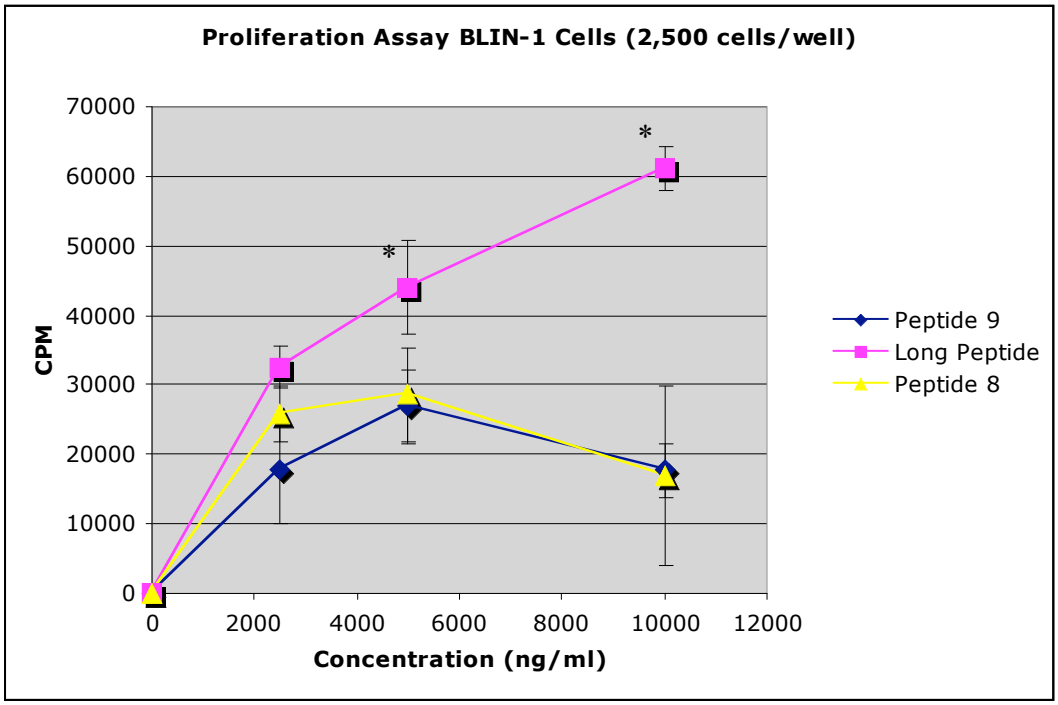

Proliferation Assay NALM-6 Cells (2,500 cells/well)
CPM
70000
60000
50000
40000
30000
20000
10000
0
0
2000
4000
6000
8000
10000
12000
Concentration (ng/ml)
*
*
Peptide 9
Long Peptide
Peptide 8

4.11 Effect of CD23 peptides on BLIN-1 cells.

Proliferation assay graph showing effect of peptides 9, long and 8 on BLIN-1 cells.

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 between the corresponding concentrations of non-binding peptide 8 and peptides 9 and LP.

These data are consistent with experiments repeated twice.

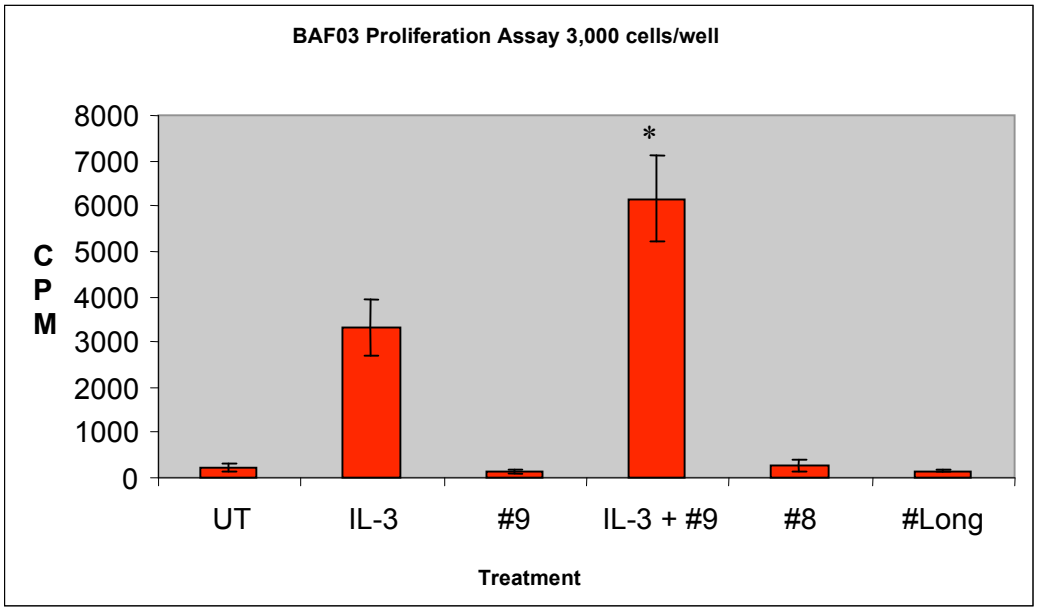

Proliferation Assay BLIN-1 Cells (2,500 cells/well)
CPM
70000
60000
50000
40000
30000
20000
10000
0
0
2000
4000
6000
8000
10000
12000
Concentration (ng/ml)
*
*
Peptide 9
Long Peptide
Peptide 8

4.12 Effect of CD23 peptides on BAF03 cells.

Proliferation assay graph showing effect of IL-3 (1ng/ml) and peptides 9, long and 8 (all at 5μg/ml) on the mouse cell line BAF03.

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 between IL-3 treated cells and cells treated with peptide 9 in addition to IL-3.

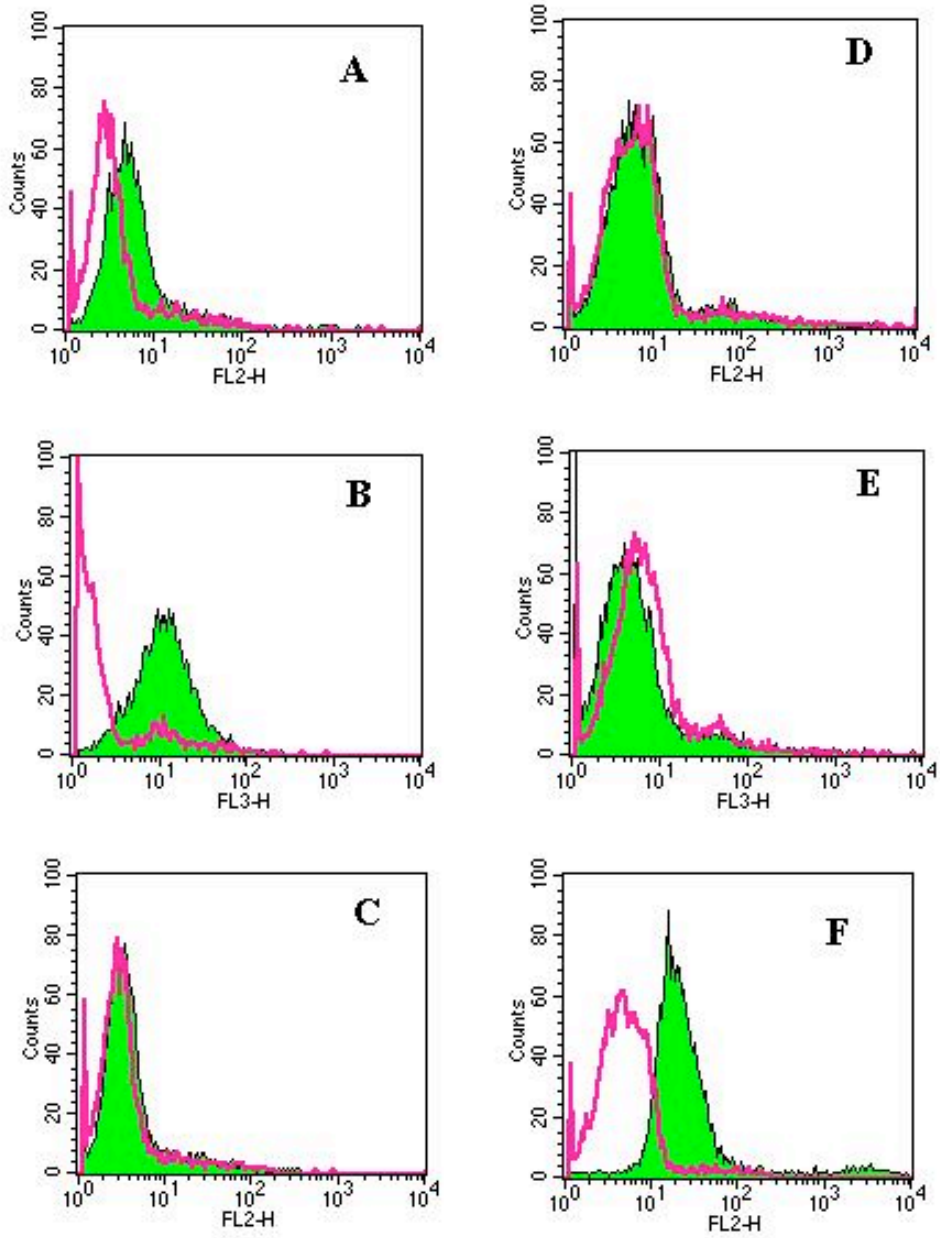
The data shown represent experiments performed 3 times.


BAF03 Proliferation Assay 3,000 cells/well
8000
7000
6000
5000
4000
3000
2000
1000
0
CPM
*
UT
IL-3
#9
IL-3 + #9
#8
#Long
Treatment

4.13 Expression of αvβ5 and αvβ3 integrins on myeloma cell lines H929 and KMS11.

KMS11 cells are shown on the left and H929 cells are shown on the right. 15F11 are A & D, P1F6 are B & E and 23C6 are C & F. 15F11 and P1F6 are both antibodies directed against αvβ5 and 23C6 antibody recognises αvβ3. Antibody staining is the filled area and isotype control is the pink line.

These data are representative of n=2.

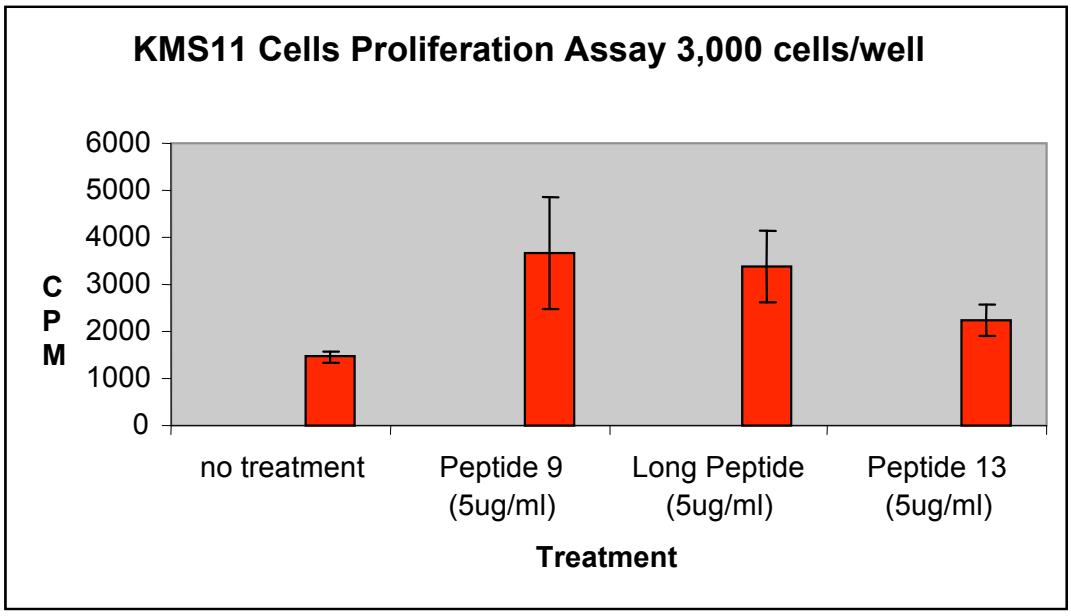

A
B
C
D
E
F
Counts
FL2-H
FL3-H

4.14 Effect of CD23 peptides on KMS11 cells.

Proliferation assay graph showing effect of peptides 9, long and 13 (all at 5μg/ml) on KMS11 cells plated at 3,000 cells/well.

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 between non-binding peptide 13 and peptides 9 and LP.

These data are representative of n=5.

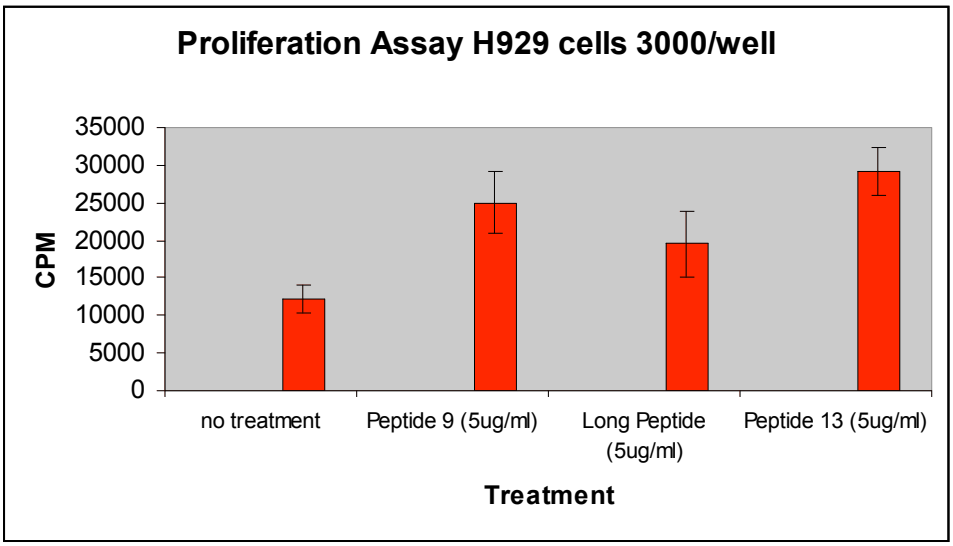

KMS11 Cells Proliferation Assay 3,000 cells/well
6000
5000
4000
3000
2000
1000
0
CPM
no treatment
Peptide 9 (5ug/ml)
Long Peptide (5ug/ml)
Peptide 13 (5ug/ml)
Treatment

4.15 Effect of CD23 peptides on H929 cells.

Graph showing proliferation data from an experiment carried out on H929 cells plated at 3,000 cells/well using peptides 8 and 9 and the long peptide (all at 5μg/ml).

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 between non-binding peptide 8 and peptides 9 and LP.

The data shown are representative of experiments performed 5 times.

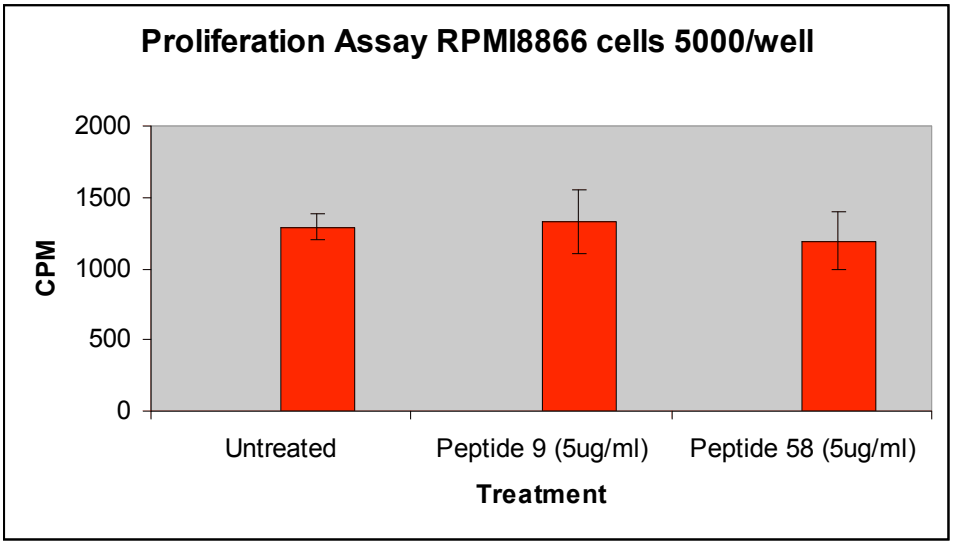

Proliferation Assay H929 cells 3000/well
CPM
35000
30000
25000
20000
15000
10000
5000
0
no treatment
Peptide 9 (5ug/ml)
Long Peptide (5ug/ml)
Peptide 13 (5ug/ml)
Treatment

4.16 Effect of CD23 peptides on RPMI8866 cells.

Graph showing proliferation assay data from an assay performed on the plasma cell line RPMI8866. Cells were plated at 5,000/well and peptides 9 and 58 were the treatments used (at 5ug/ml).

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 between non-binding peptide 58 and peptide 9.

The data shown are representative of experiments performed 5 times.


Proliferation Assay RPMI8866 cells 5000/well
2000
1500
1000
500
0
CPM
Untreated
Peptide 9 (5ug/ml)
Peptide 58 (5ug/ml)
Treatment

4.17 Expression of αvβ5 and αvβ3 integrins on RPMI8866 cells.

Flow cytometry data showing integrin expression. Panel A shows αvβ5 expression, the line represents isotype control and the filled area is integrin staining. Panel B shows αvβ3 expression. As before, the line is isotype control and the filled area is integrin staining.

The data shown are representative of experiments performed 2 times.

A B

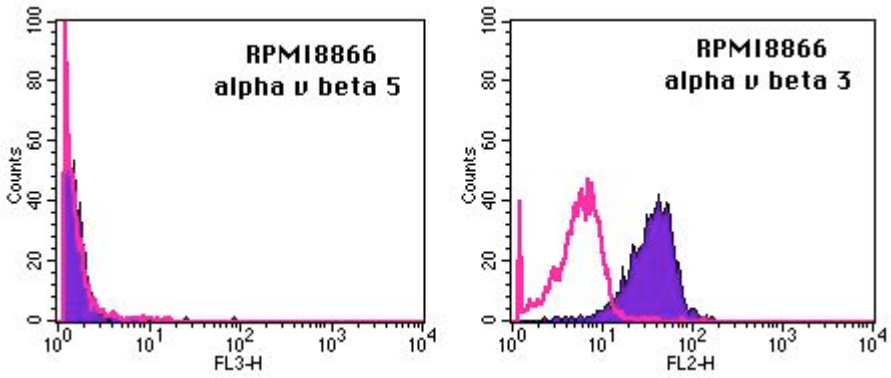
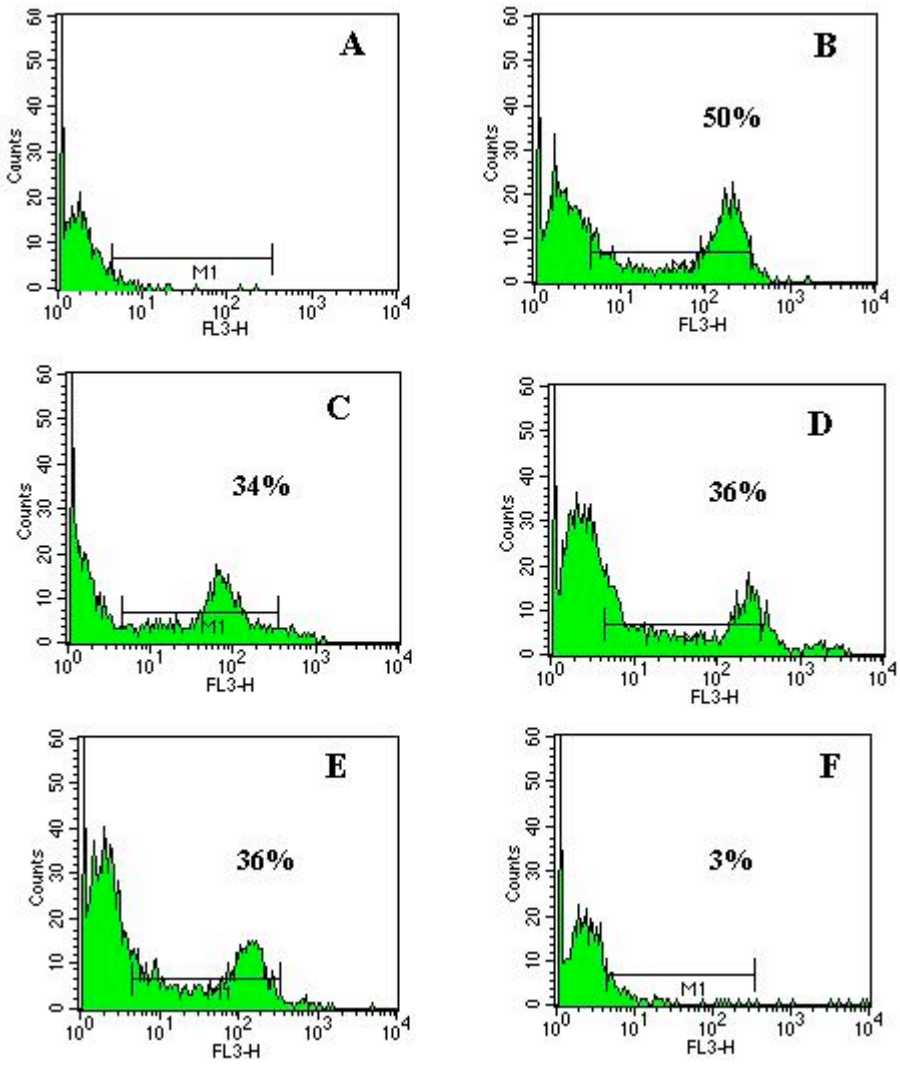

RPMI8866
alpha v beta 5
Counts
FL3-H
RPMI8866
alpha v beta 3
Counts
FL2-H

Figure 4.18 Peptide Binding to RPMI8866 cells.

As before, this assay uses biotinylated peptides and a streptavidin-Cy 5 conjugated secondary capture antibody. Panels A to F show data of peptides 8, 9, 10, 11, 12, and 58 respectively with peptide 8 being used as the negative control marker. Peptides 9, 11 and 12 bind most strongly to the NALM-6 cells.

The data shown are representative of experiments performed 3 times.


A
B
50%
C
34%
D
36%
E
36%
F
3%
Counts
FL3-H
M1

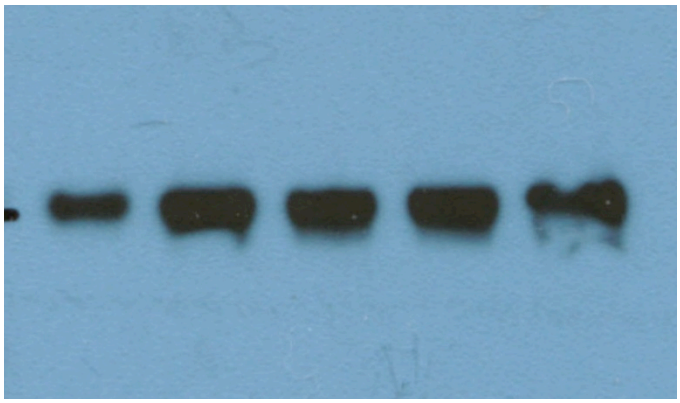
4.19 Expression of phospho-Akt-1 in SMS-SB cells.

Immunoblot showing protein expression from IPs performed on SMS-SB cells treated with peptide 9. Panel A shows phospho-Akt protein at times 0, 10, 30, 60 and 90 and panel B shows total Akt protein at times 0, 10, 30, 60 and 90.

This result was achieved twice.

A.

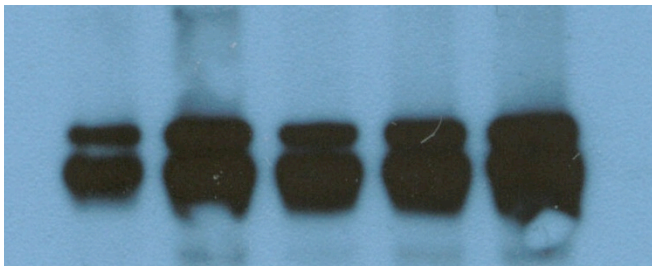
Time 0 10 30 60 90



Phospho-Akt

B.

Time 0 10 30 60 90



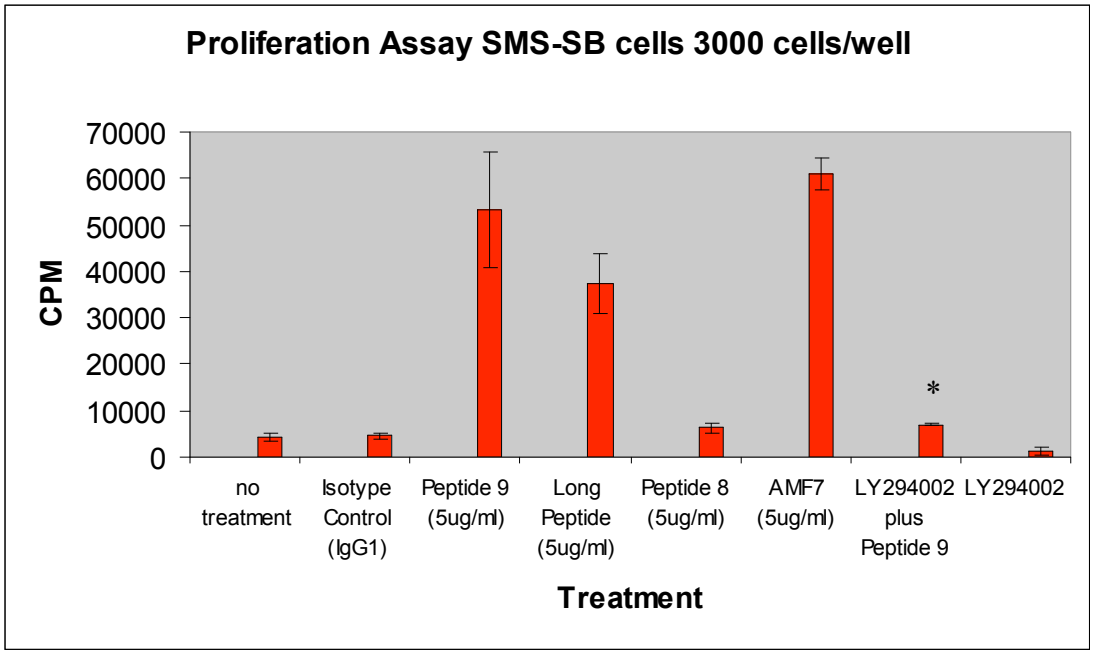
Total Akt

4.20 Effect of PI3Kinase (PI3K) inhibitor on SMS-SB cells.

Graph showing data from a proliferation assay performed using the P13K inhibitor LY294002 on SMS-SB cells.

The data displayed represent average values of triplicate treatments. Error bars denote standard deviations and statistics were calculated using the Student's T test. Asterisks signify P values of < 0.05 between inhibitor plus peptide 9/peptide 9 and inhibitor plus peptide 9/AMF7.

This assay was performed twice and these data are representative of these.


Proliferation Assay SMS-SB cells 3000 cells/well
CPM
70000
60000
50000
40000
30000
20000
10000
0
*
no treatment
Isotype Control (IgG1)
Peptide 9 (5ug/ml)
Long Peptide (5ug/ml)
Peptide 8 (5ug/ml)
AMF7 (5ug/ml)
LY294002 plus Peptide 9
LY294002
Treatment

CHAPTER 5

INTEGRIN ASSOCIATED PROTEIN (CD47)

5.1 Introduction

Integrin associated protein (IAP), also known as CD47, is known to form a signalling complex with the CD23 receptor αvβ3. This complex regulates leukocyte activation and mediates the phagocytosis of aging apoptotic leukocytes. This function resolves inflammation by removing these leukocytes before they disgorge their potentially harmful contents and trigger an inflammatory reaction [243].

Experiments using the B6H12 monoclonal antibody directed against CD47 have been performed by Mateo and colleagues upon various cell types and in soluble and immobilised format [233,234]. To date, results have indicated that ligation of CD47 has an effect on apoptosis – in some cell types CD47 stimulation initiates apoptosis and other cell types are resistant to apoptosis via this signal. Apoptosis occurring through CD47 has been found to take place via a caspase-independent pathway. The end results of CD47 ligation appear to be dependent upon the cell type involved and the presentation of the antibody, i.e. whether the antibody is soluble or immobilised [234]. Precursor and mature B cell lines were investigated for their apoptotic response following ligation of CD47 by the B6H12 antibody and the expression of CD47 was also determined to investigate whether a pattern could be established. Also, given that some of the B cells express αvβ3 and some do not, stimulation with the antibody was thought to be interesting as perhaps it would indicate whether the effect of the antibody was dependent upon the presence of the αvβ3/CD47 signalling complex or whether its effects were only due to signalling through CD47 alone. Apoptosis was measured following overnight incubation by staining with Annexin V and propidium iodide and CD47 expression was determined by flow cytometry. When analysing the flow cytometric data cells were not pre-gated on the basis of size and granularity so all events were included in the analysis. Quadrant gates were established following examination of singled-stained cells and an example of this is located in the materials and methods section (2.26). It should be noted that an observation of cells subjected to this assay is that extreme adherence occurs following the overnight incubation. Repeated pipetting up and down dislodged the majority of the cells but it is not possible to retrieve 100% of the cells initially plated out. This means that the

apoptotic status of these cells cannot be included in the data generated and it is not possible to be completely certain that the data represent accurately what is actually occurring in the cells.

5.2 Results

5.2.1 KMS 11 and H929 cells

These cell lines are derived from multiple myeloma patients and both have the chromosomal translocation, t(4:14). Previous studies of primary cells taken from multiple myeloma patients had shown that the cells underwent apoptosis upon CD47 ligation.

Figure 5.1 shows the pattern of expression of αvβ3 and αvβ5 by KMS11 and H929 cells as determined by flow cytometry. The KMS11 cells express αvβ5 (Figure 5.1 panels A and B) but not αvβ3 (Figure 5.1 panel C), whereas the H929 cells express little αvβ5 (Figure 5.1 panels D and E) but do express αvβ3 (Figure 5.1 panel F). The contrasting expression of integrins makes these cells useful for investigating apoptotic responses via anti-CD47 antibody as one line expresses the CD47 signalling partner αvβ3 and the other does not. It could be postulated that if the apoptotic stimulus requires CD47 to be partnered to αvβ3 then these two cell types should not respond identically. The antibody used is not identical to the stimulus used previously by another group on myeloma cells – they used bivalent single chain antibody fragments in an *in vivo* model [232].

All haematopoietic and non-haematopoietic cells express CD47 [244]. The KMS11 cells and H929 cells were stained with anti-CD47 and analysed cytometrically to ascertain their CD47 expression. Figure 5.2 shows the results of this analysis and it can be seen that both cell lines express high levels of CD47 – the KMS11 cells (panel B) express more than the H929 cells (panel A). Figure 5.3 shows data obtained from KMS11 cells treated overnight with anti-CD47, or suitable controls, and stained with both Annexin V and propidium iodide and it can be seen that these cells respond to both soluble anti-CD47 and immobilised anti-CD47. In both cases, the CD47 treatments performed on the KMS11 cells result in increased apoptosis when compared with untreated and isotype control treated cells. This is consistent with previous findings by Kikuchi and colleagues working with primary cells from myeloma

patients [232]. A detailed analysis of the quadrant percentages shows that there is a decrease in viable cells (lower left quadrant) from the control soluble treatments to the soluble anti-CD47 treatments of 17.5% and this difference is due mainly to an increase in cells positive for both annexin V and propidium iodide (cells undergoing apoptosis). Examination of the experiments performed with anti-CD47 in an immobilised form again shows a decrease in viable cells (55% to 34%) and this decrease is due for the most part to an increase in cells only positive for propidium iodide (late apoptotic, dying cells) – 12% change versus 7% change in the upper right quadrant.

Figure 5.4 shows data from the same experiment performed on H929 cells. Similar results were recorded with the anti-CD47 treatments, with both increasing apoptosis. Inspection of the upper right quadrant reveals that when comparing the soluble control treatment with the soluble anti-CD47 treatment that the CD47 stimulation has caused a 7% increase in cells double positive for annexin V and propidium iodide (i.e. cells actively undergoing apoptosis). The cells treated with immobilised antibodies had a different profile from the control cells and treated cells having upper right quadrant percentages of 14.5% and 14% respectively. Here the main difference was in the upper left quadrant – the propidium iodide positive, annexin V negative cells. A 17% increase in these cells was observed between the control and the CD47 ligation. These cells are late apoptotic, dying cells. The difference between the two cell types was minimal with slightly more apoptosis in KMS11 cells treated with soluble anti-CD47 and slightly more apoptosis in H929 cells treated with immobilised anti-CD47. No pattern connecting the expression levels of CD47 or the presence/absence of $\alpha v\beta 3$ is revealed by these experiments.

5.2.2 SMS-SB cells and 697 cells

As shown previously, these cells express only $\alpha v\beta 5$ as a CD23 receptor and do not express CD47's signalling partner $\alpha v\beta 3$. Any effect on apoptosis via CD47, it could therefore be postulated, would be due to signalling through only CD47 and not the signalling complex formed with $\alpha v\beta 3$. These cells are representative of an early stage in B cell development – the pre-B phase.

Figure 5.5 shows a flow cytometric analysis of the expression of CD47 on these two cell types and it can be seen that, again, both express high amounts of CD47 on their surfaces and that it is the 697 cells which have the most CD47. Overnight treatments with anti-CD47 were applied and the results are shown in Figures 5.6 and 5.7. In Figure 5.6 it was observed that when SMS-SB cells were exposed to soluble anti-CD47 an increase in apoptotic cells occurred but when the cells were placed upon immobilised anti-CD47 the opposite happened and more viable cells were detected. Comparing the control soluble antibody to the soluble α-CD47 treatment there has been an increase in cells in the upper right quadrant (5% increase) and an increase in the upper left quadrant (4% increase). As for the immobilised antibody data, when comparing control to treatment this time there was a decrease of 6% of cells staining positive for annexin V and propidium iodide and a slight increase in cells positive for only propidium iodide (2.5%). The viable cell percentage increased by 5% from 39% in the control to 44% in the immobilised α-CD47 treatment.

The same experiment was carried out using 697 cells and the data collected are represented in Figure 5.7. Looking again at soluble control versus soluble α-CD47 cell stimulations, what is seen is that with the upper left quadrant (propidium iodide only positive cells) there has been a change from 40% to 34% - a decrease of 6%. The upper right quadrant data has shifted in the opposite direction - from 34.5% to 42% (7.5% increase). The cells treated with immobilised control antibody and immobilised α-CD47 antibody have responded with an increase in late apoptotic/dying cells of only 1% and a decrease in cells undergoing apoptosis from 22% to 15%. When compared to the data from the untreated cells which have been incubated for the same amount of time as the treated cells, a large change in the upper right quadrant has occurred in the soluble α-CD47 treated cells. This change amounts to 26%. These cells appear to be responding with increased apoptosis when stimulated with the soluble CD47 stimulation and with less apoptosis (1% less than untreated cells) when treated with immobilised αCD47. In terms of CD47 expression and expression of αvβ3 and αvβ5 integrins these two cell types are very similar and from the data generated, they appear to respond similarly to stimulation through CD47. It appears to be crucial that the ligation of CD47 is via the soluble phase to enable apoptosis to take place. A tentative suggestion could be that perhaps the absence of αvβ3 prevents induction of apoptosis via the CD47

mechanism when the antibody is immobilised. This type of interaction is perhaps similar to the action of a cell-bound CD47 ligand with CD47 rather than a soluble contact and would allow for variation in response to soluble and membrane-bound CD47 partners. Another possibility is that response to CD47 stimulation depends on the differentiation stage of the cells hence the mature B cells respond differently to the pre-B cells.

5.2.3 NALM-6 and BLIN-1 cells

NALM-6 and BLIN-1 cells are also pre-B cells but are thought to represent a slightly later phase in development than the SMS-SB cells and 697 cells. These cell lines also express αvβ3 as well as αvβ5 as CD23 receptors and therefore signalling via the CD47/αvβ3 complex is possible. Figure 5.8 shows FACScan data confirming the expression of the two integrins and Figure 5.9 shows CD47 expression. The data collected following analysis of apoptosis in BLIN-1 cells treated overnight with anti-CD47 is shown in Figure 5.10. These cells do not respond to treatments with the antibody in either of its two presentations. Figure 5.11 shows the consequence of treatment of the NALM-6 cells and little response was noted in the cells exposed to soluble anti-CD47 antibody (increases of only 2% in upper right quadrant and 7% upper left quadrant). However, treatment with immobilised antibody had the same effect here as it had on the SMS-SB cells and 697 cells, i.e. apoptosis decreased and the cells were more viable in comparison with the control. This increase in the number of viable cells from 66% to 72% is a consequence of a 7% decrease in dead cells and a 6% increase in early apoptotic cells so although cells positive for annexin V increased the overall viability increased with a drop in dead cells. So what has been observed is that NALM-6 cells, which express αvβ3 and αvβ5 respond similarly to SMS-SB cells and 697 cells which only express αvβ5 and appear to express less CD47 than the NALM-6 cells. It is not possible to provide an explanation for this in terms of identifying a pattern between integrin and CD47 expression.

5.3 Conclusions

Different B cell types respond in different ways to stimulation via CD47. Some cell types do not respond (BLIN-1 cells), whereas other cells respond by undergoing apoptosis regardless of whether the stimulation is from a soluble or immobilised antibody (myeloma cell lines KMS11 and H929). SMS-SB cells, our model cell line, and 697 cells respond with an increase in apoptosis following stimulation by soluble antibody when compared with immobilised antibody treatment. NALM-6 cells also respond in this way to the immobilised antibody but their response to the soluble antibody is less obvious at only a 4% difference in viable cells compared with the control. The NALM-6 cells treated in this way respond with an increased amount of dead cells, in the upper left quadrant which are staining only for propidium iodide, which amounts to 10%.

Apoptosis induction via CD47 is thought not to be dependent upon signalling via the complex it forms with αvβ3 and the work shown in this thesis does not refute this since, with regard to the cells which responded with a decrease in overall apoptosis, one cell type expressed αvβ3 and the other did not. If an apoptotic response was depe ndent upon the formation of this signalling complex it would be more likely that the cells expressing αvβ3 would have responded with an overall increase in apoptosis. Cells which only express αvβ5 are capable of responding in an apoptotic manner to soluble anti-CD47 stimulation (SMS-SB and 697 cells) so it would follow that the signalling complex is not required to facilitate transmission of the death signal via CD47.

Figure 5.1 Expression of αvβ5 and αvβ3 integrins on myeloma cell lines H929 and KMS11. KMS11 cells are shown on the left and H929 cells are shown on the right. 15F11 are A & D, P1F6 are B & E and 23C6 are C & F. 15F11 and P1F6 are both antibodies directed against αvβ5 and 23C6 antibody recognises αvβ3. Antibody staining is the filled are and isotype control is the pink line.

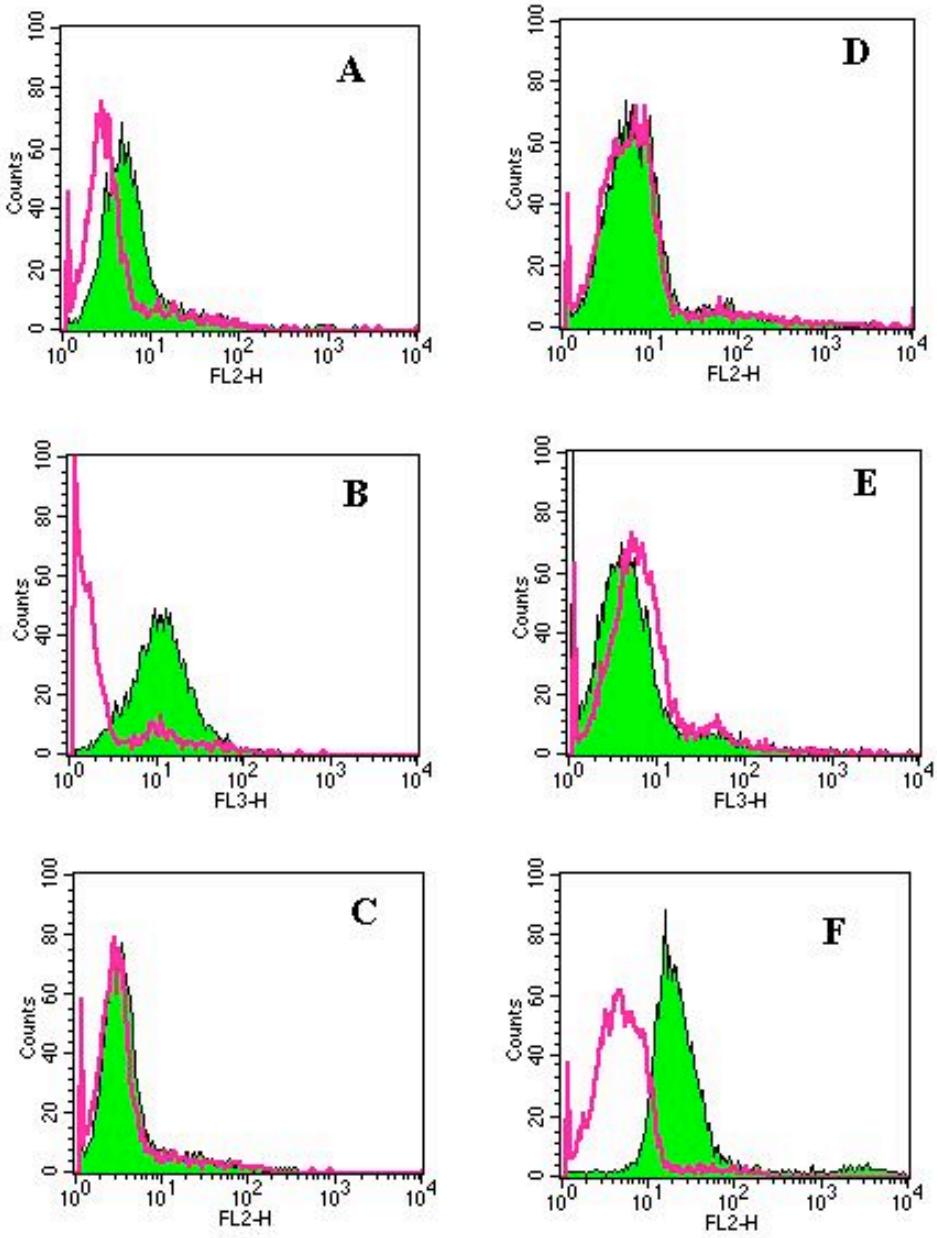
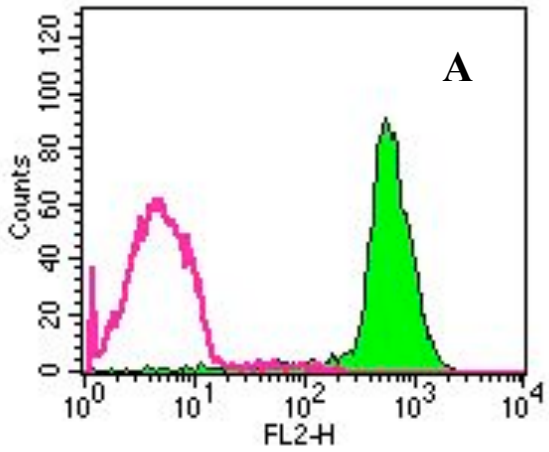

A
B
C
D
E
F
Counts
FL2-H
FL3-H

Figure 5.2. **Expression of CD47** (green solid) and isotype control (pink line) on A. H929 cells and B. KMS11 cells.


A
Counts
0
20
40
60
80
100
120
10^0
10^1
10^2
10^3
10^4
FL2-H

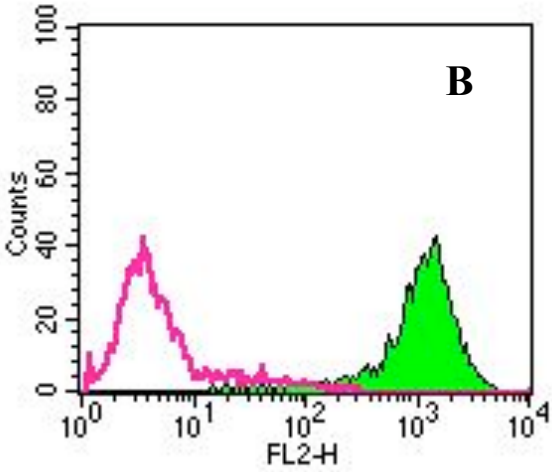

B
Counts
0
20
40
60
80
100
10^0
10^1
10^2
10^3
10^4
FL2-H

Figure 5.3 Analysis of CD47-regulated apoptosis in KMS11 cells.

A - Untreated

B – Isotype control (soluble)

C – Anti-CD47 (soluble)

D – Isotype control (immobilised)

E – Anti-CD47 (immobilised)

Quadrant percentages are marked on each plot and represent as follows:- lower left quadrant - cells which do not stain for either propidium iodide or annexin V and are not undergoing apoptosis; lower right - cells which stain positive for annexin V only and are undergoing early apoptosis; upper right – cells which stain positive for both markers and are in late apoptosis and upper left – cells which stain positive for propidium iodide only and represent dead cells.

These are data for a single representative experiment repeated a minimum of 3 times.

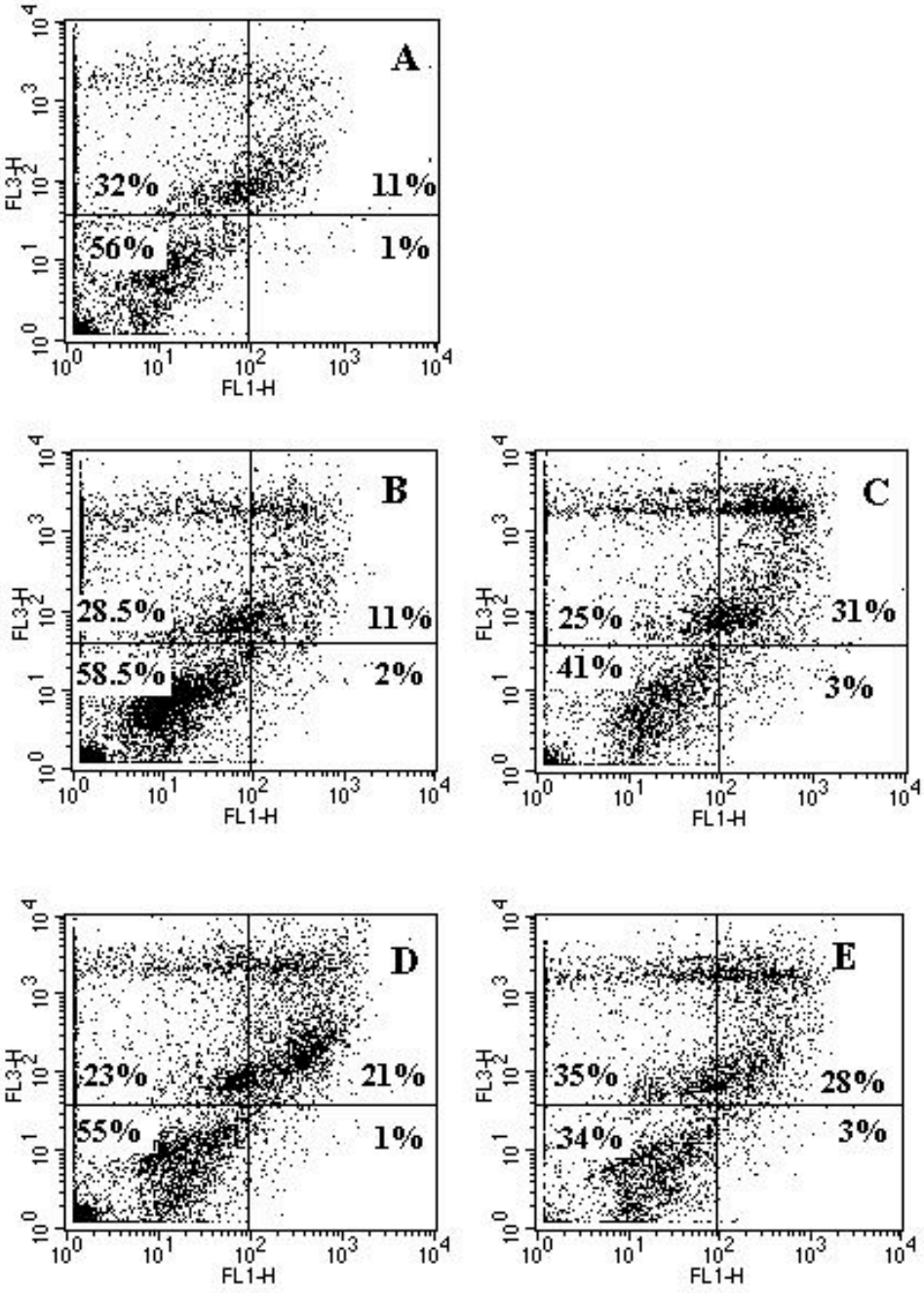

A
32%
11%
56%
1%
B
28.5%
11%
58.5%
2%
C
25%
31%
41%
3%
D
23%
21%
55%
1%
E
35%
28%
34%
3%
FL1-H
FL3-H

Figure 5.4 Analysis of CD47-regulated apoptosis in H929 cells.

A - Untreated

B – Isotype control (soluble)

C – Anti-CD47 (soluble)

D – Isotype control (immobilised)

E – Anti-CD47 (immobilised)

Quadrant percentages are marked on each plot as before.

These are data for a single representative experiment repeated a minimum of 3 times.

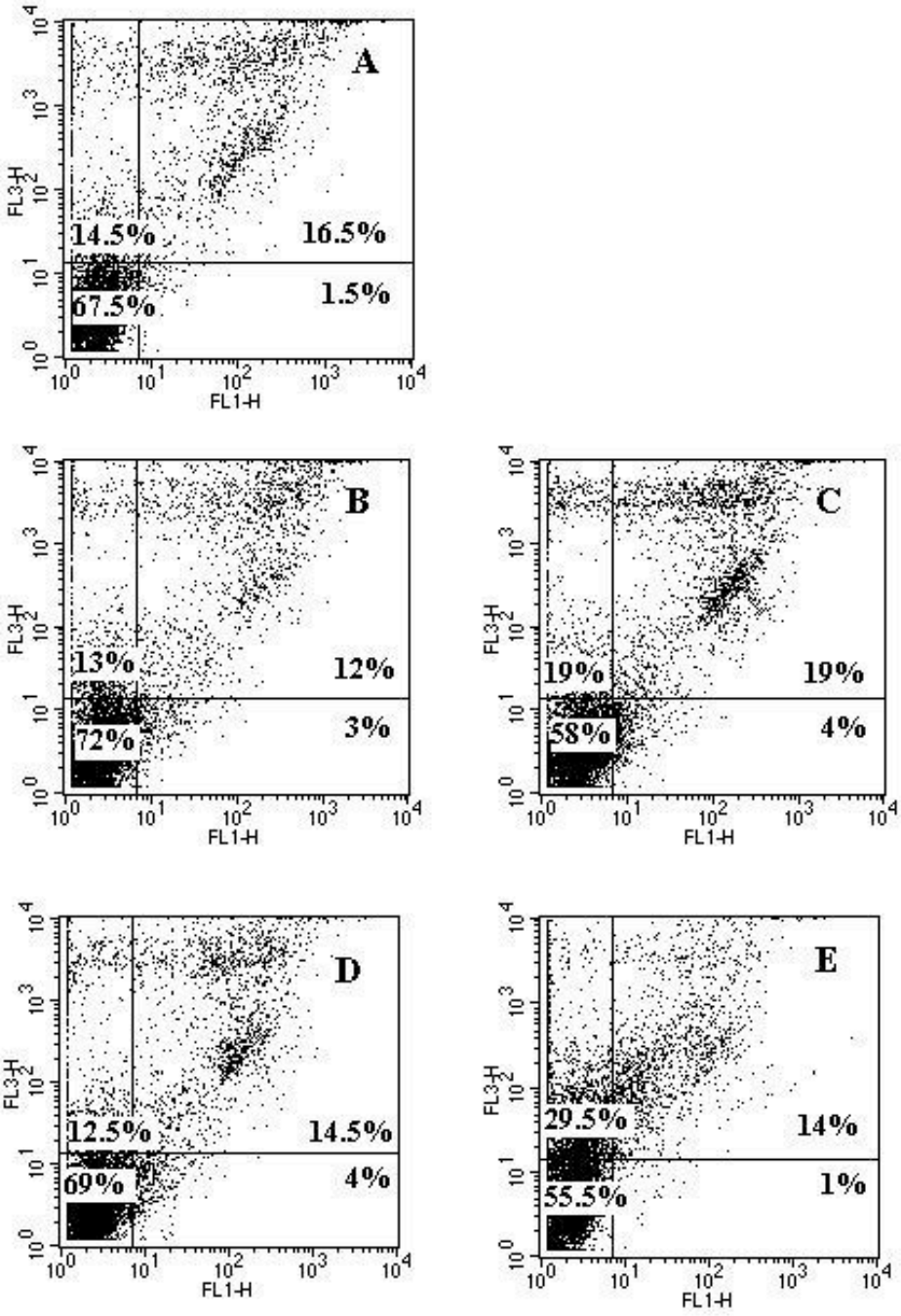

A
14.5%
16.5%
67.5%
1.5%
B
13%
12%
72%
3%
C
19%
19%
58%
4%
D
12.5%
14.5%
69%
4%
E
29.5%
14%
55.5%
1%
FL1-H
FL3-H

Figure 5.5. **Expression of CD47** (green solid) and isotype control (pink line) on A. SMS-SB cells and B. 697 cells.

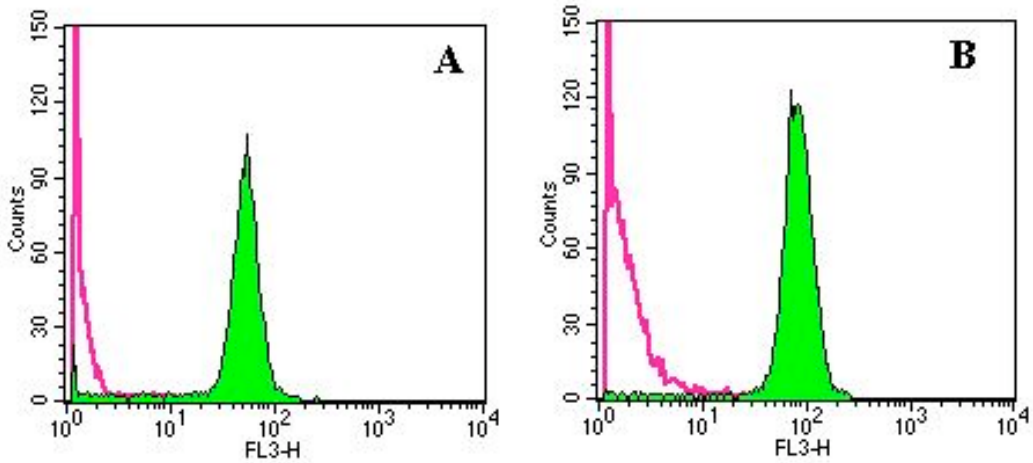

A
B
Counts
FL3-H
0
30
60
90
120
150
10^0
10^1
10^2
10^3
10^4

Figure 5.6 Analysis of CD47 regulated apoptosis in SMS-SB cells.

A - Untreated

B – Isotype control (soluble)

C – Anti-CD47 (soluble)

D – Isotype control (immobilised)

E – Anti-CD47 (immobilised)

Quadrant percentages are marked on each plot as before.

These are data for a single representative experiment repeated a minimum of 3 times.

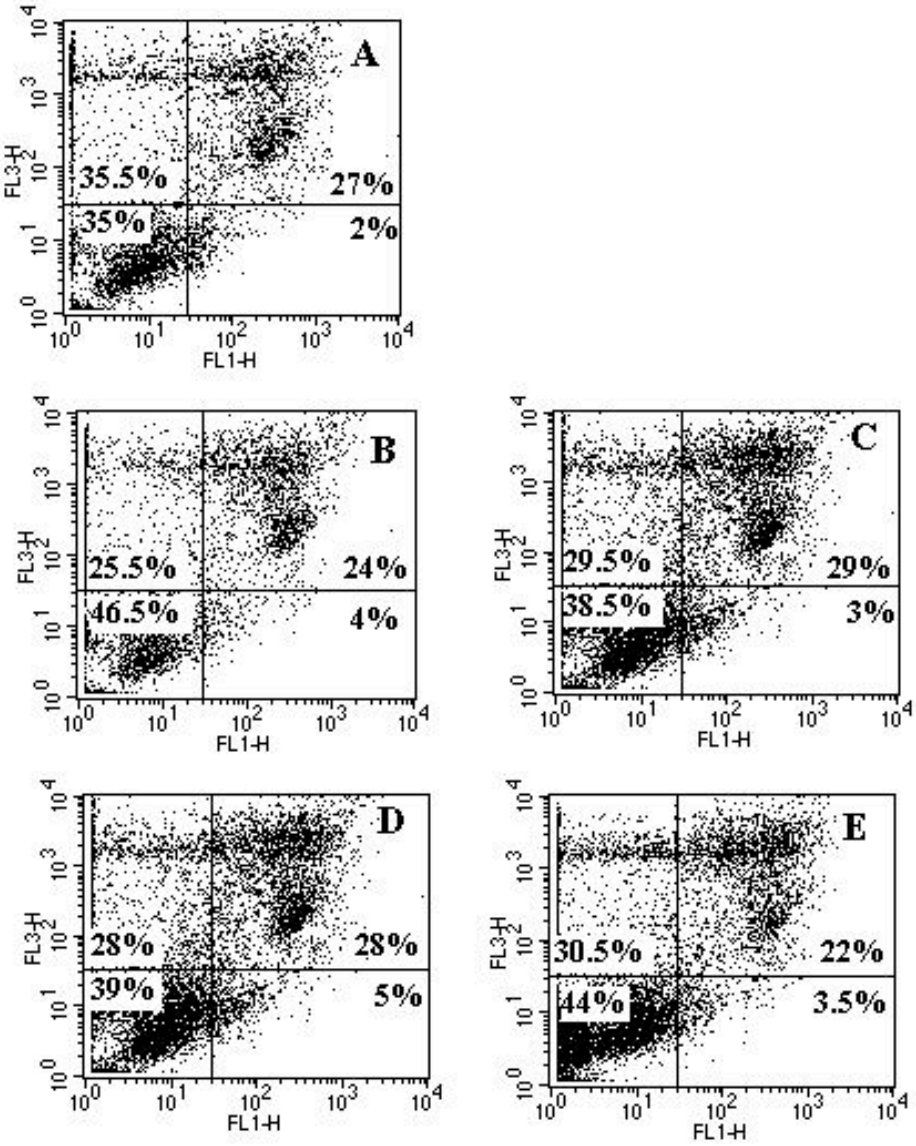

A
35.5%
27%
35%
2%
B
25.5%
24%
46.5%
4%
C
29.5%
29%
38.5%
3%
D
28%
28%
39%
5%
E
30.5%
22%
44%
3.5%
FL1-H
FL3-H

Figure 5.7 Analysis of CD47-regulated apoptosis in 697 cells.

A - Untreated

B – Isotype control (soluble)

C – Anti-CD47 (soluble)

D – Isotype control (immobilised)

E – Anti-CD47 (immobilised)

Quadrant percentages are marked on each plot as before.

These are data for a single representative experiment repeated a minimum of 3 times.

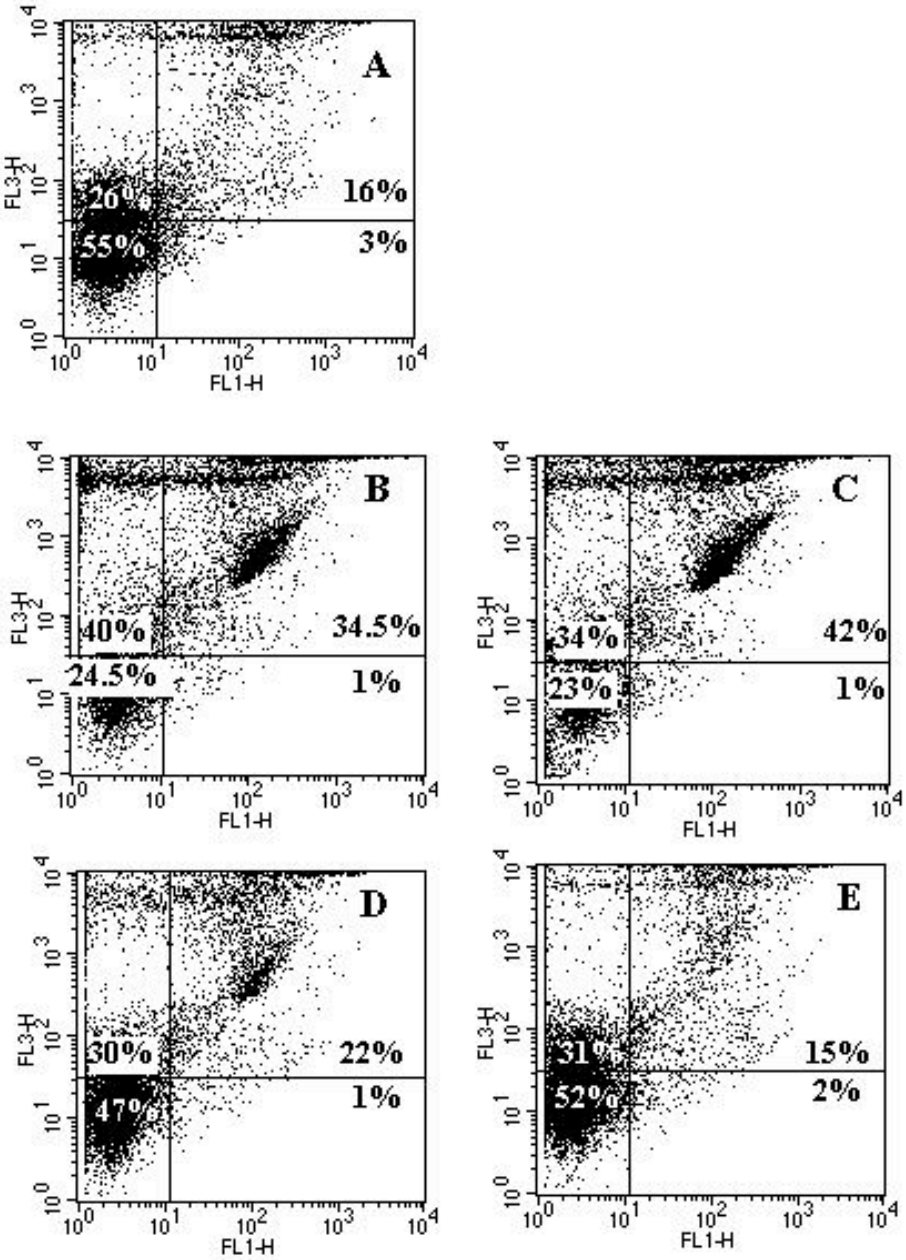

A
26%
16%
55%
3%
B
40%
34.5%
24.5%
1%
C
34%
42%
23%
1%
D
30%
22%
47%
1%
E
31%
15%
52%
2%
FL1-H
FL3-H

Figure 5.8 Expression of αvβ3 and αvβ5 on BLIN-1 and NALM-6 cells. Green solid represents integrin staining and pink lines isotype controls. A and B show BLIN-1 staining of αvβ5 and αvβ3 respectively and C and D show NALM-6 staining of αvβ5 and αvβ3 respectively.

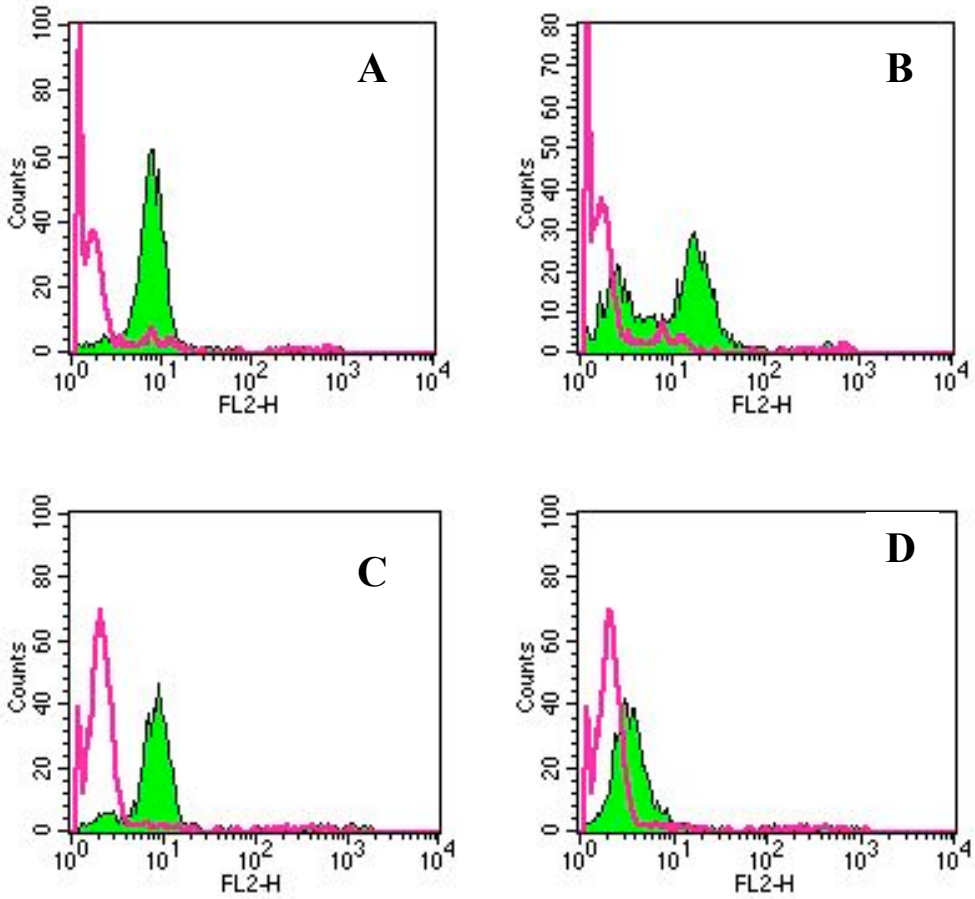

A
B
C
D
Counts
FL2-H

Figure 5.9. **Expression of CD47** (green solid) and isotype control (pink line) on A. BLIN-1 cells and B. NALM-6 cells.

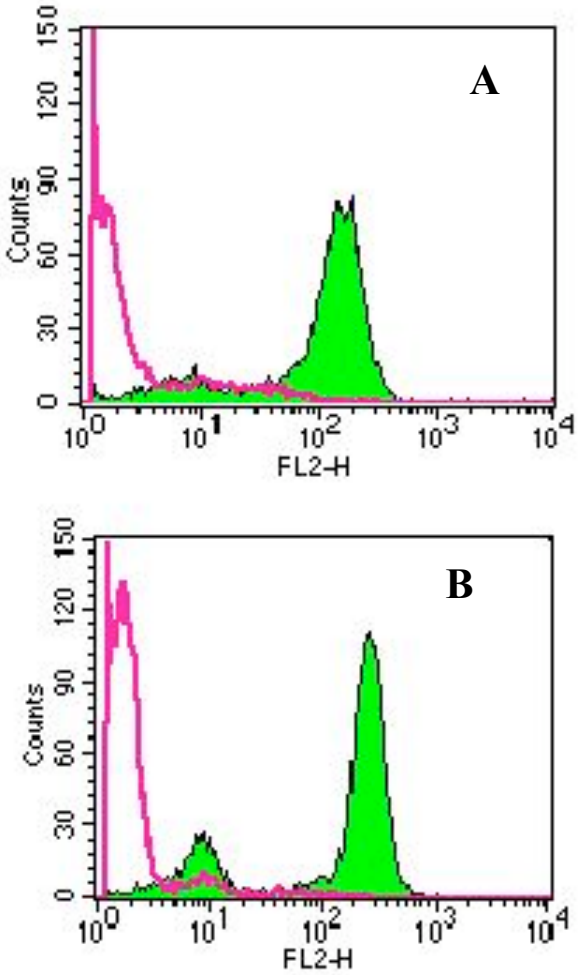

A
Counts
FL2-H
B
Counts
FL2-H

Figure 5.10 Analysis of CD47-regulated apoptosis in BLIN-1 cells.

A - Untreated

B – Isotype control (soluble)

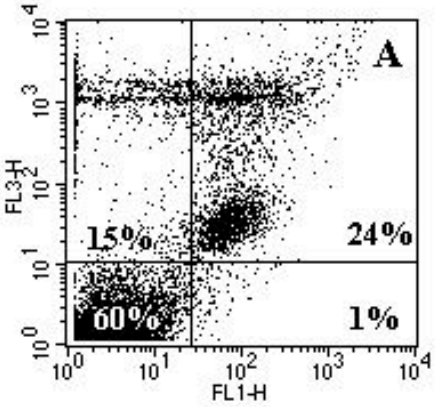
C – Anti-CD47 (soluble)

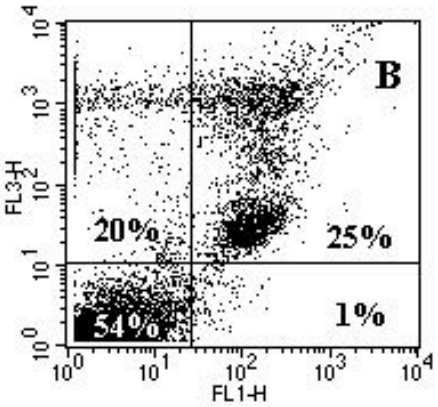
D – Isotype control (immobilised)

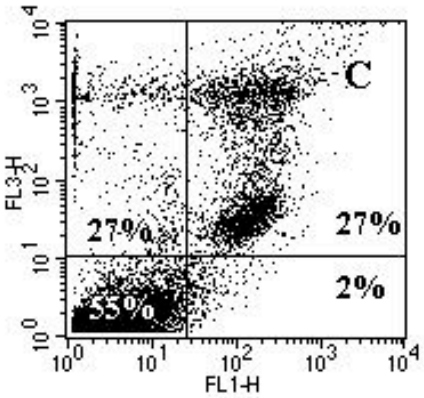
E – Anti-CD47 (immobilised)

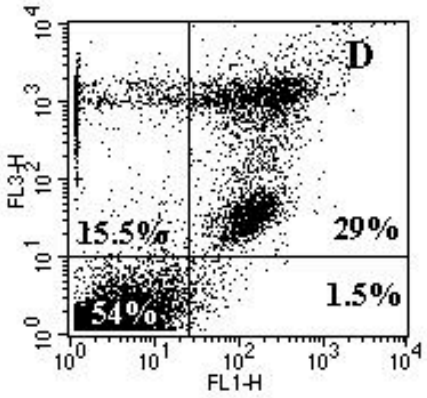
Quadrant percentages are marked on each plot as before.

These are data for a single representative experiment repeated a minimum of 3 times.


A
15%
24%
60%
1%
FL3-H
FL1-H


B
20%
25%
54%
1%
FL3-H
FL1-H


C
27%
27%
55%
2%
FL3-H
FL1-H


D
15.5%
29%
54%
1.5%
FL3-H
FL1-H

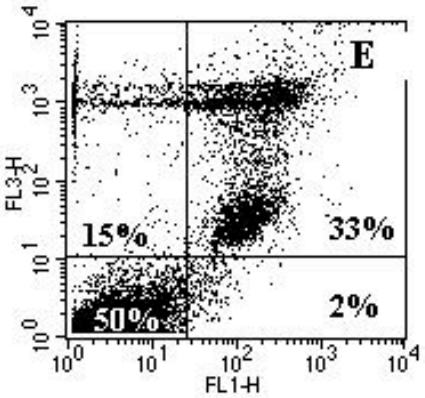

E
15%
33%
50%
2%
FL3-H
FL1-H

Figure 5.11 Analysis of CD47-regulated apoptosis in NALM-6 cells. A - Untreated

B – Isotype control (soluble)

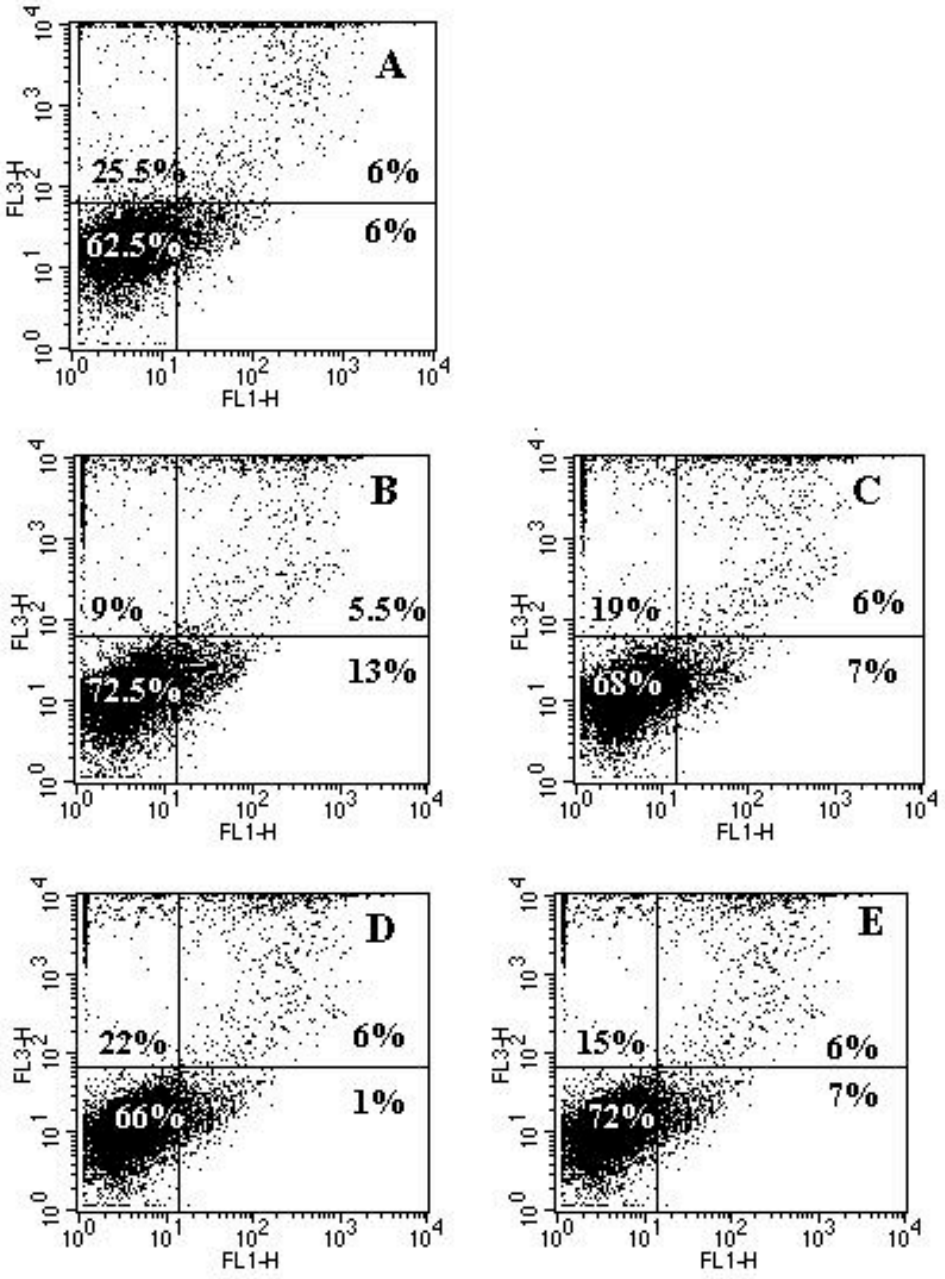
C – Anti-CD47 (soluble)

D – Isotype control (immobilised)

E – Anti-CD47 (immobilised)

Quadrant percentages are marked on each plot as before.

These are data for a single representative experiment repeated a minimum of 3 times.


A
25.5%
6%
62.5%
6%
B
9%
5.5%
72.5%
13%
C
19%
6%
68%
7%
D
22%
6%
66%
1%
E
15%
6%
72%
7%
FL3-H
FL1-H

CHAPTER 6

DISCUSSION

6.1 Main Conclusions

CD23 has been described as a multi-functional protein which can be expressed in membrane and soluble forms [111]. In this thesis its interaction with the integrin αvβ5 and the effects thereof were investigated. CD23 expression on hMSCs has been described [249] but endeavours to confirm this in a telomerised stromal cell population were unfruitful. The possibility that these cells are not producing CD23 protein has to be considered when taking results attained here into account. Only CD23 message was detected following investigation of hMSCs RNA although real-time PCR was not carried out to fully characterise the gene expression of CD23. Since CD23 protein was not found on the hMSCs it followed that interpretation of attempts to block the αvβ5 interaction with CD23 in cell culture experiments was precluded. These experiments were designed to exploit the observed growth pattern between hMSCs and SMS-SBs. The hypothesis was that CD23 on the hMSCs and αvβ5 on the SMS-SB cells interacted to produce a pro-survival signal to the precursor cells. Data confirming the presence of αvβ5 on the SMS-SBs is displayed in Chapter 3. Antibodies directed against various CD23 domains had no effect on the growth of SMS-SB cells on hMSC feeder layers. Experiments (not shown) designed to ascertain whether soluble factors are solely responsible for the positive growth effect were carried out. This involved taking conditioned media from the hMSCs and using this to culture the SMS-SB cells. This media was not able to sustain SMS-SB growth. In vivo in the bone marrow microenvironment strong adherence of B cell precursors to stromal cells occurs so logically it would follow that adhesions are very important to viability. The hMSCs obviously provide all of the contacts and growth factors required to sustain growth of the B cell precursors but a clear role for CD23 could not be established. Another interesting observation was made regarding this co-culture system. It was noted that the hMSC cells enjoy better viability in the presence of the SMS-SB cells and without the presence of these cells the stromal cells lose adherence and die.

Use of RKC-containing CD23-derived peptides revealed that growth was stimulated in cell lines which (a) express either αvβ5 alone or αvβ5 and αvβ3 together and (b) were of a precursor B cell phenotype. Cells belonging to a later stage in development, e.g. myeloma

cell lines which are plasma cell-like, did not respond to these peptides. The growth factor action of RKC-containing CD23 peptides appears to be confined to a particular stage in B lymphopoiesis and the presence of the integrins which are acting as receptors for the peptides is not sufficient to drive this proliferation. Although the αvβ5 integrin is the main candidate as the receptor these peptides are acting through [251] it has to be considered that they are recognizing αv. The anti-αv antibody AMF7 has a positive effect on SMS-SB growth and the peptides display varying binding and growth profiles when tested on other precursor B cell lines. Follow-up experiments to clarify this could include the use of the CD23 peptides on cell lines which express or have been engineered to express αv but not β5 or BIACORE analysis on αv and other αv integrins. If the CD23-derived peptides have a truly representative effect to that of the entire CD23 molecule it would be reasonable to predict that they should interact with established CD23 receptors. The αv integrins identified as CD23 receptors are αvβ3 and αvβ5. Perhaps the CD23-derived peptides are able to interact beyond the CD23 receptor repertoire and this is what is being observed here.

In SMS-SB cells ligation of the αvβ5 integrin signals via phosphorylation of the kinase Akt and this occurs following activation of PI3K. Due to time constraints and technical difficulties further elucidation of this signalling pathway was not possible.

CD23 also interacts with the integrin αvβ3 and this integrin has been described as part of a signalling complex with CD47 or IAP. Signalling through CD47 has been reported to induce apoptosis in various cell lines and primary cells but this signalling mechanism has not been fully defined at present. Of the cell lines investigated in this thesis some express αvβ3 alone, some express αvβ5 alone and some express both αvβ3 and αvβ5 so they are suitable for analysis to determine whether CD47 requires αvβ3 to induce apoptosis. All cell lines express high amounts of CD47. This investigation revealed that myeloma cell lines responded to stimulation via CD47 with increased apoptosis regardless of the presentation of the antibody. The responses of other cell lines were less decisive. The SMS-SB cells and 697 cells underwent increased apoptosis when the anti-CD47 antibody was in soluble form but not when it was in immobilised form and the NALM-6 cells also responded in this manner. SMS-SB cells and 697 cells express αvβ5 but not αvβ3 and NALM-6 cells express both αvβ3 and

αvβ5. BLIN-1 cells also express both of these integrins and these cells were the only cell line which did not exhibit any increase in apoptosis when treated with either soluble or immobilised anti-CD47.

6.2 Effect of RKC-containing CD23-derived peptides on proliferation

Treatment of various cell lines with RKC-containing CD23-derived peptides revealed that a positive growth response depended upon two factors: the presence of the αvβ5 integrin; and a precursor phenotype. Cell lines with a plasma cell-like phenotype were non-responsive to the peptides despite the presence of the αvβ5 integrin. Other approaches such as testing of a precursor B cell line expressing only αvβ3 or using RNAi to knock down expression of αvβ5 in NALM-6 or BLIN-1 cells would have confirmed whether stimulation via this integrin would produce an increase in cellular proliferation. In the absence of other evidence it is possible that the effect on cellular proliferation could occur via either of the αvβ3 and αvβ5 integrins in NALM-6 or BLIN-1 cells but that in SMS-SB cells and 697 cells this can only occur via αvβ5 as this is the only CD23 receptor expressed by these cells.

A pattern of integrin expression emerges when analysing these cells in that it appears αvβ5 is expressed at early stages with αvβ3 being expressed as development progresses. The 697 and SMS-SB cells express αvβ5 but not αvβ3 whereas the NALM-6 and BLIN-1 cells which are representative of a slightly more advanced stage in B lymphopoiesis express both of these integrins. A precedent for such an integrin switch expression model exists in osteoclast development [222]. It is possible that as cells lose their αvβ5 expression whilst they progress through normal development they no longer respond to stimulation by CD23. The fact that normal peripheral blood B cells do not express αvβ5 and ALL cells universally express αvβ5 supports this assertion and suggests a role for this proliferative interaction in neoplasia.

Signalling through αvβ5 in lymphoid precursors occurs via PI3K. Changes in levels of phospho-Akt (phosphorylation site serine 473) were detected by immunoblotting. Further eludication of the signalling pathways upstream and downstream of this kinase was not possible due to time constraints but examination of proteins such as Erk, the BH3 only

protein Bad or Mdm2 and p53 would have been feasible due to their activation/inhibition by Akt and prominent role in proliferation and apoptosis. Use of inhibitors of these proteins or antibodies for immunoblotting/flow cytometric analysis would have been some of the methods included in any further investigations undertaken. It would also have been interesting to attempt to induce some of the cell lines to further differentiate to investigate whether αvβ3 expression would have been stimulated. This would have revealed whether an expression pattern analogous to that seen in osteoclast development [222] exists in B cells.

6.3 CD47-induced apoptosis

Data already in the literature has shown that the ability of CD47 ligation to induce apoptosis is dependent upon the cell type involved and the presentation of the antibody used to recognise CD47. It has been found that cell lines such as Jurkat T cells and U937 cells are susceptible to apoptosis induced by the B6H12 anti-CD47 antibody [234]. In this thesis it was found that using this antibody clone KMS11 and H929 myeloma cell lines underwent apoptosis whether the antibody was in soluble or immobilised format. Of these two cell lines only one (H929) expresses the CD47 co-receptor αvβ3 and hence the presence of this integrin does not appear to affect induction of apoptosis in these cell lines. Expression of CD47 on these two cell lines, as determined by flow cytometric analysis, was found to be higher than that found in other cell types with KMS11 expressing more CD47 than H929. Since these two cell lines were found to be the most responsive to anti-CD47-mediated apoptosis it is possible that the amount of CD47 expressed on the cell is relevant to whether or not apoptosis is triggered.

SMS-SB cells, NALM-6 cells and 697 cells only responded to the apoptotic stimulus when the antibody was presented to the cells in soluble format. When treated with immobilised anti-CD47 the cells were resistant to apoptosis. Analysis of these cells reveals that SMS-SB cells and 697 cells do not express αvβ3 whereas NALM-6 cells do express αvβ3. BLIN-1 cells express αvβ3 and were resistant to apoptosis regardless of whether the antibody was in soluble or immobilised form. It would appear that, with regard to these experiments, no correlation exists between expression of αvβ3 and induction of apoptosis via CD47 ligation.

This was also the conclusion reached by Mateo et al [233]. Apoptotic induction is not thought to be mediated by the complex formed with αvβ3.

The generation of apoptosis via ligation of CD47 has previously been described as occurring via a caspase-independent pathway [233]. Time constraints prevented experiments being carried out to confirm whether the same is true in these cell lines but further investigation should be undertaken to ascertain the mechanism occurring in the KMS11 and H929 cells. Analysis of caspase activity and mitochondrial studies would be useful in such an endeavour.

6.4 Concluding remarks

The studies of stromal cells and SMS-SBs failed to further knowledge of the interaction between CD23 and αvβ5 but use of RKC-containing CD23-derived peptides revealed that ligation of αvβ5 and/or αvβ3 by these peptides has a positive effect on proliferation. This growth factor action of the RKC-containing CD23 peptides was exclusive to precursor B cells. Preliminary studies revealed that the signalling mechanism underlying this growth promotion includes activation of PI3K and subsequent phosphorylation of Akt. Further analysis is required to elucidate the complete signalling cascade responsible for this proliferative effect.

Induction of apoptosis via CD47 stimulation appears to occur in the absence of CD47's signalling complex partner αvβ3. Whether or not apoptosis occurs appears to be dependent upon cell type and the way CD47 is engaged. Further analysis is required to confirm that apoptosis generated in the myeloma cell lines H929 and KMS11 takes place via a caspase-independent mechanism and to elucidate the mechanism being utilised.

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APPENDIX

SOLUTIONS AND BUFFERS

Unless otherwise stated all were made up in distilled water.

10 X PBS STOCK	NaCl	1.37M
(pH 7.2)	KCl	26.8M
	Na_2HPO_4	42mM
	KH_2PO_4	14.7mM
10 X TBS STOCK	Tris base	200mM
	NaCl	1.4M
	pH to 7.2 with HCl	
RIPA BUFFER	Tris-HCl (pH7.4)	50mM
	NP40	1% v/v
	Na deoxycholate	1mM
	NaCl	150mM
	EGTA	1mM
	Na_3VO_4	1mM
	NaF	1mM
(added just prior to use)	PMSF	1mM
	Leupeptin	2μg/ml
	DTT	0.5mM
2 X PROTEIN LOADING DYE	Tris-HCl pH 6.8	125mM
	SDS	4%
	Bromophenol blue	0.01%
	Glycerol	20% v/v
	2-mercaptoethanol	5% v/v
TRANSFER BUFFER	Tris base	25mM
	Glycine	0.2M
	Methanol	20%v/v
WASH BUFFER	TBS	1 X
	Tween 20	0.1% v/v
ANTIBODY DILUTION BUFFER	TBS	1 X
	Bovine Serum	

	Albumin	5%w/v
	Tween 20	0.1%
BLOCKING BUFFER	TBS	1 X
	Tween 20	0.1%v/v
	Non fat milk	5% w/v
BINDING BUFFER	HEPES/	
	NaOH pH7.4	10mM
	NaCl	140mM
	$CaCl_2$	2.5mM