Expression and function of the αVβ5 integrin during human B lymphopoiesis

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Abstract

The integrin αVβ5 is a receptor for sCD23 molecule and the αVβ5-CD23 interaction sustains proliferation of the pre-B cell line SMS-SB. This thesis describes further investigation into the role of αVβ5 integrin during human B cell development. B cell development in the bone marrow involves stepwise maturation of progenitor cells through different defined stages. A tight regulation of proliferation and differentiation mediates the progression of progenitor cells through this developmental pathway. A variety of signals from soluble molecules and adhesive interactions regulate this balance of proliferation and differentiation. The main aim of this work was to assess the importance of the integrin αVβ5 during B cell development by defining its expression and function during specific stages of B cell development in the bone marrow.

The αVβ5 integrin was expressed by B cell precursors in the bone marrow, by different pre-B cell lines and the αVβ5-CD23 interaction sustained proliferation of some pre-B cell lines. The pre-B cell lines SMS-SB, RS4;11 and 697 showed a significant proliferative response to αVβ5 stimulation by sCD23, a sCD23-derived long peptide and anti-αVβ5 MAb 15F11. Transitional and more mature B cell lines down-regulated αVβ5 expression and did not show a proliferative response. Both αVβ5 and αVβ3 integrin could be detected on normal bone marrow B cell precursor populations, though αVβ5 was the more highly expressed integrin. In preliminary functional experiments, stimulation of $CD19^{+}/\kappa^{-}$ cells with sCD23 induced cell proliferation whereas equivalent treatment of $CD19^{+}/\kappa^{+}$ cells did not. The data are consistent with the interpretation that αVβ5 integrin is expressed in B cell precursors but that its ability to sustain growth of these cells wanes as the cells mature towards a membrane immunoglobulin-positive state.

The αVβ5-mediated proliferation was enhanced by the chemokine SDF-1 and PDGF but not by the cytokines IL-3, IL-4, IL-7 or IL-11. This effect was apparently restricted to earlier B cell precursors and was independent of levels of expression of both αVβ5 and CXCR4. Stimulation of SMS-SB cells with CD23, 15F11 or CD23-derived long peptide provoked ERK phosphorylation and co-stimulation with SDF-1 promoted a more rapid and sustained ERK activation. PDGF induced a similar effect on αVβ5-mediated activation of

ERKphosphorylation. These data suggest that ligation of αVß5 by soluble, adhesion-independent stimuli activates ERK phosphorylation and this pathway can be modulated by inputs from G-protein-coupled and tyrosine kinase receptors.

The murine pro-B cell line BAF03 also displayed αVß5-mediated proliferation in response to human CD23. Murine CD23 is generally regarded as lacking the cytokine-like activities found in human CD23. The αVβ5 integrin binds an arg-lys-cys motif beginning at arginine-172 (arg-172) in human CD23 and the equivalent murine sequence is gln-lys-cys. Studies using CD23-derived peptides indicated that arg-172 is not required for binding to murine $\alpha V\beta 5^+$ BAF03 cells, but that it is required for full biological activity. Preliminary experiments showed that human CD23 sustained growth of murine bone marrow B cell precursors. Therefore, these data suggest that murine B cells can also use αVβ5 integrin to sustain their growth and the studies described here in human cell lines could be translated into *in vivo* murine models.

Further work is needed to confirm the proliferative response due to the αVß5-CD23 interaction in normal B cell precursors in the bone marrow and to define the exact stage of development where this interaction is critical. In addition to its expression in the bone marrow B cell precursors, previous work has also demonstrated the expression of αVß5 integrin in B cells from patients with ALL. Therefore, the αVß5-CD23 interaction could have important implications not only in proliferation of normal B cell precursors but also in proliferation of neoplastic B cells. These data identify the αVß5-CD23 interaction as a potentially important interaction during early B cell development, as αVß5 expression and function is stage-specific, regulated by other molecules and can be demonstrated in both human and murine cell lines.

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For my parents; thank you for everything.

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Declaration

I hereby declare that the work presented in this thesis is my own, except where otherwise cited or acknowledged. No part of this thesis has been presented for any other degree.

Mridu Acharya

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ALL	Acute lymphoblastic leukaemia
AML	Acute myelogenous leukaemia
BCR	B cell receptor
BSA	Bovine serum albumin
BL	Burkitt's lymphoma
CALLA	Common acute lymphoblastic leukaemia antigen
CIA	Collagen induced arthritis
CLL	Chronic lymphocytic leukaemia
CLP	Common lymphoid progenitor
CPM	Count per minute
EBV	Epstein-Barr virus
EBNA2	Epstein-Barr virus nuclear antigen 2
ECL	Enhanced chemilluminescence
ERK	Extracellular signal-reuglated kinase
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate
GPCR	G-protein-coupled receptor
HPC	Haematopoietic progenitor cell
HSC	Haematopoietic stem cell
Ig	Immunoglobulin
ICAM-1	Intracellular cell adhesion molecule
IL	Interleukin
LFA-1	Lymphocyte function-associated antigen-1

LP	Long peptide
LCD	Low cell density
LCL	Lymphoblastoid cell line
MAb	Monoclonal antibody
NOD/SCID	Non obese diabetic/Severe combined immunodeficiency
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PFHM	Protein-free hybridoma medium
PI3K	Phosphatidylinositol-3-kinase
RA	Rheumatoid arthritis
RAG	Recombinase activating gene
RT-PCR	Reverse transcriptase-polymerase chain reaction
RTK	Receptor tyrosine kinase
sCD23	Soluble CD23
SDF-1	Stromal cell-derived factor-1
SLE	Systemic lupus erythematosus
TdT	Terminal deoxynucleotidyl transferase
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule
VLA-4	Very late antigen-4
VLA-5	Very late antigen-5

1 Introduction

The mammalian immune system is a highly complex network of specialized cells and soluble factors which cooperate to protect the body against invasion by pathogens. Lymphocytes (T or B lymphocytes) are types of cells of the immune system that are able to mount a specific immune response against virtually any foreign antigen. This is possible because each individual T or B lymphocyte matures bearing a unique variant of a prototype antigen receptor resulting in a population of T and B lymphocytes that collectively bears a huge repertoire of receptors that are highly diverse in their antigen binding specificities.

The B lymphocytes express membrane immunoglobulin (Ig) on their surface as the B cell receptor (BCR) and are activated when this receptor binds its specific antigen. To develop into plasma cells which secrete antibodies, B cells usually require T cell help, in the form of secreted interleukins and cell-cell surface interactions. Antibodies mediate humoral immune responses by binding to specific antigens and aiding their elimination. Antibodies possess numerous protective biological effects including: neutralisation of soluble antigens (viruses and bacteria); activation of the classical complement pathway; opsonization of particulate matter to allow enhanced phagocytosis; and antibody-dependent cell-mediated cytotoxicity (ADCC). In addition, antigen-specific B cells are highly efficient at presenting their cognate antigens and therefore act as antigen-presenting cells to activate T-helper cells. Thus, B lymphocytes play a central role in the immune system and inappropriate cell activation or proliferation of B cells can lead to development of immunodeficiencies, allergy, autoimmunity or malignancies such as lymphomas and leukaemias.

1.1 B cell development

Haematopoietic stem cells (HSCs) with extensive self-renewal potential generate all blood cell types and as they differentiate into progenitor cells they become more restricted in their differentiation potential. B lymphocytes develop from pluripotent HSCs in specialised microenvironments such as foetal liver and adult

bone marrow. During this process the progenitor cells undergo stepwise maturation through defined stages of development. This dynamic process of growth, survival and differentiation is strictly regulated by multiple mechanisms including sequential changes in gene expression, signals from cytokines and other soluble molecules and interactions between the progenitor cells and bone marrow adherent cells [1].

Several classification schemes for human and murine B cell development have been described, based on the expression of cell surface markers on the progenitor cells. CD19 is a signal transduction molecule which is the earliest marker known to be expressed on B-lineage cells and is expressed throughout B cell development except on plasma cells [2]. Other markers that define early developmental stages include CD34, CD22 and CD10 in humans and B220 (CD45R), the tyrosine kinase c-kit, CD43 and CD24 in mouse. A model of human B-cell development and its corresponding counterparts described in mouse is represented in Figure 1.1. Although differences exist between murine and human B cell progenitors the key events occurring during B lymphopoiesis are known to be similar. The common lymphoid progenitor (CLP) arising from the HSC is defined as the progenitor with the capacity to develop into T, B-lymphocytes or NK (natural killer) cells. These cells do not express the T, B or NK lineage markers (CD2, CD4, CD8, CD16, CD19, CD20, CD58) but are $CD34^+$, $CD10^+$ (in humans) [3].

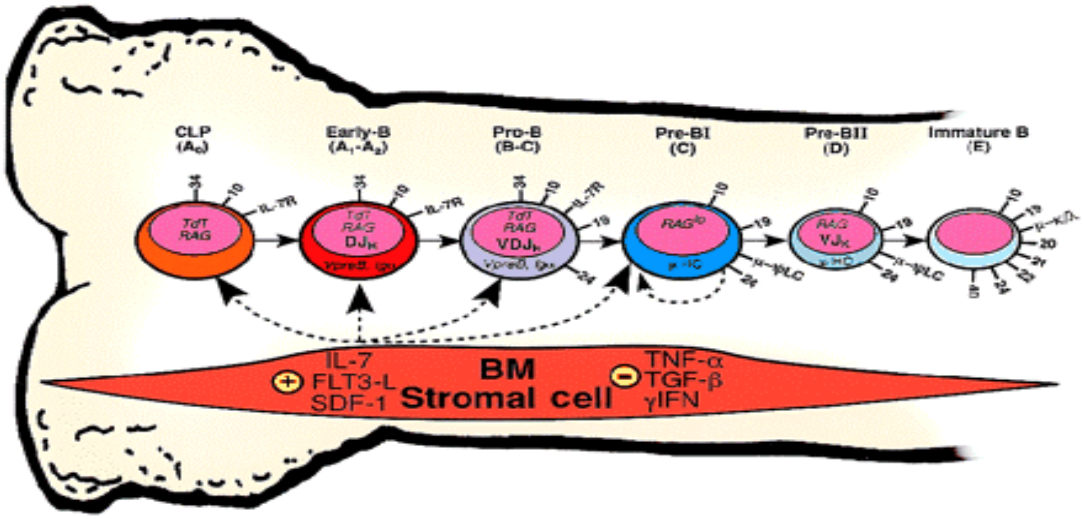

Figure 1.1 Schematic representation of a model of B cell development in human bone marrow.

The B cell developmental stages are shown beginning from the CLPs to immature B cells with the key surface molecules expressed in each stage. Expression of the intracellular enzymes such as TdT and RAG and stages in immunoglobulin gene rearrangement are also indicated. The pre-BCR is indicated as the μ-ψLC and BCR as μ-k/λ. The approximate counterpart stages in murine B-cell development are represented in parenthesis as A-E using the nomenclature of Hardy and colleagues [4,5]. The arrows indicate the potential regulatory factors produced by stromal cells and the numbers represent surface expression of CD markers. Picture reproduced from LeBien 2000 [1].

1.1.1 Pro-B cells

CLPs in the bone marrow enter the B lineage as pre-pro-B cells which further differentiate into pro-B cells [1]. However there is no known surface marker that differentiates CLPs from early pro-B cells. Murine CLPs are known to be particularly sensitive to IL-7 signalling and signalling through the interleukin 7 receptor (IL-7R) is required in the mouse to generate $CD19^+$, $CD43^+$ $B220^+$ pro-B cells [6,7]. IL-7 signalling is not as crucial in humans, as patients with mutations in the IL-7Rα chain have normal numbers of peripheral blood B cells [8,9]. However IL-7 is known to induce pro-B cells to expand and has been shown to up- regulate the expression of CD19 and the transcription factor Pax5 [6]. Pro-B cells represent the irrevocably committed B cell precursors and in humans are characterised by the surface expression of the B cell marker CD19 along with CD10 and CD34 [2]. Some studies have shown that pro-B cells express neither cytoplasmic nor cell-surface μ heavy chain (μHC) [10,11] while others studies have shown that cytoplasmic μHC can be detected on 5-10% of pro-B cells [12]. Pro-B cells have also been shown to express a precursor form of the BCR composed of immunoglobulin α (Igα), Igβ and calnexin termed pro-BCR [13-15]. Cross-linking the pro-BCR with anti-Igβ was shown to induce pro-B cells to acquire some of the cell surface markers of pre-B cells, therefore indicating that the pro-BCR is potentially a functional receptor in pro-B cells [15].

One of the factors guiding B lymphopoiesis is immunoglobulin (Ig) gene rearrangement leading to the generation of a functional antigen receptor. The antigen receptor (or B cell receptor) is a complex of an antigen recognition structure composed of two identical heavy and light chains associated non-covalently with a signal transduction heterodimer of Igα and Igβ. Each of the light and heavy chains can further be divided into the variable region (variable heavy V_H and variable light V_L regions) and constant regions. Variable regions for both the heavy and light chains are encoded by separate gene segments. These segments are brought together by somatic recombination to make a complete V region gene and are joined with the constant region genes by RNA splicing after transcription. This recombination of Ig genes is of fundamental importance for generation of diverse antigen receptor repertoires. The variable (antigen recognition) domain of Ig heavy chain (IgH) is encoded by many scattered germline gene segments of three types-variable (V), diversity (D) and joining (J)

and the variable region of Ig light chain is encoded by V and J gene segments. These gene segments are appropriately rearranged before expression [16].

Immunoglobulin gene recombination begins with D to J_H segment rearrangements in pro-B cells [17-19]. The vast majority of pro-B cells express the enzyme terminal deoxynucleotidyl transferase (TdT) essential during immunoglobuline gene recombination [20-22]. Although VDJ_H gene rearrangement is easily detected on many pro-B cells, a minority of pro-B cells have both heavy chain (HC) alleles in the germline configuration [10]. The D_H-J_H rearrangement of immunoglobulin heavy chain gene segments is already initiated at the pre–pro-B cell stage and pro-B cells express B lineage specific proteins such as the Igα-Igß heterodimer and VpreB [23]. A variety of transcription factors play important roles in B lineage commitment and progression by activating or repressing certain genes. Transcription factors such as E2A, EBF and Pax5 are required for up-regulation of pro-B cell stage- specific proteins as well as for expression of lymphoid-specific recombinase activating genes, RAG1 and RAG2 [24,25]. These enzymes are essential for rearrangement of the immunoglobulin genes. Human pro-B cells may correspond to Hardy fraction B ± fraction C (murine counterpart) based on analysis of VDJ_H rearrangement [10].

1.1.2 Pre-B cells

A functional VDJ_H rearrangement of the gene segments coding for the variable region of immunoglobulin heavy chains is essential for the progression of pro-B cells into the pre-B cell stage[1]. This rearrangement results in the surface expression of µHC (heavy chain). Expression of surface µ results in the assembly of the pre-BCR and marks the transition to the pre-B cell stage leading to the process of allelic exclusion which results in the productive rearrangement of only one of the two parental Ig HC alleles. Expression of membrane Igµ in pre-B cells has been shown to inhibit further $V(D)J_H$ recombination [26]. µHC in association with the surrogate light chain (LC) components VpreB, λ5 and the signalling heterodimer Igα and Igß comprise the pre-BCR.

In both humans and mouse the pre-B cells can be divided into large proliferating pre-BI and small post mitotic pre-BII cells. The pre-BI cells express cytoplasmic μHC while the pre-BII cells express the pre-BCR [1]. Human pre-BI cells partially overlap with Hardy fraction C [4].

The genes encoding the two proteins VpreB and λ5 were originally identified in mouse and their organisation differs in mouse and humans [1]. Mice with a targeted disruption in the λ5 locus exhibit a block at the pro-B to pre-B transition [27] but the number of B cells in secondary lymphoid tissue gradually increases over time so this block is not absolute. Moreover, the discovery of an agammaglobulinemia patient with mutations in both λ5 and a severe disruption in B cell development underscores the importance of the surrogate light chain components in human B cell development [28]. Expression of these proteins in human cell lines has been defined using MAbs (monoclonal antibodies) that recognise the specific components of the surrogate light chains. Expression of a signalling-competent pre-BCR is an important checkpoint for developmental progression and survival. In mice, targeted mutations in the different pre-BCR components including the trans-membrane domains of Igα or Igß result in developmental arrest at the pro to pre-B cell checkpoint [28-30]. Pre-B cells lose TdT and CD34 expression. Pre-BCR signalling is shown to be required for allelic exclusion at the heavy chain locus and phenotypic changes in proliferating B cells in response to IL-7 signalling [31].

Igα and Igß are immunoglobulin superfamily members that activate cellular signalling pathways through cytoplasmic immunoreceptor tyrosine-activating motifs (ITAMS) by recruiting Src, Syk family kinases and the Tec family kinase Btk (Burton's tyrosine kinase) [32-35]. Several Src kinases, including Lyn, Fyn and Blk and variety of other signalling molecules appear to contribute to BCR or pre-BCR signalling in a redundant fashion as deletion of genes encoding individual Src kinases has little effect on B cell development [36-38]. However other components of the BCR signalling pathway such as Syk, Blnk , PI3K, Btk have been shown to be non-redundant as deletion of these components impairs B cell development [39-44]. Signalling through the pre-BCR regulate HC allelic exclusion and induce pre-B cell development. Neither allelic exclusion nor pre-B cell development

occurs in the absence of tyrosine phosphorylation of ITAM motifs in the cytoplasmic domain of Igα and Igβ [45]. B cells that fail to assemble pre-BCR fail to progress in development and are deleted. It is still debated whether pre-BCR cross-linking by an unknown ligand or simple surface expression is sufficient to trigger allelic exclusion and pre-B cell development.

Cells displaying the pre-BCR expand through a proliferative burst and initiate light chain gene (k or λ) rearrangements. After clonal expansion the pre-B cells arrest in G1 phase of cell cycle and RAG and Igκ germline gene transcripts are expressed for light chain gene recombination. Successful rearrangement of the light chain genes results in the expression of the BCR on the cell surface. These cells, termed immature B cells, express cell specific surface IgM and emigrate to secondary lymphoid organs. Here, they mature further into immunocompetent B cells (IgM^+ and IgD^+), which in response to primary antigen challenge and T cell support can differentiate into Ig secreting plasma cells.

Immature B cells are particularly susceptible to BCR-induced apoptosis [46] and it is in this compartment that self-reactive B cells failing to edit their receptors are deleted or anergized [46] . The BCR is an essential regulator of immature B cell development since in the absence of cytoplasmic domain of Igα, BCR with a single functional Igβ produce few pre-B cells and immature B cells and most of these fail to progress to mature B cell stage [47]. Immature B cells that emigrate from the bone marrow to the periphery are termed transitional B cells and can be distinguished from mature B cells by a series of cell surface markers such as CD10, CD38, IgD and IgM [48].

Transitional B cells are short-lived and only 10-30% of these cells enter the long-lived mature peripheral B cell compartment [49,50]. The mechanisms that govern selection of newly-generated transitional B cells to the long-lived B cell compartment are poorly understood but once again BCR signalling appears to be an important determinant as loss of BCR expression by conditional IgM ablation inhibits this transition to mature B cell stage [51]. In addition, in the absence of functional Igα, Syk, Btk, Vav, Lyn, CD45 or CD19 molecules BCR signalling is insufficient to induce differentiation of transitional B cells into mature B cells [47,52,53].

1.1.3 B-1 B cells

The predominant population of B lymphocytes, known as B-2 B lymphocytes, is present in the spleen and lymph nodes. These B-2 B cells are produced in the bone marrow during postnatal life and migrate to the spleen and lymph nodes where, after progressing through several transitional stages, they mature into a major follicular (FO) and minor marginal zone (MZ) B lymphocyte populations. While the MZ B lymphocytes do not circulate and respond to blood borne antigens, the FO B lymphocytes respond to protein antigens and in response to T-cell help undergo class switching and affinity maturation into B-2 lymphocytes.

A minor population of B-1 B lymphocytes (about 5%) is localised to serous cavities. Although the bone marrow retains the potential to produce B-1 B lymphocytes they are most effectively generated from progenitors that arise during foetal hematopoiesis. Similar to all B cells, B-1 cells express the B lineage antigens CD19 and CD45, although CD45R is present at lower levels on B-1 cells than on B-2 cells [54]. Murine B-1 cells can be distinguished by the phenotype $B220^{low}$ $CD23^{-}$ $CD43^{+}$ IgM^{hi} and IgD^{low}. B-1 cells in the peritoneal and pleural cavities also express CD11b. Therefore they are of the phenotype $CD11b^{+}$ IgM^{hi} IgD^{low}. These cells can further be sub-divided on the basis of differential expression of cell surface antigen CD5 as $CD5^{+}$ B-1a cells or $CD5^{-}$ B-1b cells [55]. B-1 cells can also be distinguished from conventional B cells by patterns of anatomical localisation. In mice, B-1 cells are almost completely absent from lymph nodes and make up about 5% of splenic B cells. However they constitute a substantial fraction of B cells in the peritoneal and pleural cavities [56,57]. In addition to surface phenotype, B-1 cells have a number of distinguishing properties. *In vitro* they are long lived [58], refractory to activation through BCR ligation [59-61] and in contrast to B-2 cells they can be induced to proliferate by treatment with phorbol esters [62].

B-1 cells are effectors of innate immunity and mainly respond to T-cell-independent immunogens (thymus-independent type 2 antigens) that include carbohydrate antigens, by producing mainly IgM and some IgG3 antibodies. These

cells provide a first line of defence against pathogens by spontaneously secreting IgM antibodies. B-1 cells share many phenotypic characteristics of MZ B cells and like these latter cells appear to develop in response to T-cell-independent type 2 antigens. The immunoglobulin repertoire of B-1 cells is less diverse compared to B-2 cells and this is reflected in their capacity to respond to antigens [63,64]. Studies have shown that $CD5^+$ cells produce natural polyreactive antibodies, that are often auto-reactive, of the IgM isotype [65].

Although B-1 cell function in innate immunity is generally well-established the origin of B-1 cells has been a controversial topic. The debate has centred around two models of B-1 cell development. The selection model proposes that B-1 and B-2 cells are derived from a common progenitor and antigen selection at the IgM^+ stage determines whether a B cell will follow the B-2 or B-1 differentiation pathway [66,67]. This model is based on experiments demonstrating that cross linking of the BCR in the absence of antigen or T-cell help resulted in expression of CD5 by splenic B-2 cells and conferred upon them the ability to proliferate in response to phorbol esters [68,69]. Further evidence for this model was provided by results from transgenic mice generated with the V_H gene from the V_H12 family, which is preferentially expressed by B-1-lineage cells and was inserted into the immunoglobulin heavy chain locus. The V_H12 expressing mice mainly generated B-1 cells, whereas those expressing B-2-cell-associated V_H specificities mainly generated B-2 cells [67].

By contrast, the lineage model proposes that B-1 and B-2 cells are separate lineages that derive from distinct haematopoietic progenitors. This is based on the observation that foetal tissues most efficiently repopulate B-1 cells in irradiated immunodeficient recipients while adult bone marrow preferentially repopulates B-2 cells [55,70,71]. Moreover, recent studies identifying B-1-cell progenitors [72] and showing distinct cytokine requirements for B-1-cell and B-2-cell development provide further support for this model [73,74] . The fact that the foetal B cell development is skewed to B-1 cells indicates interesting differences between foetal and adult hematopoiesis. Although the adult HSCs preferentially generate B-2 cells, they retain the potential to generate B-1 cell progenitors.

A variety of influences from transcription factors, differences in the microenvironment and cytokine expression during adult and foetal haematopoiesis will have important regulatory roles in guiding B-1 or B-2 cell development pathways.

Another important factor in B-1 cell differentiation is the BCR. It is known that B-1 cell development is promoted by strong signalling through the BCR [75]. Blockade of most of the positive regulators of BCR signalling such as BLNK, PLCγ2, VaV, Rac, PI3K, BCAP and CD19 leads to absence of peritoneal B-1 cells [40,42,76-78], while disruption of some negative regulators of BCR signalling such as CD22 and PIRB which leads to greater strength of BCR signalling, leads to a greater number of peritoneal B-1 cells only [79,80]. This indicates that the strength of BCR signalling regulates which mature subsets can develop.

The murine B-1a cells have been considered the murine equivalent of human $CD5^+$ B cells. In humans, the CD5 marker was first identified on malignant B-CLL (chronic lymphocytic leukaemia) cells and only subsequently demonstrated as a marker for normal B cells [81]. Because B-1 cells often produce auto-reactive antibodies, although non-pathogenic and of a low affinity, this has led to interest in these cells as possible sources of high affinity pathogenic autoantibodies seen in polysystem autoimmune diseases. Indeed, there are examples, in both humans and mice, of an association between autoimmune disease and B-1 cells. $CD5^+$ B cells emerge at a crossroad between malignant and auto-reactive B cells. A monoclonal expansion of $CD5^+$ B cells is a feature of malignant diseases such as B-CLL and MZ lymphoma [82], while a polyclonal expansion is observed in systemic autoimmune diseases like RA, SS (systemic sclerosis) and occasionally SLE (systemic lupus erythematosus) [83,84 85]. Therefore a study of B cell differentiation into B-1 or B-2 subsets could provide important information in understanding malignant transformations as well as autoimmunity.

1.2 Haematopoietic microenvironment

Hematopoiesis is known to take place in the intersinusoidal spaces of medullary cavities in the bone. HSCs are located at the stem cell niche in the endosteal surface in association with osteoblasts [86] and the HSCs destined to generate the different blood cell types ultimately exit from this niche and associate with stromal cell populations that form a three-dimensional network between venous sinusoids [87,88]. The bone marrow microenvironment is composed of a heterogeneous population of stromal cells and their products, such as extra cellular matrix components and various cytokines and chemokines. These cytokines, chemokines and adhesive molecules play a critical role in regulating the survival, proliferation and differentiation of the precursor cells. Studies on mice and patients with human immunodeficiencies have led to the identification of some important regulatory molecules including IL-7, Flt3 ligand, the chemokine SDF-1 (stromal cell derived factor-1) and many still remain to be identified. The binding of Flt3 ligand (FL) to Flt3 receptor is important in normal B lymphopoiesis as the number of pre-pro-B and pro-B lymphocytes is reduced in $FL^{-/-}$ and $Flt3^{-/-}$ mice [89,90]. Murine but not human B cell development is absolutely dependent on the presence of stromal cell-derived IL-7 and the expression of its receptor on developing B cells [91,92]. Stromal cell derived factor is a chemokine that has been shown to have established roles in B cell development.

1.2.1 SDF-1

Stromal cell interactions and mobilisation and trafficking of haematopoietic precursors are thought to be controlled by cytokines, particularly chemokines [93]. In addition, some chemokines have been reported to modulate cell growth and survival [94-99]. Chemokines are a family of low molecular weight chemoattractant cytokines that bind specific G protein-coupled transmembrane receptors on target cells.

Stromal cell derived factor-1 (SDF-1) or CXCL12 is a member of the CXC chemokine family [100] and binds to its receptors CXCR4 and CXCR7 [101,102]. Two alternate splice variants of the SDF-1 gene give rise to SDF-1α and SDF-1ß which

are identical except for an additional four amino acid residues in the carboxy terminus of SDF-1ß. Gene inactivation of SDF-1 and CXCR4 *in vivo* leads to embryonic lethality due to abnormal cerebellar and gastrointestinal vasculatures and haematopoietic development [103-105]. Studies by Ma, *et.al.,* in knockout mice and in chimeric mice that transmitted the CXCR4 mutations in the genome, show that mice deficient for CXCR4 die perinatally and display profound defects in the haematopoietic and nervous systems. CXCR4-deficient mice have severely impaired B lymphopoiesis and reduced myelopoiesis in the foetal liver and a virtual absence of myelopoiesis in the bone marrow [106].

SDF-1 and CXCR4 are expressed constitutively in a wide range of tissue types including brain, heart, kidney, liver and spleen. CXCR4 is expressed not only on lymphocytes and monocytes in peripheral blood, but also on a variety of other cell types including microglia, astrocytes and neurons. SDF-1 is constitutively produced by many cell types including immature osteoblasts and endothelial cells within the bone marrow as well as by epithelial cells in many organs such as in the central nervous system [107,108].

The role of SDF-1 in migration and homing of haematopoietic progenitors is well established. It is known to be the most powerful chemoattractant for undifferentiated human $CD34^+$ haematopoietic progenitors [109,110]. A number of studies have shown that SDF-1 is essential for activation of the integrins LFA-1, VLA-4 and VLA-5 [111,112] leading to enhanced integrin-mediated adhesion to ligands such as ICAM-1, VCAM-1 and in facilitating transendothelial migration as well as homing and engraftment of precursor B cells in the bone marrow of NOD/SCID mice [113,114]. In addition to its established role in regulating cell motility, a number of studies have shown that SDF-1 can also play a role in cell survival [93,115] and proliferation [116] often in synergy with other cytokines. SDF-1 has been directly implicated in survival of pre-B cells *in vitro* [115]. Lataillade, *et. al.,* showed that SDF-1 enhances circulating $CD34^+$ cell proliferation with other cytokines and that SDF-1 acts as a survival factor in counteracting peripheral blood $CD34^+$ cell apoptosis [117].

CXCR4 ligation by SDF-1 has been shown to induce receptor internalisation, elevation of cytoplasmic Ca^{2+} levels, activation of phosphatidylinositol 3-kinase (PI3K), phosphorylation of MEK/ERK and phosphorylation of components of focal adhesion complexes including paxillin, $p130^{cas}$, and focal adhesion kinase in many cell types [118-121]. Work by Arai, *et.al.,* showed that SDF-1 and IL-3 synergistically increase the number of viable cells and induce a chemotactic response of haematopoietic cells, in an ERK-dependent manner [122]. SDF-1-driven proliferative signals are thought to be mediated via the MEK/ERK and PI3K/AKT signalling pathways [123,124] and survival signals through PI3K/AKT [125]. Recent work by Bendall, *et. al.,* also shows that defective p38 MAPK signalling impairs chemotactic but not proliferative responses to SDF-1 in acute lymphoblastic leukaemia (ALL) cells indicating a distinct role for SDF-1 in leukemic B cell survival [126].

1.2.2 Signalling pathways mediating B cell survival

B cell survival, differentiation and maturation involves a delicate balance between apoptosis and proliferation. The complex mixture of growth factors, extracellular matrix and stromal cells provides extrinsic signals that can activate a variety of signalling pathways leading to growth, differentiation and survival of B cell precursors. Survival stimuli activate intracellular signalling through the ligation of transmembrane receptors which either possess intrinsic tyrosine kinase activity, are indirectly linked to tyrosine kinases (such as integrins) or are coupled to seven transmembrane G –protein-coupled receptors. Activation of these receptors results in the recruitment of signalling molecules that activate signalling pathways such as the ERK/mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway.

1.2.2.1 ERK- mitogen activated protein kinase (MAPK) pathway

It is well established that many of the cytokines and growth factors regulating hematopoiesis activate MAP kinase sigalling pathways to generate their effects including phosphorylation of transcription factors, regulation of transcription, nuclear chromatin remodelling, immediate gene induction as well as regulation of apoptosis and cell cycle progression [127].

Three major groups of MAP kinases (MAPKs) exist: the p38 MAP kinase family, the extracellular signal-regulated kinase (ERK) family, and c-Jun NH_2-terminal kinase (JNK) kinase family. Biological activities vary with specific family of MAPKs activated and stimulus initiating the activation. In general, the Ras/ERK pathway mediates primarily cell growth and survival signals and in certain circumstances is known to promote cell differentiation [127-132]. The p38 and JNK pathways are known to mediate pro-apoptotic and growth inhibitory signals as well as proinflammatory signals[128-132]. However, the p38 MAPK pathway may also be involved in inducing anti-apoptotic, proliferative and survival signals under certain conditions, depending upon the tissue-specific isoforms involved.

One of the most extensively studied MAPK pathways is the Raf/MEK/ERK cascade and it has been implicated in many chronic and acute leukaemias. The upstream activators of ERK are the MEK (or Map kinase kinase) family of kinases which are dual-specificity kinases that can phosphorylate both serine/threonine and tyrosine residues on the MAPKs. A kinase responsible for activation of MEK is Raf and upstream of Raf is the GTPase Ras. Ras is an oncoprotein and has been shown to be mutated in approximately 20% of all human malignancies [133]. ERK1 and ERK2 are 43/44 and 41/42 kDa kinases, respectively, that are directly downstream of the MEKs. Phosphorylated ERKs in turn phosphorylate and activate a number of transcription factors such as ELK-1, CREB (cAMP) response element binding protein, c-Myc, Nuclear factor-κB, Activator protein family transcription factors-1(AP-1), and Ets family members. They also activate other protein kinases such as $p90^{RSK}$ and enzymes such as cytoplasmic phospholipase A_2[127]. In addition, ERK1/2 are also known to regulate apoptosis by phosphorylation of the pro-apototic protein BIM (Bcl-2-interacting mediator of cell death) [134]. The duration and degree of MAPK activation is dependent on a delicate balance between the activating signals and inactivation mechanisms. Removal of a phosphate from the phosphorylated residues (tyrosine and threonine), through the action of phosphatases results in decreased activity.

Constitutive ERK1/2 activation has been reported frequently in solid tumors [135,136]as well as in hematological and neoplastic disease, particularly AML (acute myelogenous leukaemia) [137]. In relation to ALL, constitutive ERK1/2 activation

was reported in ALL cell lines and in a limited number of clinical ALL specimens [138] Gregorj, *et. al.,* demonstrated constitutive ERK activation in leukemic samples and showed that ERK1/2 phosphorylation is an independent predictor of complete remission in newly-diagonsed adult ALL [139]. Furthermore R115777, an enzyme-specific inhibitor of farnesyl protein transfterase (FT), developed as a potential inhibitor of Ras signalling in ALL, was shown to have important anti-leukemic activity in phase 1 clinical trials [140].

1.2.2.2 Phosphatidylinositol 3-kinase (PI3K)/ AKT pathway

The PI3K/AKT pathway often transduces signals that are similar in nature to that of the Raf/MEK/ERK pathway. Phosphatidylinositol-3-kinase (PI3K) is found as a dimer of two subunits (p85 regulatory subunit and p110 catalytic subunit) in resting cells. Upon activation of receptor tyrosine kinases, and subsequent autophosphorylation, the SH2 domains of the p85 subunit bind to the receptor's phosphorylated residue and PI3K then phosphorylates PIP_2 (phosphatidylinositol 4,5 bisphosphate) generating PIP_3 (phosphatidylinositol 3,4,5 triphosphate) at the cytoplasmic membrane [141]. PIP_3 in turn activates and phosphatidyl inositol dependent kinases (PDKs) and their downstream substrate AKT/PKB [142]. AKT, activated by phosphorylation by the PDKs, in turn phosphorylates a variety of downstream substrates including the pro-apoptotic BCL-2 family member BAD [143]. BCL-2 is the prototype for a large family of related proteins that regulate cell death in mammalian cells [144]. Some of these proteins such as BCL-2 and BCL-XL promote cell survival whereas other proteins in the family such as BAX and BAD promote cell death. AKT phosphorylation of BAD on serine-136 results in its inactivation and cell survival. When BAD is dephosphorylated in the absence of survival signals it interacts with the pro-survival BCL-2 family members and inactivates them causing cell death. Two pharmacological inhibitors of PI3K have been used extensively: Wortmannin and LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopryran-4-one] [145,146]. Although both of these inhibitors are useful in blocking PI3K signalling neither of them has the capacity to directly inhibit AKT. Abrogation of oncogenic signals from PI3K occurs mainly via the tumour supressor gene product PTEN which is a phosphatase [147].

Cross-talk between PI3K and Raf/MEK/ERK pathways has been reported on multiple levels [148-150]. In addition to controlling Raf kinases, Ras has also been shown to interact and stimulate PI3K activity leading to AKT activation. Some studies suggest that the PI3K pathway enhances and/or synergises with Raf/MEK/ERK pathway to provide robust signalling through ERK [151-153], while there is also conflicting evidence that AKT phosphorylates Raf abrogating downstream signalling pathway activation [153-155]. However, this effect may be dependent on the differentiation state of the cell [156]. Regardless of the cell type, differentiation state or assay conditions used it is apparent that there is a varying degree of cross-talk between these two pathways important in cell proliferation and survival. Study of the activation and inhibition of these pathways in B cell proliferation and survival is instrumental in the development of new agents designed to treat leukaemias.

1.3 Precursor cells and stromal cell interactions

A second general means by which stromal microenvironment regulates B cell development is via direct cell contact. Direct contact of the progenitor cells with bone marrow stromal cells is particularly important during the early B cell developmental stages [157]. Developing B cells have been observed to grow in close association with stromal cells and the contact between the progenitor cells and bone marrow adherent layers is maintained by a combination of adhesion molecules including VCAM-1, ICAM-1 on bone marrow stromal cells and the β_1 integrins VLA-4, VLA-5 and β_2 integrin LFA-1 on the B cell surface [158,159].

Stromal cell and B cell interactions possibly involve a variety of mechanisms important for regulating growth survival and differentiation of B cell precursors. CD23 is a glycoprotein known to be expressed on human bone marrow stromal cells [160] and the membrane bound or soluble CD23 could potentially play an important regulatory role in growth and survival of B cell precursors.

1.3.1 CD23

CD23 was initially identified as a B cell differentiation antigen and subsequently established as the low-affinity receptor (FcεRII) for immunoglobulin E (IgE). It is a surface antigen expressed by haematopoietic cells and some epithelial cell subsets. CD23 is expressed weakly on a minority of freshly-isolated resting B cells from peripheral blood or tonsils and is both dramatically and rapidly up-regulated on the B cell surface following activation by stimuli such as EBV (Epstein Barr Virus), phorbol esters and interleukin 4 (IL-4), IL-3 and CD40 [161-165]. Furthermore, CD23 expression is a requisite marker for susceptibility to growth transformation by EBV and indeed all EBV-immortalized B cell lines are CD23 positive [166,167]. CD23 expression was said to be a feature restricted to primary B cells expressing IgM/IgD [168], however CD23 was also shown to be expressed by pro-B cells after triggering of CD40 in the presence of IL-3 [169].

CD23 functions both as a receptor and a ligand. As the low-affinity receptor for IgE, it is a focus for IgE immune complexes leading to modulation of allergic responses. As an adhesive molecule, CD23 interacts with CD21 to regulate IgE production, germinal centre B cell survival and presentation of soluble protein antigen by B cells to T cells. Membrane-bound CD23 can be cleaved into soluble fragments that act as cytokines *in vitro*. Potentially different forms of the membrane-bound and soluble forms of CD23 have been described, making it an important player in allergic, autoimmune and lymphoproliferative disorders.

1.3.1.1 CD23 Structure

CD23 is the only FcR which does not belong to the immunoglobulin super-family [170]. The CD23 gene on chromosome 19 [171] codes for two isoforms (a and b) differing in their 5' untranslated sequence and in their intracytoplasmic region. The CD23a isoform expression is restricted primarily to B cells but CD23b isoform is inducible by IL-4 on a variety of haematopoietic cells [172]. Human CD23 is a 45 kDa glycoprotein member of the C-type lectin family, with a long C-terminal extracellular domain (277 residues), a short cytoplasmic N-terminus (23 residues) and is anchored by a single transmembrane domain (20 residues). As a type II integral transmembrane protein it has the COOH terminus oriented extracellulary and the short NH_2 terminus oriented intracellularly [173-176].

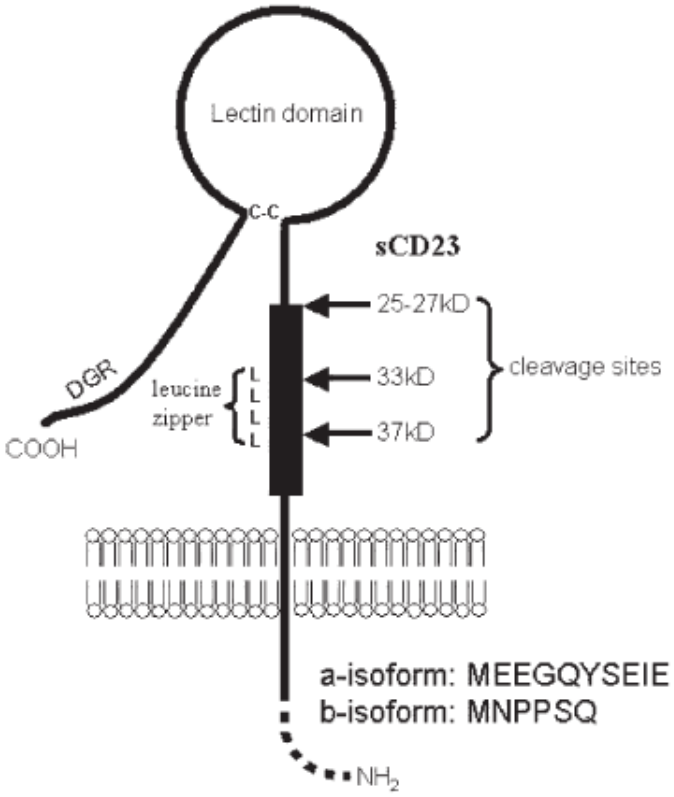

Figure 1.2 Schematic representation of membrane bound CD23.

The important structural features shown are the lectin head domain, leucine zipper motif that mediates formation of trimers and the arrows indicate the cleavage sites for soluble CD23 fragments. Sequences differing in the two isoforms at the N-terminal region are also shown. Figure reproduced from Schwarzmeier J. D, *et.al.,* (2005) [177].

A model of membrane bound CD23 is represented in Figure 1.2. The extracellular region of CD23 comprises three important features:

(a) The leucine zipper motif near the transmembrane domain consists of seven amino-acid motifs that are repeated five times in case of the human CD23 and forms an α-helical coil stalk region. This region mediates the formation of trimers.

(b) The lectin head domain that comprises IgE binding site [178] consists of four highly conserved and two partially conserved cysteine residues that interact by disulphide bonds and contain the Ca^{2+} and sugar binding amino acids. The region delimited by Cys163 and Cys282 encodes all the structural information required to form the IgE binding site [179]. The cytokine effects of CD23 are mediated by an epitope distinct from the IgE binding site [180].

(c) A DGR sequence, which in inverse is a common recognition site for integrins is located near the C-terminus of the molecule.

Similar to most FcR, CD23 exists in soluble and membrane-bound forms. Membrane-bound CD23 is cleaved into soluble fragments [181] by mechanisms involving matrix metalloproteases. Soluble CD23 is first released as an unstable 37-33 kDa molecule which is subsequently transformed into more stable 25 kDa fragments [182].

IgE plays a central role in allergic diseases and the first receptor identified for this molecule, termed FcεRI, binds IgE with such a high affinity (Ka~ $10^{10}M^{-1}$) on cells bearing FcεRI are permanently coated with IgE and thus sensitized for rapid release of molecules promoting immediate inflammatory response when cross-linked with allergen. CD23 binds to IgE with lower affinity (Ka~ $10^{7}M^{-1}$) and this interaction is involved in both IgE regulation and antigen presentation by B cells.

Binding of IgE to CD23 is calcium-dependent and appears to involve protein-protein interactions since deglycolysated native IgE peptides are able to bind CD23 [183]. However, Delespesse, *et. al.,* 1992 showed an ability of fucose-1-phosphate to inhibit binding of native IgE to soluble CD23, therefore CD23-IgE binding probably involves protein-protein and protein-carbohydrate interactions [184]. IgE-CD23 interactions have been implicated in many IgE dependent roles, including combating parasitic infestations [185], induction of IgE synthesis [186] and maintenance/modulation of IgE responses [187].

CD23 is known to play a regulatory role in IgE production with positive and negative effects. CD23 acts as a buffer in negative feedback regulation of IgE, as membrane-bound CD23 delivers a down-regulatory signal for IgE synthesis by B cells when engaged by IgE-allergen complexes [188], while sCD23 is known to act as a B cell growth factor up-regulating the production of IgE when in trimeric forms. It has been proposed that trimeric sCD23 promotes IgE synthesis by cross-linking membrane IgE and CD21 on B cells committed to IgE synthesis [189]. CD21, the EBV and complement receptor-2 (CR2) were identified as second ligands for CD23 [190] . Engagement of B cell CD21 by either anti-CD21 antibodies or by recombinant CD23 leads to IgE secretion by B lymphocytes in the presence of IL-4 [190,191]. The CD23-CD21 interaction is known to be critical for enhancing contacts between T and B cells and other molecules such as CD40 provide co-signals for this interaction [192,193].

CD23 has been shown to be physically associated with the MHC class II molecule, HLA-DR on B cells which indicated that it could act as an accessory molecule in T cell interactions with antigen presenting cells [194,195]. CD23 occupancy by anti-CD23 antiboides (MHM6 and EBVCS4) and IgE was shown to prevent B cells from stimulating allogeneic T cells [194] and further investigation showed that the CD23/CD21 interaction is required for presentation of conventional, soluble protein antigens by B cell lines to $CD4^+$ T cell clones [196]. This antigen presentation function requires the binding of antigen-IgE immune complexes to CD23, internalization of the complexes and transport to compartments of the endosomal network containing proteolytic enzymes and MHC class II antigens.

CD23 on macrophages, eosinophils and platelets mediates IgE-dependent cytotoxicity and promotes phagocytosis of IgE-coated particles [185]. Various studies have shown that engagement of membrane CD23, on monocytes/macrophages leads to production of important inflammatory modulators; for example, production of cyclic AMP (cAMP), TNF-α and lipid mediators after ligation of CD23 in IL-4 activated monocytes [197,198], nitric oxide production (NO) in human monocytes, macrophages and eosinophils [198-200]. Furthermore, CD23 engagement in pro-monocytes was shown to induce differentiation as well as activation of the transcription factor NF-κB, induction of the proto-oncogenes c-fos, c-jun and junB and production of TNF-α [201].

The fact that not all of the activities of CD23 can be attributed to its interaction with IgE or CD21 has led to research on novel ligands for CD23. ß2 integrins CD11b/CD18 and CD11c/CD18 and the vitronectin receptor αvß3 integrins were identified as receptors for CD23 [202,203]. Triggering CD11b and CD11c on monocytes with recombinant CD23 was shown to cause an increase in production of proinflammatory cytokines IL-1α, IL-1ß, Interferon-γ (INF-γ), IL-6 and tumour necrosis factor α (TNFα) as well as marked increase in nitrite (NO_2^-) and H_2O_2 production [202,204]. Another study showed triggering of the ß2 integrins on primary human monocytes provides activation signals leading to nuclear translocation of NF-kB and subsequent secretion of MIP1α (macrophage inflammatory protein) and MIP-1ß [205]. This may have important roles in recruitment of other inflammatory cells during initiation of an inflammatory response. The vitronectin receptor αvß3 in association with CD47 was shown to induce sCD23-mediated pro-inflammatory function by production of TNF-α, IL-12 and IFN-γ [203].

1.3.1.2 sCD23 and B cell growth

CD23 can be described as a multifunctional cytokine. Soluble fragments of CD23 retain the capacity to bind IgE but have also been ascribed numerous cytokine-like activities. CD23 induces various growth promoting and differentiation effects on haematopoietic cells in synergy with the cytokine IL-1α. *In vitro* experiments show that sCD23 in synergy with IL-1α prevents centrocyte apoptosis and drives the cells towards a plasmacytoid phenotype [206], induces

maturation of precursor T cells in the thymus [207,208] as well as increasing proliferation of mature $CD4^+$ T cells in response to mitogens such as PHA (phytohemagluttinin) [209] and drives proliferation and maturation of bone marrow-derived $CD34^+$ myeloid precursors [210]. The significance of these observations is underlined by *in situ* detection of CD23 producing cells in the thymus (on a subset of epithelial cells) [207], in bone marrow (on stromal cells) [160] and in the light zone of germinal centres (on follicular dendritic cells) [203].

1.3.1.3 CD23 and EBV

Growth-promoting effects of the CD23 molecule are closely related with EBV transformation. EBV belongs to Herpesvirus family [211] and has been closely associated with endemic forms of Burkitt's lymphoma (BL), while the majority of sporadic BL are not EBV related. Although this virus is known to be the causative agent in endemic BL its precise role is not fully understood. CD23 is associated with the immortalising properties of the virus and as mentioned above the non-immortalising strains of this virus do not up-regulate CD23 expression. The Epstein-Barr virus nuclear antigen (EBNA-2) and latent membrane protein-1(LMP-1) are two latent genes essential for immortalisation of human B cells and both of these genes are involved in CD23 expression [212,213]. B cell infection by EBV is often considered as a model for B cell activation [167]. Following transformation by EBV, B cells are activated to release sCD23 that contains autocrine growth-promoting activity [214,215]. *In vitro* experiments clearly demonstrated that sCD23 acts as a growth factor for both EBV-immortalised cell lines and antigen receptor stimulated normal B lymphocytes [215].

Apart from the cases of EBV transformed lymphblastoid cell lines (LCLs,) Gordon, *et. al.,* have shown that anti-CD23 MAb provided a progression signal to human B cells primed by phorbol ester to enter the G1 phase of cell cycle [216]. CD23 was also shown to enhance growth promoting effects of IL-4 and antibodies to CD40 [214,217]. Work by Fournier, *et. al.,* showed that CD23 could play a role in leukemic B cell disease, especially chronic lymphocytic leukaemia (CLL); since IFN-α and IL-2 selectively upregulated CD23b and stimulated growth of malignant B cells while IL-4 and IL-2 upregulated CD23a and had no growth promoting activities but suppressed B-CLL apoptosis [218,219].

1.3.1.4 CD23 in disease

CD23 is involved in a variety of allergic diseases owing to its role in IgE regulation. However sCD23 levels are known to be increased in diseases in which IgE is not implicated. As mentioned above, a characteristic feature of CLL is high serum concentrations of sCD23 which can be 500 times higher than in normal individuals and sCD23 appears to be an independent prognostic variable in this disease [220]. Interestingly it was also shown that CD23 expression is markedly stronger in proliferating CLL cells located in splenic white pulp and proliferating centres in lymph nodes in comparison to CLL cells outside these proliferation centres [221]. Increased CD23 levels have been reported in various chronic inflammatory diseases including systemic lupus erythematosus (SLE) [222], inflammatory bowel disease [223], Sjogren's syndrome [222] and rheumatoid arthritis (RA)[224,225]. In the case of RA, successful disease modulation was achieved when arthritic mice were treated with either MAb or polyclonal antibodies to mouse CD23 in the typeII collagen-induced arthritis (CIA) mouse model [226]. This clearly establishes the therapeutic importance of CD23 in these disease conditions. Although the disease amelioration in the CIA mouse by using anti-CD23 antibodies is attributed to blocking of cytokine production by macrophages, the variety of roles for CD23 in B cell activation and growth indicate a strong role for CD23 on B cells in diseases where there is clonal expansion of malignant B cells such as CLL or in autoimmune diseases such as SLE.

The pleiotropic activities of soluble CD23 fragments and particularly the activities relating to B cell activation and growth, have not been shown *in vitro* in mouse cell lines or in genetically-engineered mice. Studies in mice have confirmed the negative regulatory effects of CD23 in IgE production. Targeted deletion of both CD23 alleles ($CD23^{-/-}$) in mice was shown to produce higher levels of IgE than wild-type equivalents on immunisation with thymus-dependent antigens [227]. Moreover, over-expression of CD23 under certain genetic backgrounds showed reduced IgE production in response to antigenic stimulation [228]. Recently, a naturally occurring dominant negative CD23 allele in NZB mice was shown to have reduced IgE binding and failed to produce negative feedback regulation for IgE responses [229]. However these mice had normal B lymphocyte growth and maturation indicating perhaps the differences in the role of human and murine CD23 in modulating B cell growth.

Several differences in structure and cellular expression may account for the differences in function between the human and murine CD23. Murine CD23 has 2 instead of 1 N-linked glycoslation sites, 4 instead of 3 consensus repeats in the leucine zipper motif [173,178] and is also not known to form trimers as it lacks the DGR motif by a naturally occurring truncation and the sCD23 fragments bind IgE with much lower affinity [230]. Murine CD23 is expressed only on B cells, follicular dendritic cells and some T-cells. Interestingly the mouse CD21 does not bind EBV and mouse and human CD23 could also have evolved with different function in relation to EBV infection [231].

Previous work from our laboratory identified the vitronectin receptor αvβ5 as a CD23 receptor on SMS-SB cells. SMS-SB cells do not express CD21 or any other CD23 receptors identified and do not produce CD23 mRNA for autocrine CD23 activity. αVβ5 was shown to bind to an RKC motif (arg-lys-cys) on human CD23 and this interaction sustained proliferation of the pre-B cell line, SMS-SB [232]. The growth-promoting effects of sCD23 described so far have been in the mature B cells whether it is EBV-transformed LCLs, germinal centre B cells or normal mature B cells and these effects have been attributed to CD23-CD21 interactions. With regard to haematopoiesis, a role for CD23 has been suggested either in the myeloid precursors or T cell precursors. CD40 ligation together with IL-3 was indeed shown to induce CD23 expression [169] in human B cell precursors and induce proliferation [169,233]. Considering the important role of CD23 in mature B cells and other haematopoietic precursors and the expression of CD23 by bone marrow stromal cells, it is conceivable that CD23 plays some role during B cell development in the bone marrow. However, the precursor B cells do not express CD21 and therefore any effects of CD23 on these cells would have to be via another receptor. As shown by the previous work in our laboratory on SMS-SB cells, the CD23-αVβ5 integrin interaction could be an important mechanism for survival of B cell progenitors in the bone marrow, independently or together with other cytokines and adhesion molecules.

1.3.2 Integrins

Integrins are heterodimeric transmembrane adhesion receptors composed of non covalently bound α and ß subunits [234-237]. They mediate cell-to-cell and cell-to-extracellular matrix (ECM) interactions and are involved in fundamental cellular processes such as cell proliferation, migration, differentiation and apoptosis [237]. The integrin family is composed of 18α and 8ß subunits and each α and ß combination has its own binding characteristics. The integrins are expressed in a cell type-specific manner and despite a high degree of redundancy most integrins have specific biological functions and differences exist in signalling between integrins.

Ligands for integrin receptors are diverse and ligand binding can induce structural changes in the integrin's interaction with other molecules. For example, vitronectin inhibits sCD23 binding to the integrin αVß3 [203]. Integrins mediate adhesion upon binding to specific adhesion proteins such as vitronectin, fibronectin and collagen, often through the recognition of the tripeptide arg-gly-asp acid (RGD) motif. In addition, they also bind to soluble ligands such as fibrinogen or counter receptors on adjacent cells such as intracellular adhesion molecules (ICAMs) [234]. Although many integrins recognise the RGD sequence in their matrix ligands they are also capable of distinguishing different RGD-containing proteins such that some integrins bind primarily to fibronectin and others to vitronectin.

1.3.2.1 Integrin Signalling

Integrins not only transduce signals to cells in response to the extracelluar environment but they also respond to intracellular cues that alter the integrin response to the extracellular environment. Ligand binding leads to integrin clustering, assembly of cytoskeletal proteins (such as vinculin, talin, paxilin), reorganisation of the actin cytoskeleton, all resulting in the formation of focal adhesions [238]. In addition to forming a structural link between ECM and the cytoskeleton, focal adhesions are also important sites of signal transduction. The signalling pathways activated by integrins are similar to the kinase cascades activated by growth factor receptors. Depending on the composition of the ECM,

integrins activate one or more intracellular pathways. Because integrins, like all adhesion receptors, do not have intrinsic kinase activity, activation of signalling pathways requires recruitment of non-receptor kinases. These kinases typically involve phosphorylation of focal adhesion kinase (FAK) or the related kinase PyK2 and activate a number of Src-homology domain SH2 and SH3 containing adapter proteins [238]. FAK is activated by integrin-mediated adhesion and localises to focal adhesions, creating a binding site for Src family of tyrosine kinases and adaptor proteins such as Grb-2, and subsequent activation of mitogen activated protein kinase (MAPK) cascades such as the Ras/ERK pathway [239,240]. FAK is also known to bind to PI3K and can activate PI3K-mediated pathways for cell survival [241].

Signals from the integrin receptors are also integrated with those originating from growth factor and cytokine receptors and association of integrins with other membrane proteins has been shown to provide additional coordinate signals to cells that are specific for individual integrins [242]. Integrin-dependent adhesion and signalling is important in regulation of multiple processes such as tissue development, inflammation, angiogenesis, tumour cell growth and metastasis [237].

1.3.2.2 Vitronectin receptors

The vitronectin receptors (VnRs) are members of the integrin family comprising the αV chain in non-covalent association with one of five ß subunits ß1, ß3, ß5, ß6 and ß8 and bind a broad spectrum of ligands, including fibrinogen, fibronectin and vitronectin, typically by recognition of the arg-gly-asp (RGD) motifs [236,237]. The integrins αVß5 and αVß3 are the vitronectin receptors currently defined as CD23 receptors.

The αVß3 and αVß5 integrins have been studied for their roles in tumour progression, metastasis, angiogenesis and inflammation[237]. Moreover, mice lacking these integrins have been shown to have enhanced tumour growth and angiogenesis [243]. Studies using antibodies to ß3 and ß5 domains have shown that antibodies to the region of ß3 proximal to the ligand binding site failed to recognise ß5 [244]. This may relate to the fact that αVß5 displays a more restricted

ligand binding, primarily to vitronectin, while αvß3 binds to vitronectin, fibronectin, fibrinogen and von Willebrand factor [245]. These integrins may also have different signalling functions as both αVß3 and αvß5 localise to focal adhesions but only ß5 containing complexes undergo endocytosis and are capable of sustaining focal adhesion kinase activity at high levels in a Src-dependent manner [245]. These differences in ligand specificity and activation patterns indicate that these integrins may have specialised function in different cellular processes.

The integrin αVß3, consists of 125kDa αV subunit and 105 kDa ß3 subunit and has been a focus of intensive research because of its major role in several distinct processes, such as osteoclast-mediated bone resorption, angiogenesis, pathological neovascularisation and tumour metastasis [246]. The engagement of this integrin is shown to enhance cell invasion in human multiple myeloma [247] and it has also been shown to be involved in proliferation and migration of lymphoid tumours [248]. The crystal structure of αVß3 binding an RGD ligand has been determined, giving more insight into integrin conformation and activation states [249].

1.3.2.3 Integrin Structure

Integrins are large glycoproteins with multiple domains and integrin ligand binding is tightly linked to molecular conformation. Each of the α and ß subunits comprises a large extracellular domain, single transmembrane domain and a short cytoplasmic tail. The N-terminal portions of the α and ß subunits fold into a globular headpiece (containing the ligand binding site) which is connected through the α and ß tailpiece domain to the membrane (Figure 1.3). Activation of the integrin results in rearrangement in the orientation of these domains [236].

Structural studies have revealed that the integrins exist in at least three different conformational states. A bent conformation, an extended conformation with a closed headpiece and an extended conformation with an open headpiece[250]. It has been suggested that the bent conformation is the low affinity state while the open conformation corresponds to the ligand-bound high affinity state. Many antibody epitopes that are buried in the bent conformation

become exposed in the extended conformation and ligand binding induces the high affinity conformation [250]. For example, cyclic RGD peptides bind to the low affinity bent conformation of αVß3 and induce conversion to the extended conformation with open headpiece [250]. The model proposed is that the bent conformation represents an inactive physiologically-relevant conformation and that activation would be accompanied by a switchblade-like opening of the headpiece and tailpiece interface into an extended state [236]. The crystal structure of αVß3 revealed a bent conformation where the αV and ß3 subunits are bent acutely at the knee region (in between the head piece and tail piece). The ligands were shown to bind at the major interface between the αV and ß3 subunits making extensive contacts with both [249].

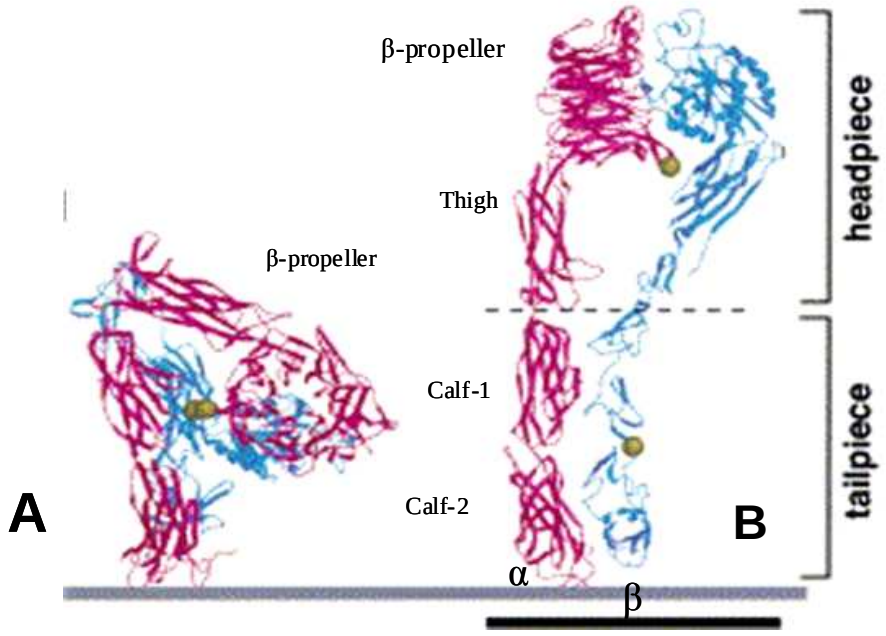

Figure 1.3 Representation of bent and extended conformation of extracellular regions of integrin αvβ3.

(A) Shows the ribbon diagram of the integrin in the bent conformation with closed headpiece and **(B)** shows the extended conformation with open headpiece. The headpiece and the tailpiece region are indicated. The β-propeller region in the headpiece which is found in the ligand binding site and the thigh and calf regions are also indicated. This representation is based on the crystal structure of αvβ3 and the figure is reproduced from Takagi *et.al.,* (2002)[250].

In solution or on the cell surface, the integrins are thought not to be fixed in a particular conformation but are in equilibrium between them [250]. Whether equilibrium favours the bent or extended conformation depends on the presence of activating intracellular factors and concentration of extracellular ligands. Activation by signals within the cell, known as inside-out signalling, induces straightening and stabilises the extended form and binding of the extracellular ligand also stabilises the extended form enhancing the separation of integrin tails, resulting in transmission of signals to the cytoplasm (outside-in-signalling) [250].

1.3.2.4 Integrins in hematopoiesis

Interactions of haematopoietic precursors with the stromal microenvironment are critical for controlling their migration, self-renewal and differentiation, and many of these interactions are mediated by integrins [251]. Aside from retaining stem and progenitor cells in the bone marrow, engagement of adhesion receptors on these cells also plays an important role in modulating growth and survival signals[252]. Among the integrins, ß1 and the ß2 'leucocyte integrins' have been identified on haematopoietic progenitor cells. The β1 integrins, particularly α4ß1 (VLA-4) and α5ß1 (VLA-5) and ß2 integrin LFA-1 have been studied extensively for their roles in cell adhesion and migration in haematopoietic cells. Both ß1 and ß2 integrins are responsible for interactions between cells and ECM components such as fibronectin, collagen, laminin and thrombospondin or the cell surface expressed adhesion molecules VCAM-1 and ICAM-1 [158,253-257]. A number of studies have assessed the expression of both the ß1 and ß2 integrins in $CD34^+$ HSCs as well as cells from different types of B cell leukaemias.

In vitro experiments demonstrated that adhesion of HSCs and HPCs (haematopoietic progenitor cells) to fibronectin is mediated by α4ß1 and α5ß1 [257,258]. α4ß1 also mediates binding to VCAM-1 expressed on bone marrow stromal cells[259]. Contact between normal and leukemic hematopoetic progenitors and bone marrow adherent layers has also been shown to be maintained *in vitro* by a combination of both ß1 and ß2 integrins [112,126,255,260]. *In vivo* experiments have shown a dominant role for these integrins in retention of stem cell progenitors in

the bone marrow and for trafficking of stem cells and, as mentioned above, these functions are modulated by the cytokine SDF-1 [113,261]. Moreover, α4ß1-mediated attachement of HPC to fibronectin was also shown to promote proliferation [262,263] and prevent apoptosis [264].

Gene knockout studies have also indicated important roles for both the α4 and ß1 subunits during hematopoiesis. Studies by Hirsch, *et. al.,* and Potocnick, *et. al.,* have demonstrated that ß1 integrin is an essential adhesion receptor for homing and retention of HSCs in the foetal and adult haematopoietic tissuses [265-267]. Similarly, genetic studies from α4 null chimeric mice revealed that α4 integrins are essential for normal development of lymphoid and myeloid lineages and homing of lymphocytes to Peyer's patches [268,269].

1.3.2.5 αVβ5 integrin in B lymphopoiesis

The importance of VnRs in general, and αVß5 in particular, in lymphopoiesis is unclear. The interaction between CD23 and integrin αVß5 suggests a role for this integrin in B cell development. The ß5 and ß3 knock out mice do not indicate any perturbances in B cell development [270]; however, the murine and human system may have differential requirements for αvß5 function as murine CD23 does not show the cytokine-like activities shown by the human counterpart. Moreover, the functioning of the integrins may be different in normal systems and in knock out mice.

Previous results for the staining of αVß5 integrins on B lymphocytes derived from peripheral blood, bone marrow and ALL patients demonstrate that αVß5 is expressed exclusively on normal or leukaemic B cell precursors (*Borland, et. al., 2008 manusript submitted*). No αVß5 expression was seen on $CD19^+$ B lymphocytes in peripheral blood from normal individuals while normal human bone marrow showed $CD19^+$ cells that were $\alpha V\beta5^+$. αVß5 was shown to be expressed universally on ALL-derived samples whereas no αVß5 expression was shown on B–CLL samples. No αVß3 expression was seen on the precursor cells (normal or leukaemic) indicating that αVß5 is the only CD23 receptor expressed on these cells. Expression of the integrin αVß5 and ubiquity of αVß5 expression

by ALL cells strongly suggests a significant role of αVβ5 expression and function during B cell development.

1.4 Research aims

The main aim of the research work presented in this thesis was to assess the role of αVβ5 integrin during B cell development by testing the hypothesis that the integrin αVβ5 delivers a pro-survival signal at all stages of antigen-independent B cell development in the bone marrow via the αVβ5-CD23 interaction.

The specific aims were to:

(a) Define the expression of αVβ5 integrin (and related αV integrins) during specific B cell developmental stages. This was investigated by using cell lines representative of different B cell developmental stages and B cells from human bone marrow. Cell lines expressing αVβ5 integrin were used to address αVβ5 function by assessing proliferation response stimulated by the αVβ5-CD23 interaction.

(b) Explore the role of cytokines chemokines and growth factors important in B cell development in regulating αVβ5 function and expression. Cytokines (IL-7, IL-3, IL-4, IL-7), the chemokine SDF-1 and platelet-derived growth factor (PDGF) were used to assess αVβ5 expression and αVβ5 mediated cell proliferation as well as signalling pathway activation in different pre-B cell lines.

(c) Determine whether murine B cell precursors could respond to soluble stimuli delivered by the αVβ5 integrin. This series of experiments would demonstrate whether the data from the human studies could be transferred to a murine model that would allow an analysis of the *in vivo* biology of αVβ5 integrin in the haematopoietic system.

2 Materials and Methods

2.1 Antibodies and Reagents

All routine chemicals used were purchased from Sigma Chemicals Co. Poole, UK unless otherwise stated. Recombinant cytokines human (IL-7, IL-3, IL-11, IL-4) and mouse (IL-3) were purchased from R&D Systems, Abingdon, UK. Biotinyl-murine IgG1 and recombinant SDF-1β were purchased from Chemicon, Hampshire, UK. PI-3K inhibitor LY294002 and platelet-derived growth factor (PDGF-β) were supplied by Cell Signalling Technologies, Beverly, MA, USA. Streptavidin-PE, streptavidin-APC and annexinV-FITC were from BD Bioscience, Oxford, UK. Anti-actin and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulins, AMD3100, propidium iodide (PI), Trypan blue, NHS-LC biotin, mouse IgG1 and IgG2a isotype control antibodies, IgG1-PE secondary antibody, DMEM (Dulbecco's Modified Eagle's Medium), L-Glutamine 200mM and Penicillin-Streptomycin solution were purchased from Sigma Chemicals Co, Poole, UK. Isotype control antibody IgG1-FITC was purchased from DAKO, Cambridgeshire, UK. Normal Armenian hamster IgG1-FITC was purchased from Autogen Bioclear, Witshire, UK. Foetal Calf serum was supplied by TCS Cellworks, Buckingham, UK The MEK inhibitor U0126 and Access RT-PCR System kit were purchased from Promega, Southampton, UK. Tritiated thymidine was obtained from Amersham International plc, Amersham, Buckinghamshire, UK. Recombinant 25-kDa sCD23, encompassing residues Met^{151}-Ser^{231} with an N-terminal His_6 tag, was purchased from R&D Systems, Abingdon, UK. A CD23-GST fusion protein preparation comprising residues Asp^{48}-Gly^{248} of CD23 (referred to as $sCD23^{48-248}$) was obtained from Assay designs (Cambridge Bioscience, Cambridge, UK). Der CD23 was a kind gift from Dr. Jim McDonnell (University of Oxford, UK). RPMI-1640 medium and protein-free hybridoma medium-II (PFHM) were from GIBCO-BRL (Paisley, Scotland, UK).

The antibodies used and their suppliers were as follows:

Target Antigen	Monoclonal or polyclonal	Clone	Isotype	Supplier	Other
Human CXCR4	Mouse Monoclonal	12G5	IgG2a	R&D Systems, Abingdon, Oxford, UK	Biotinylated
Human αVβ5	Mouse Monoclonal	P1F6	IgG1	Chemicon, Hampshire, UK	
Human αVβ5	Mouse Monoclonal	15F11	IgG2a	Chemicon, Hampshire, UK	
Human αVβ3	Mouse Monoclonal	23C6	IgG1	Chemicon, Hampshire, UK	
Human αVβ3	Mouse Monoclonal	LM609	IgG1	Chemicon, Hampshire, UK	
Human αV	Mouse Monoclonal	AMF7	IgG1	Chemicon, Hampshire, UK	
Human αV	Mouse Monoclonal	LM142	IgG1	Chemicon, Hampshire, UK	
Human αVβ6	Mouse Monoclonal	10D5	IgG2a	Chemicon, Hampshire, UK	
Human β1	Mouse Monoclonal	47BR	IgG1	Autogen Bioclear, Buckinghamshire, UK	
Human αV	Rabbit polyclonal	-	IgG1	Chemicon, Hampshire, UK	
Human β5	Rabbit polyclonal	-	-	Chemicon, Hampshire, UK	
Human β3	Goat polyclonal	-	-	Autogen Bioclear, Buckinghamshire, UK	
Human Phospho-p44/42 MAP kinase (Thr202/Tyr204)	Rabbit polyclonal	-	-	Cell Signalling Technologies, Beverly, MA, USA	
Human p44/42 MAP kinase	Rabbit polyclonal	-	-	Cell Signalling Technologies, Beverly, MA, USA	
Human IgM (μ-chain)	Mouse Monoclonal	SA-DA4	IgG1	Southern Biotech, Birmingham, Alabama, USA	
Human κ-chain	Mouse Monoclonal	L1C1	IgG1	Ancell Corporation, Bayport, USA	FITC conjugated
Mouse β3	Armenian hamster-Monoclonal	2C9.G2	IgG1	Autogen Bioclear, Buckinghamshire, UK	
Human IgM (μ-chain)	Goat polyclonal	-	-	Sigma Chemicals Co, Poole, UK	FITC conjugated
Human CD38	Mouse Monoclonal	HI157	IgG1	Sigma Chemicals Co, Poole, UK	
Human CD10	Mouse Monoclonal	SS2/36	IgG1	DAKO, Cambridgeshire, UK	FITC conjugated
Human CD9	Mouse Monoclonal	P/33/2	IgG1	DAKO, Cambridgeshire, UK	FITC conjugated
Human CD19	Mouse Monoclonal	HIB19	IgG1	BD Bioscience, Oxford, UK	PE conjugated

The following antibodies were used for FACS sorting experiments and were all ordered from BD Biosciences, Oxford, UK. All antibodies were mouse monoclonal IgG1 isotype.

Target Antigen	Clone	Conjugated to
Human CD19	SJ25C1	PerCP-Cy5.5
Human CD10	HI10A	PE
Human CD38	HIT2	FITC
Human CD5	UCHT2	FITC
Human CD7	M-T701	FITC

2.2 Buffers

The following buffers were routinely used:

Buffers	Compostion
PBS	13mM NaCl, 27mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4 (pH 7.2)
TBS	50mM Tris HCl (pH 7.4), 150mM NaCl
TAE	40mM Tris.acetate, 2mM Na_2EDTA pH8.5
Transfer buffer	25mM Tris, 192mM Glycine , 20% (v/v) methanol
Stripping buffer	62.5 mM Tris HCl pH 6.7, 2% (w/v) SDS, 100mM 2-mercaptoethanol
Blocking buffer	TBST (1X TBS with 0.1% (v/v) Tween) , 5% (w/v) BSA

2.3 Cell lines and cell culture

The *SMS-SB* cell line was derived from a female patient at the leukaemic phase of lymphoblastic lymphoma [271]; *SMS-SB* cells were cultured in PFHM with 2 mM fresh glutamine, penicillin (100U/ml) and streptomycin (100µg/ml) at 37°C in 5% (v/v) CO_2 in air in a humidified atmosphere, at 2.5-5 x 10^5 cells/ml ("normal cell density"). *RS4;11* [272], *697*, *Nalm6*, *Blin1*, *Raji*, *Ramos*, *Daudi*, *IB4* and *SKW* B cell lines were from laboratory liquid nitrogen stocks. Cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM fresh glutamine, penicillin (100U/ml) and streptomycin (100µg/ml), at 37 °C in a 5% (v/v) CO_2 in air in a humidified atmosphere. The *BAF03* murine

pro-B cell line was also from laboratory stocks [273] and this cell line was cultured in RPMI-1640 medium with 50μM 2-ME (mercapto-ethanol) and 1ng/ml murine IL-3. The stromal cell line *hMSC-Tert* [274] was maintained in DMEM {10% (v/v) FCS, 2 mM fresh glutamine, penicillin (100U/ml) and streptomycin (100μg/ml)} and the murine stromal cell line *OP9* [275] (a kind gift from Dr. Alison Michie, Glasgow, UK) was maintained in DMEM {20% (v/v) FCS, 2 mM fresh glutamine, penicillin (100U/ml) and streptomycin (100μg/ml) and 50μM 2ME} at 37 °C in 5% (v/v) CO_2 in air in a humidified atmosphere.

The different B cell lines used and the stages of B cell development represented by these cell lines is summarised in table 2.1. The translocations well known for each of these cell lines is indicated in the case of the cell lines where these translocations and fusion gene products have been well characterised.

Table 1 Summary of the different B cell lines used for cell culture experiments.

Cell Line	Established From	Type of Cell Line	Known Translocations
SMS-SB	Blood mononuclear leuckocytes of 16-year old girl in leukaemic phase of lymphoblastic lymphoma	Human Pre-B ALL	None
RS4;11	Bone marrow of a 32-year old woman with ALL	Human Pre-B ALL/ Mixed lineage leukaemia (MLL)	t(4;11)(q21;q23) MLL/AF4 fusion gene
697	Bone marrow of a 12-year old boy with ALL (cALL)	Human Pre-B ALL	t(1;19)(q23,p13) E2A-PBX fusion gene
Nalm6	Peripheral blood of a 19-year old man with ALL	Human Pre-B ALL	t(5:12)(q33.2;p 13.2)
Blin-1	Bone marrow aspirate of 11-year old boy with ALL	Human Pre-B ALL	-
Raji	Left maxilla of 12-year old African boy with Burkitt lymphoma	Human Burkitt lymphoma	t(8;14) translocation associated with Burkitt lymphoma
Ramos	Ascitic fluid of a 3-year old boy with American type Burkitt lymphoma	Human Burkitt lymphoma	t(8;14) translocation associated with Burkitt lymphoma
Daudi	Left orbital biopsy of a 16-year old African boy	Human Burkitt lymphoma	t(8;14) translocation associated with Burkitt lymphoma
SKW	EBV infection of B cells	EBV transformed lymphoblastoid cell line (LCL)	-
IB4	EBV infection of umbilical cord B cells	EBV transformed lymphoblastoid cell line (LCL)	-
BAF03	IL-3 dependent murine pro B cell line from peripheral blood derived from BALB/c mouse	Mouse pro B cells	-

2.4 Proliferation assays

SMS-SB cells were harvested and washed in PFHM then plated at a density of 2500 cells/100-µl culture {low cell density (LCD)}, a seeding density at which the cells are prone to apoptose [276], in 96-well microtitre assay plates in PFHM. All other cell lines were washed extensively in PFHM prior to culture at 5000 cells/100-µl cultures. Cultures were propagated in the presence or absence of cytokines, monoclonal antibodies (MAbs) or peptides, at 37 °C for 72 h followed by addition of 0.3 µCi/well ($5.5*10^{-6}$ µmol) tritiated thymidine ([^{3}H]-TdR) for 18 h prior to harvest; incorporation was determined by liquid scintillation spectrometry. Briefly, the cells from 96-well plates were harvested onto glass fibre mats (Wallac Oy, Truku, Finland) using a TomTec MachIII Cell Harvestor (PerkinElmer, Massachusets, USA). The mats were air-dried, sealed in sample bags with Betaplate Scintillin (Wallac Oy, Truku, Finland) and counted using Wallac 1450 MicroBeta Liquid Scintillation and Luminescence Counter (Wallac Oy, Truku, Finland). Tritiated thymidine incorporation was measured as count per minute (cpm). All determinations were made in triplicates and at least 3 repeats were performed for each experiment. Cytokines were used at 0.5-32nM concentration ranges and sCD23 was employed at 0.4-6nM. In experiments using inhibitors, cells were incubated with the inhibitors (5-10µM U0126, 50µM LY294002, 50µM AMD3100) for 30 min at 37°C prior to the introduction of stimuli.

Bone marrow B cell proliferation assays were performed with stromal cell support using hMSC-Tert (human telomerised stromal cell line) or OP9 (murine stromal cell line) cells. Stromal cells were grown until confluent and irradiated in solution (30 Gy [3000rad]) using a cobalt irradiation source and plated at a density of 1.5 $X10^4$ cells/well at least 3 days before the addition of B cells for hMSC-Tert and 2 days before the addition of B cells for OP9 cells in their appropriate medium. Pre-B cells, FACS sorted from human mononuclear bone marrow cells using anti-CD19 and anti-κ MAbs, were washed and plated on 96-well plate containing hMSC-Tert stromal cells at a density of 2500-3000 cells/ well with appropriate antibody, CD23 or peptide treatment in RPMI-1640 (2% (v/v) FCS and 20U/ml DNase I). Stromal cell medium was removed and cells

washed extensively with RPMI-1640 immediately before plating the B cells. Plates were incubated at 37 °C for 72 h and tritiated thymidine ([^{3}H]-TdR) analysis performed as mentioned above.

Murine bone marrow cells (a kind gift from Dr. Adam Lacy-Hulbert, Boston, USA) were harvested from bone marrow of wild type or αV conditional knockout mice by flushing the bones in PBS and the cells were then washed with red cell lysis buffer (0.83% (w/v) NH_4Cl in 50mM Tris HCl pH 7.6). To purify $CD19^+$ B cells, a single cell suspension of bone marrow cells were resuspended in MACS buffer (PBS pH 7.2, 0.5% (w/v) BSA and 2mM EDTA) (90µl buffer per 10^7 cells), with mouse CD19 micro beads (10µl/10^7 cells) (Miltenyi Biotech, Surrey UK) for 15 min at 4 °C. After incubation cells were washed and resuspended in MACS buffer. The cells were then positively selected using magnetic-activated cell separation (MACS) LS columns according to manufacturer's insctructions (Miltenyi Biotech, Surrey UK). Purified $CD19^+$ cells were plated on to OP9 cells containing appropriate treatments at 2500-3000 cells/well in RPMI-1640 (10% (v/v) FCS, 50µM 2ME, 1ng/ml IL-7). Medium was removed and OP9 cells were washed extensively with RPMI-1640 immediately before plating the B cells. Plates were incubated at 37 °C for 72 h and tritiated thymidine ([^{3}H]-TdR) analysis performed as mentioned above.

2.5 Peptide Biochemistry

We have previously shown that CD23-derived peptides containing the RKC motif recognised by αVβ5 integrin are biologically active. The peptides were used at 0.5-3.33µM and are numbered according to their position in the original screening library [232]. The sequences of the specific peptides used in this study are #9, KWINFQRKC; #10, SGSGINFQRKCYY; #12, SGSGRKCYYFGKG; #11, FQRKCYYFG; LP long peptide, KWINFQRKCYYFGKG; Peptides #8, PEKWINFQR; #41, SGSGGEFIWVDGS; #34, SGSGHTGSWIGLR #17, SGSGKQWVHARYA; and #58 SGSGGSGRWNDAF; which lack the RKC motif and do not bind to αVβ5 were used as negative control peptides. Peptides were synthesised with a common SGSG linker and N-terminal biotin tail or as unlabelled peptides lacking the linker and biotin tail (Mimotopes, Clayton, Victoria, Australia). For binding experiments

using FACS, biotinylated peptides were used and for proliferation experiments unlabelled peptides were used.

2.6 Flow cytometry

Cells were harvested and washed twice with ice-cold PBS and resuspended at 5×10^6 cells/ml. 100µl of cell suspension was stained with either biotinylated, unlabeled primary MAb or biotinylated CD23-derived peptides (10µg/ml) for 30-60 min at 4 °C; unlabeled primary antibody was visualized using a secondary PE-conjugated anti-mouse IgG or, in the case of biotinylated antibody or peptides, using streptavidin-PE. Cells were incubated in secondary antibody for 30-60 min at 4 °C and washed twice with ice-cold PBS before anlaysis. For assay of apoptosis, approximately 1 X 10^6 cells were washed twice with ice-cold PBS and stained with 100µl labelling buffer (10mM HEPES-NaOH pH7.4, 150mM NaCl, 5mM $CaCl_2$, 5µg/ml propidium iodide and 5µl annexinV-FITC) and incubated at room temperature for 20 min. The labelling buffer was aspirated and the cells were resuspended in 400µl binding buffer (10mM HEPES-NaOH pH7.4,150mM NaCl, 5mM $CaCl_2$). Cells were analyzed on a *FACScan* flow cytometer, using *CellQuest* software.

For bone marrow analysis and cell sorting frozen human monounclear bone marrow cells (Lonza Walkersville, Inc, Walkersville, MD, USA) were thawed in RPMI -1640 (10% (v/v) FCS and 20U/ml DNase-I) and washed twice with ice-cold PBS and processed as mentioned above. The cells were processed under sterile conditions. All antibodies were monoclonal IgG1 isotype except µ-FITC, which was a goat polyclonal antibody. Bone marrow mononuclear cells were counted and resuspended in PBS and stained with appropriate antibody mixtures and all cells were analysed and sorted on BD FACSAria cell sorter (BD Bioscience, Oxford, UK). Cells were sorted on the basis of $CD19^+/\kappa^+$or $CD19^+/\kappa^-$ and isotype control antibodies and control beads (BD Bioscience, Oxford, UK) were used to draw appropriate gates for positive and negative cell populations.

2.7 RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated using the Trizol reagent (GIBCO-BRL, Paisley, Scotland) according to manufacturer's instructions. Briefly, 10^6-10^7 cells were harvested and resuspended in 1ml Trizol/10^7 cells and RNA solubilised by passing the lysates through a pipette a few times. Chloroform was added (0.2ml/1ml Trizol) and the sample was shaken vigorously for 15 seconds, left on ice for 5 min followed by centrifugation at 12000 x ***g*** at 4 ºC for 15 min. The colourless aqueous phase containing the RNA was removed from the red phenol-chloroform phase and RNA was precipitated by incubating the sample in equal volume of ice-cold isopropanol for 10 min followed by centrifugation at 12000 x ***g*** at 4 ºC for 15 min. RNA was then washed with 70% (v/v) ethanol, air-dried and resuspended in 50-100µl of RNAse-free dH_20 (Promega, Southampton, UK). Sample concentrations and purity were determined by spectrophotometric readings of a 1/500 dilution of the sample and using the absorbance at 260nm and 280nm wavelengths.

Total RNA extracted was used for RT-PCR reactions to amplify transcripts using the Access RT-PCR system kit (Promega, Southampton, UK) according to the manufactuer's instructions. Briefly 1µg RNA was added to a mixture of AMV Tfl reaction buffer, dNTP mix (10mM each dNTP), 1µM each of forward and reverse primer, 25mM $MgSO_4$, AMV reverse transcriptase (5 units), Tfl DNA polymerase (5 units) and RNAse-free water to make the volume up to 50µl.

The primers used were as follows (MWG Biotech, London UK):

Target transcript	Forward Primer	Reverse Primer
GAPDH	5'-TCCACCACCCTGTTGCTG-3'	5'-ACCACAGTCCATGCCATCAC-3'
Integrin αV	5'-CGGTTGCCGGGCAAGGTGAG-3'	5'-CAGGCATGGGCGCTGGCTGA-3'
Integrin β5	5'-GGCAGTGCCAATGCACGGAG-3'	5'-GGCCCTGGATCGCTCGCTCT-3'
Integrin β3	5'-GACTGCCTGTGTGACTCCGAC-3'	5'-TGAGCAGGGCGGCAAGGCCA-3'
Integrin β6	5'-TGGAGTGCTCTGCAGCGGGC-3'	5'-GGTTCCCGTTTGCCACTTGGC-3'
Integrin β8	5'-TGCACCGATCCCAGGAGCATC-3'	5'-CTCACGTCGGTAGGTGACTGC-3'

The following programme was used on the thermal cycler

- For reverse transcription:
  45 minutes at 45ºC for 1 cycle and
- For AMV RT inactivation and RNA/cDNA primer denaturation
  2 minutes at 94ºC for 1 cycle.

- For PCR amplification:
  30 seconds at 94ºC, 1 minute at 60 ºC and 2 minutes at 68ºC for 40 cycles
  7 minutes at 68ºC for 1 cycle
  Storage at 4ºC

For RT-PCR with the ß5 primers in some cell lines, the annealing temperature was changed to 62 ºC for 1 minute to obtain a single specific band. PCR products were analysed by agarose gel electrophoresis using 2% (w/v) agarose gel in 1XTAE buffer.

2.8 Western blot analysis

Approximately 3 X 10^7 cells in PFHM were stimulated with either SDF-1 (250ng/ml) sCD23 (160ng/ml), Long peptide (1µg/ml), Peptide #9 (5µg/ml), Peptide 8 (5µg/ml), 15F11 MAb (5µg/ml) alone or in combination for specified time periods at 37 ºC. Stimulation was stopped by addition of RIPA buffer without detergents (50mM Tris-HCl pH7.4, 150mM NaCl, 1mM EGTA, 1mM Na_3VO_4). Cell pellets were lysed in RIPA buffer with protease inhibitors (RIPA buffer as above plus 1% (v/v) NP40, 1mM Na deoxycholate and protease inhibitors 1mM PMSF and 2µg/ml Leupeptin) and clarified by centrifugation at 13,000 x ***g*** for 15 min. Protein concentrations were measured using the Bradford reagent (BioRad Laboratories Ltd, Hampstead, UK) with BSA as a standard. Equal amounts of protein (30-40µg) were loaded per lane and run on a 4-12% NuPAGE Novex Bis-Tris gradient gels according to manufacturere's instruction (based on standard SDS-PAGE method as described by Laemmli, 1970) [277] using the MOPS buffer supplied (Invitrogen, Paisely, UK). Proteins on the gel were transferred onto nitrocellulose (Amersham, Buckinghamshire, UK) using 1X transfer buffer

and the membrane was blocked for 1 h in blocking buffer. Phosphorylated protein was detected using specific primary antibodies used at 1/1000 dilution with overnight incubation; Blots were washed extensively in TBST and incubated for 1 h in secondary antibodies (1/10000 dilution) conjugated to HRP. All antibody dilutions were made in TBST with 1% (w/v) BSA. Enhanced chemiluminescence (ECL) (Pierce, Rockford, USA) was used according to manufacturer's instruction to develop the blots and blots were exposed to Kodak MXB film for appropriate time depending on the siganl to detect antibody binding. The same membrane was stripped using stripping buffer and total protein was detected using specific primary and secondary antibodies as with the phoshphorylated proteins. Bands were quantified by densitometry using the ImageJ software.

2.9 Biotinylation and immunoprecipitation of cell surface integrin β5

10^8 cells were harvested and washed extensively in PBS. The cells were resuspended at 10^7 cells/ml in 5mM sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin)/PBS (Sigma Chemicals Co, Poole, UK) and incubated on a roller for 30-60 min at room temperature in the dark. The cell pellet was resuspended and washed in 50mM Tris HCl pH 7.4/150mM NaCl and then resuspended in 5ml RIPA buffer with protease inhibitors (see section 2.7) for 30 min on ice. The cell extract was pelleted by centrifugation 12000 x ***g*** for 15 min. 1ml of the cell lysate was added to 100µl of packed streptavidin beads (in RIPA buffer) and incubated at room temperature for 1 h with gentle agitation. The beads were pelleted and washed with RIPA buffer and resuspended in 25µl 5X SDS-PAGE loading dye (0.225M Tris-HCl pH 6.8, 50% (v/v) Glycerol, 5% (w/v) SDS, 0.05% (w/v) Bromophenol blue), boiled for 5 min followed by centrifugation at 12000 x ***g*** for 1-3 min and the supernatant was loaded on 4-12% NuPAGE Novex Bis-Tris gels and transferred onto nitrocellulose membrane. Membranes were probed separately using primary anti-αv, ß5 and ß3 antibodies to detect biotinylated integrins. Secondary antibodies conjugated to HRP and enhanced chemiluminescence were used to visualise the appropriate bands as mentioned in section 2.7.

2.10 Staistical Analysis

Results are shown as mean plus or minus standard deviation (SD) or standard error of the mean (SEM) of triplicate results. Statistical comparision was performed using the Students paired *t* test. The minimal level of significance was $P<0.05$. Each result is representative of at least 3 experiments unless otherwise stated.

3 αV Integrin Expression and Function in Human B Lymphocytes

3.1 Introduction

The proliferation and differentiation of B lymphocytes is regulated by numerous growth factors and adhesive interactions. During development, B cells undergo stepwise maturation through different defined stages. Each stage of this developmental process is regulated by stage-specific signals that modulate B cell growth/survival and differentiation. HSCs in the bone marrow committed to the B lineage go through the pro-B, pre-B and immature B cell stages of development in the bone marrow. Perturbation of this developmental process in the bone marrow leads to malignancies such as ALL. Leukaemias represent a heterogenous group of disorders and ALL is a malignant disease of the bone marrow in which proliferating early lymphoid progenitors replace the normal haematopoietic population. It is a common malignancy in children and approximately 80% of childhood ALL constitutes pre-B ALL. In pre-B ALL the malignant cells are arrested at an early stage of development [278].

Previous work in the laboratory demonstrated that the αVβ5 integrin is a receptor for the sCD23 molecule and the αVβ5-CD23 interaction sustains proliferation of pre-B cell line SMS-SB [232]. Furthermore it was also shown that αVβ5 is expressed significantly in B cells from patients with ALL (*Borland, et. al., 2008 manuscript submitted*). This led to the hypothesis that the αVβ5 integrin expression and function has important roles during B cell development and malignancies that arise at this stage. Therefore the expression of different αV integrins and function of particulary αVβ5 integrin was investigated in different pre-B cell lines and some bone marrow B cell precursors.

3.2 Results

3.2.1 Phenotypic characterisation of different pre-B cell lines and mature B cell lines

During development, B cells undergo stepwise maturation through defined stages. Various cell surface markers have been identified that allow the precise identification of B cells at specific stages in the developmental process. B cell lines established from patients with different types of leukaemias are arrested at a certain stage in the developmental process and therefore provide a good model to study each developmental stage. From a range of cell lines that were available in the laboratory, cell lines were chosen as representative of major B cell developmental stages. Cell lines were classified as *early pre-B, late pre-B, immature, transitional or mature B* depending on cell surface marker expression.

CD19 expression was used to confirm all the cell lines as B lineage cells and surface expression of the μ chain was used to identify the cell lines as B cells in Figure 3.2. Surface expression of CD9 was seen in all the pre-B cell lines and the pre-B/immature B cell line 1E8 while the transitional and more mature B cell lines did not express this molecule (Figure 3.1 and 3.2). The CD38 marker was expressed in all the cell lines from pre-B to through to transitional B cell stages although the SMS-SB cell line showed lower levels of CD38 expression than other pre-B cell lines (Figure 3.1). The mature cell lines SKW and IB4 were CD38 negative (Figure 3.2).

SMS-SB and RS4;11 cells were both negative for CD10 (CALLA antigen) (Figure 3.1) which is a marker for pre-B cells [279,280]. SMS-SB cells expressed low levels of surface immunoglobulin heavy chain (μ) and RS4;11 cells also were low/negative for surface expression of μ chain (Figure 3.1). Therefore, these cell lines were designated as early *pre-B cells*. The 697, Blin1 and Nalm6 cell lines were positive for CD10, expressed high levels of surface μ chains and were designated as *late pre-B cells*. The 1E8 cell line was derived originally from Blin1 cells [281] after spontaneous re-arrangement of the kappa (κ) light chain genes, leading to

differentiation of surface κ-negative pre-B cells into surface kappa positive B cells. This is depicted by down-regulation of CD10 expression and the expression of immunoglobulin κ light chain by 1E8 cells and these cells were termed as *immature B cells* (Figure 3.1). The cell lines Daudi and Ramos were considered to be *transitional B cell lines* because they were $CD10^+$ and $CD38^+$ but had lost CD9 expression, which was characteristic of pre-B cell lines (Figure 3.2). Both of these three cell lines express immunoglobulin light chains κ (Daudi) or λ (Ramos) (Figure 3.2). These cell lines were, however, $CD21^-$ and IgD^-. CD21 and IgD expression is a characteristic of transitional and mature B cells. CD21 expression was not noted even in the mature B cell lines SKW and IB4. SKW cells were κ positive but the IB4 cells were not clearly positive for either a clear kappa or lambda light chains (Figure 3.2). This could be because they were more mature immunoglobulin-secreting cell types. Both these cell lines were $CD38^-$ and lacked CD9 or CD10 expression (Figure 3.2) and therefore were designated as mature B cells.

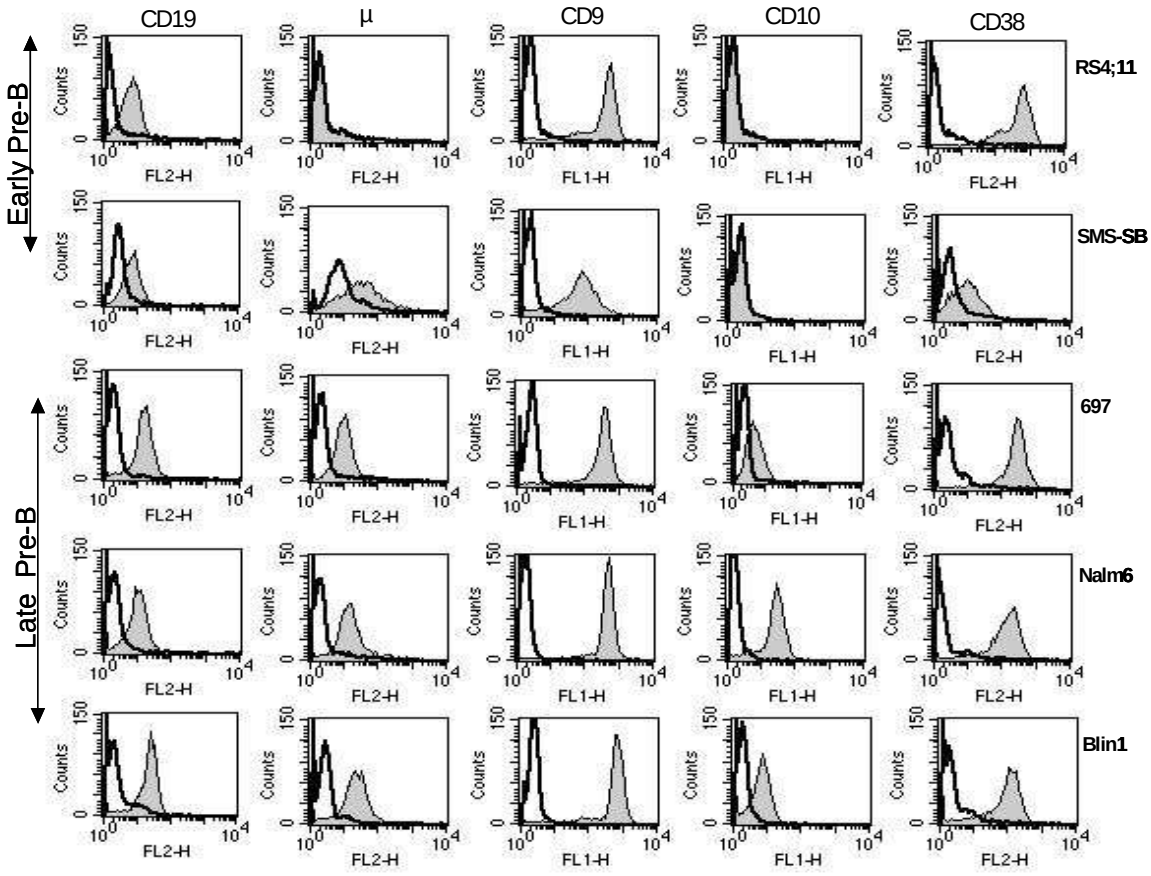

Figure 3.1 Phenotypic characterisation of different human pre-B cell lines.

Pre-B cell lines were categorised into different stages of B cell development (early pre-B or late pre-B) based on expression of surface markers CD19, CD9, μ, CD10 and CD38 using monoclonal antibodies (MAbs). FACS staining for the each MAb is shown as grey shaded area and the isotype control for the specific antibody is shown as a black line. Data are representative of at least three independent staining procedures.

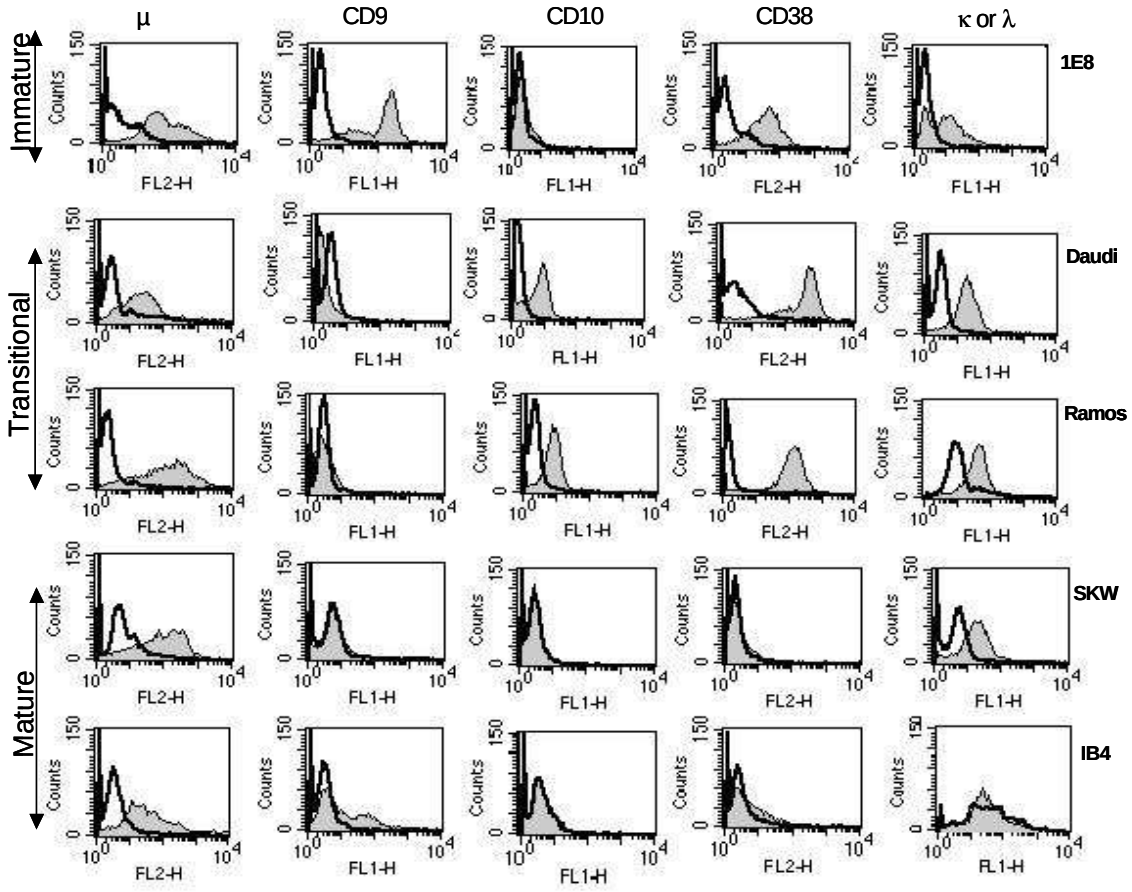

Figure 3.2 Phenotypic characterisation of different human immature and mature B cell lines.

B cell lines were categorised into different stages of B cell development (immature, transitional or mature B cells) based on expression of surface markers CD9, μ, CD10, CD38 and κ or λ light chain expression, using monoclonal antibodies (MAbs). FACS staining for the each MAb is shown as grey shaded area and the isotype control for the specific antibody is shown as a black line. Data are representative of at least three independent staining procedures.

3.2.2 αV integrin expression in pre-B cell lines and mature B cell lines

αV integrin expression was assessed in cell lines representing different stages of B cell development. The αV subunit is known to pair with 5 different β subunits (β1, β3, β5, β6, β8) and monoclonal antibodies are available for all of these heterodimers except for αVβ8. P1F6 and 15F11 are two monoclonal antibodies which bind the αVβ5 heterodimer at different epitopes [282], so both antibodies were used to assess αVβ5 expression. The 23C6 antibody was used to assess surface expression of the αVβ3 heterodimer and the 10D5 antibody detected αVβ6 expression. The anti-β1 antibody (4B7R) was used to assess β1 integrin expression. However, since the β1 subunit can pair with other α subunits, such as α4 and α5, staining with 4B7R could not be considered to demonstrate αVβ1 expression definitively.

Figure 3.3 clearly shows that all the pre-B cell lines from RS4;11 through to Blin1 show high surface expression of αVβ5 as shown by P1F6 antibody staining. However, the pattern of staining using 15F11 antibody was very different to the P1F6 staining. In general, P1F6 staining was much higher than the 15F11 staining. Although 15F11 antibody showed staining for αVβ5 in all the pre-B cell lines, the cell lines RS4;11, SMS-SBs and 697 cell lines had higher 15F11 staining when compared with Nalm6 and Blin1 cell lines. In fact, the cell line 697 had the highest expression of the 15F11 epitope.

αVβ3 integrin expression was more difficult to define accurately as there seemed to be very low-level expression in most of the cell lines and this made it difficult to conclude whether the cell lines were negative or positive for this integrin (Figure 3.3). The three cell lines staining well with 15F11 antibody showed very low or negative staining with the 23C6 antibody, while Blin1 and Nalm6 cell lines had clearly started to express αVβ3. (Figure 3.3). All the pre-B cell lines were clearly negative for αVβ6 integrin and positive for β1 integrin expression.

Figure 3.3 also depicts an obvious decrease in staining for αVß5 using P1F6 antibody in the immature B 1E8 cell line when compared to the pre-B cell line Blin1. A similar decrease is seen with the 15F11 antibody although this is less pronounced than P1F6 staining. αVß5 integrin expression is significantly decreased in the transitional and more mature B cell lines as shown by P1F6 staining (Figure 3.4). The transitional cell line Daudi shows low but positive staining for αVß5, as does the mature B cell line SKW, while the cell lines Ramos and IB4 are clearly negative for αVß5. Among the transitional cell lines, Daudi is slightly positive and Ramos is either negative or very slightly positive for αVß3 expression, while the more mature cell lines SKW and IB4 very clearly show high surface expression of αVß3. Interestingly, the Ramos cell line seemed to express low levels of αVß6 which could not be detected on the other cell lines (Figure 3.4). Staining for the ß1 integrin was significantly reduced in the transitional as well as the mature B cell lines (Figure 3.4).

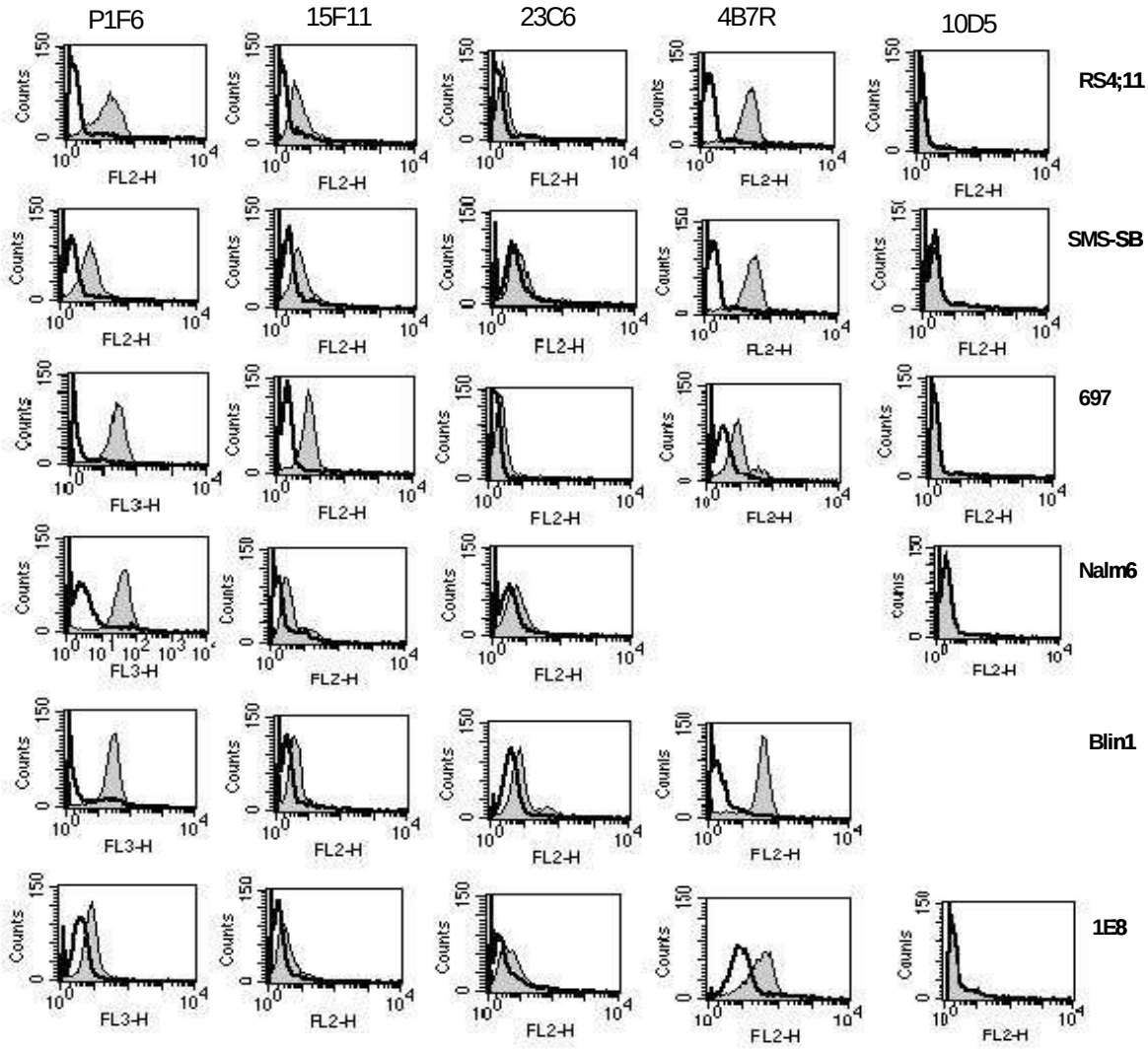

Figure 3.3 Expression of different αV integrins on human pre-B cell lines.

Surface expression of αV integrins was assessed by FACS staining using monoclonal antibodies P1F6 and 15F11 for αVβ5, 23C6 for αVβ3, 10D5 for αVβ6 and 4B7R for the β1 subunit, in the different pre-B or immature B cell lines. Histograms illustrate staining for the integrin antibody as grey shaded area and staining for the specific isotype control antibody as a black line. Staining data was not available for β1 staining of Nalm6 cells and β6 staining of Blin1 cells. Data are representative of at least three independent staining experiments.

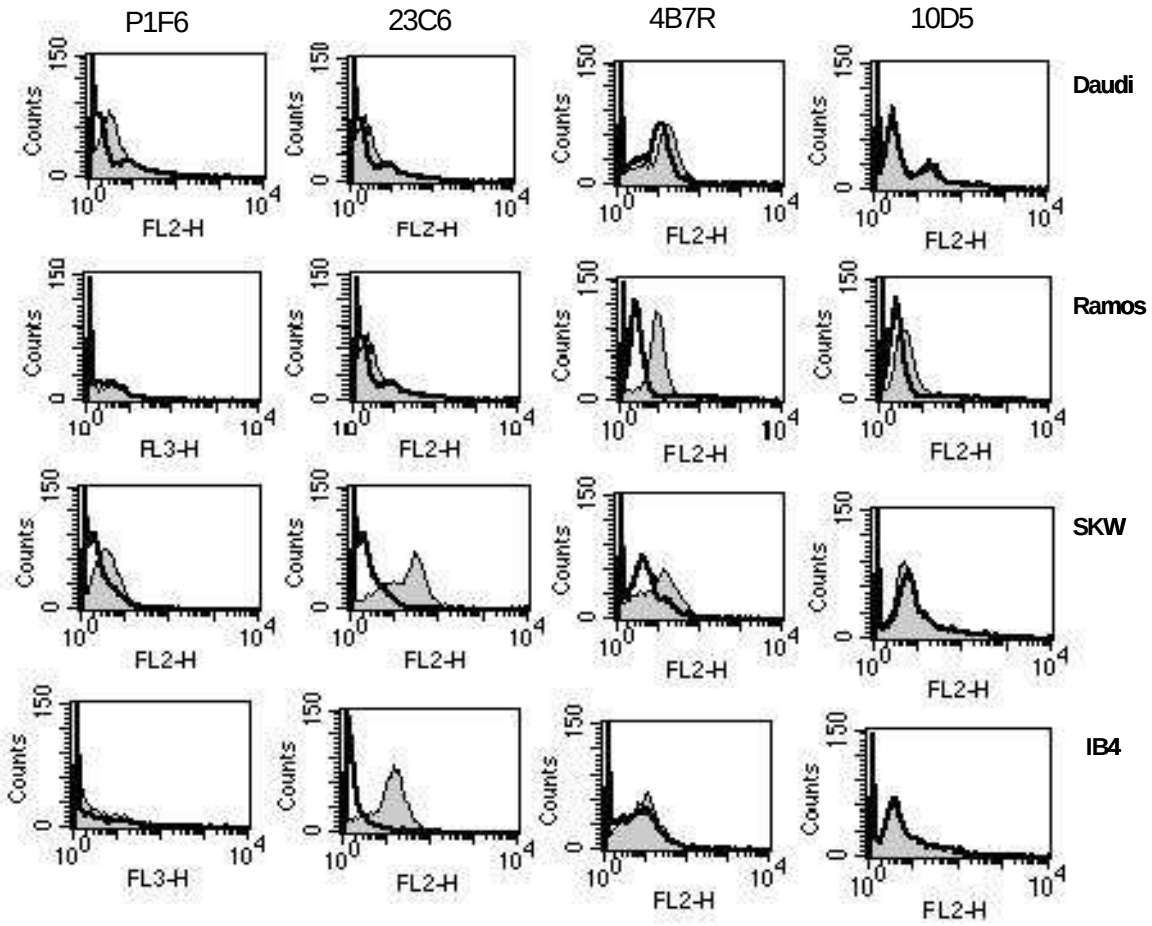

Figure 3.4 Expression of different αV integrins on human mature B cell lines.

Surface expression of αV integrins was assessed by FACS staining using monoclonal antibodies P1F6 for αVβ5, 23C6 for αVβ3, 10D5 for αVβ6 and 4B7R for the β1 subunit, in the different mature B cell lines. Histograms illustrate staining for the integrin antibody as grey shaded area and staining for the specific isotype control antibody as a black line. Data are representative of at least three independent staining experiments.

3.2.3 RT-PCR analysis of integrin transcripts in different B lineage cell lines

The low levels of αVß5 and αVß3 integrin expression found in certain cell lines (Figure 3.5) were further investigated using primer pairs specific for integrin subunits by RT-PCR. Figure 3.5 shows an RT-PCR product of 460 bp for the ß5 integrin subunit in SMS-SB, 697, RS4;11, Daudi, SKW and IB4 cells and the expression was compared to the PCR product from the ß5 plasmid. Daudi cells showed a very faint band at 460bp but there was a more prominent larger band of a higher size. Only the Ramos cell line showed no expression for the ß5 subunit and the IB4 cell line, which showed no cell surface expression of αVß5 by FACS, had transcripts for ß5 subunit. The pre-B cell lines SMS-SB, 697 and RS4;11 showed no transcript for the ß3 subunit while the pre-B cell line Blin1 possesed transcripts of 504 bp (just above 500bp marker) for ß3 subunit (Figure 3.5); interestingly, there was no transcript for the ß3 subunit detected in the 1E8 cell line although we observed staining for αVß3 by FACS. Nevertheless there is a product of higher size in the lane for 1E8. In the transitional cell line category, Daudi cells expressed transcript for the ß3 subunit but Ramos cells did not; both the mature B cell lines SKW and IB4 clearly showed transcripts for the ß3 subunits and this correlated with the results from FACS analysis. The product for ß6 subunit was not of the correct size so no conclusions could be made from them (*data not shown*). Using the ß8 primers a faint band for the correct size of transcript was observed in all the cell lines however, very prominent band of 400 bp was seen in both the mature B cell lines SKW and IB4 (Figure 3.5).

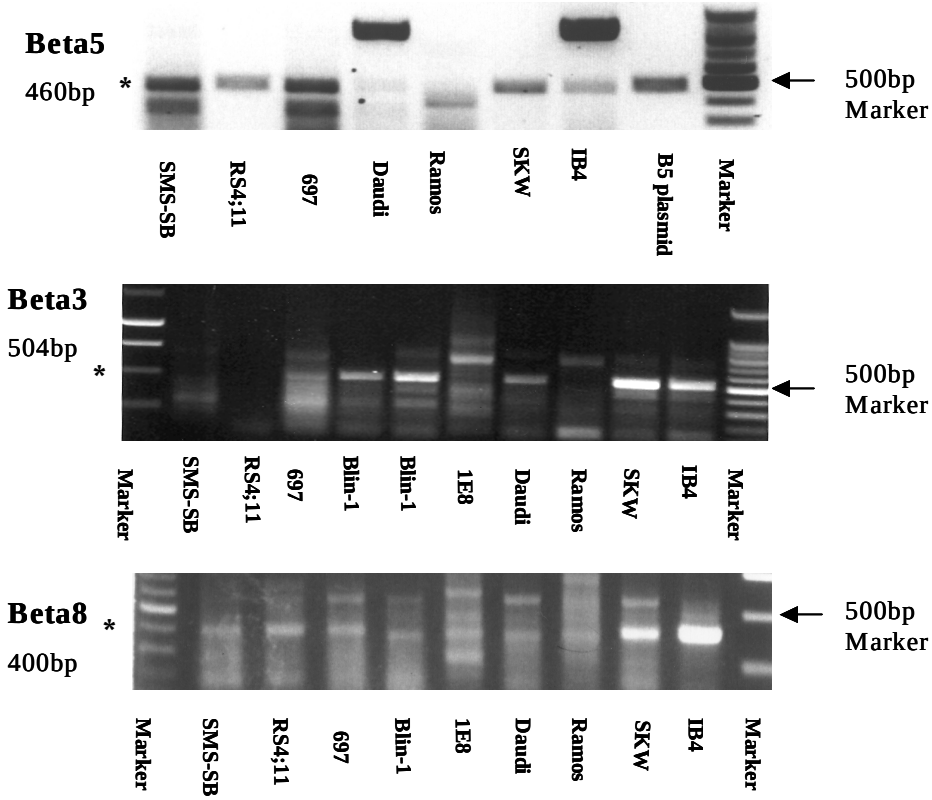

Figure 3.5 RT-PCR analysis for αV integrins β subunit expression in the different B cell lines.

Total RNA isolated from the different B cell lines was used as template for the RT-PCR reaction using primer pairs specific for each β subunit and PCR products were separated by agarose gel electrophoresis. For the β5 subunit a PCR product was amplified from β5 plasmid for comparison of the size. All the cell lines were previously shown to express the αV integrin subunit (*data not shown*). The size of each subunit was compared with a DNA ladder. * Indicates the position of the appropriate sized bands. RT-PCR analysis with GAPDH primers were performed with each cell line as a positive control. Data represent three independent RT-PCR results.

Table 2 Summarises the expression of different cell surface markers (from FACS analysis data) for characterisation of different cell lines together with pattern of expression of individual αV integrins.

	CD9	CD10	CD38	αvβ5	αvβ3	αvβ6	β1	μ-chain	κ or λ
SMS-SBs	+++	-	+	+++	-	-	+++	+	-
RS4;11	+++	-	++	+++	-/+	-	+++	-	-
697	++	+	++	+++	-/+	-	+++	+	-
Nalm-6	++	+++	+++	+++	++	-	NA	+	-
Blin-1	++	+++	+++	+++	++	NA	+++	+	-
1E8	++	-	++	++	+	+	++	++	+
Ramos	-	++	+++	-	-	-	+++	++	++
Daudi	-	++	+++	+	-	-	+++		++
SKW	-	-	-	+	+++	-	+	++	++
IB4	-	-	-	-/+	+++	-	+	++	?

****NA-** Not available

3.2.4 αV Integrin function in human B cell Lines

Previous data have demonstrated that the integrin αVß5 is a receptor for sCD23 on precursor B cells [232]. αVß5 binds an RKC motif on CD23 and this interaction was shown to sustain proliferation of the SMS-SB cell line. This effect on proliferation was mimicked by CD23-derived peptides containing the RKC motif and by specific monoclonal antibodies (MAbs) known to bind to αVß5. The AMF7 MAb, which binds the αV subunit, and the 15F11 MAb, which binds the assembled αVß5 heterodimer, sustained proliferation in manner similar to sCD23; however, the P1F6 MAb that also binds to the αVß5 heterodimer had no effect on cell proliferation. Therefore, sCD23, CD23-derived peptide (long peptide) and MAbs AMF7 and 15F11 were used to assess αVß5-mediated proliferation across the B cell lines representing different stages of B cell development.

3.2.4.1 Proliferation in response to sCD23

Figure 3.6 shows that SMS-SB cells proliferate in response to a range of recombinant sCD23 types. The optimal concentration for a proliferative response varies according to the type of sCD23 used and other constituents in the medium. Previous work in the laboratory showed that gel filtration-purified recombinant sCD23 induces a bell-shaped dose-response curve with maximum growth stimulation seen on addition of approximately 200-500 ng/ml (*White, LJ, PhD thesis, University of Glasgow, 1995*), therefore the concentration range of 20-250 ng/ml was chosen to assess sCD23 driven SMS-SB cell proliferation. The two commercially-available sCD23 (R&D systems) and $sCD23^{48-248}$ (Assays Designs) showed an almost identical dose-dependent increase in tritiated thymidine ($[^3H]$-TdR) uptake when added to SMS-SB cells (Figure 3.3A). However the derCD23 (*from D. J. McDonnell, University of Oxford*) showed optimal increase in cell proliferation at 20ng/ml and no effect at 250 or 120ng/ml.

Furthermore, as indicated in Materials and Methods, (Chapter 2) protein-free medium was always used to assess SMS-SB cell proliferation but some of the pre-B cell lines used did not survive in protein-free medium alone and therefore, 1% FCS was added to the medium. When sCD23-induced cell proliferation was assessed in SMS-SB cells with addition of 1% FCS to the medium, an optimal increase in cell proliferation was seen at 2ng/ml derCD23. Proliferation induced by derCD23 in the presence of 1% FCS was of lesser degree than proliferation induced in PFHM (SI of 1.6 vs. SI of 3), (Stimulation index, SI was calculated as mean of cpm in presence of treatment/mean cpm untreated cells). Constituents of serum such as vitronectin and growth factors such as PDGF will obviously have some regulatory effects on this proliferative response therefore the proliferation response may differ in the two cases.

SMS-SB cell proliferation was demonstrated with a commercially available CD23, sCD23 $^{48\text{-}248}$. This is a truncated form of the full-length sCD23 and lacks the IgE and CD21 binding sites present on the full length CD23. A structural representation of the IgE binding site (in red) and CD21 binding site (in blue) is depicted in Figure 3.3B. The RKC motif bound by the αVβ5 integrin is represented in yellow and is present on the sCD23$^{48\text{-}248}$. The proliferative response induced by this sCD23 establishes that the sCD23-induced proliferation of human B cell precursors is independent of the presence of the IgE binding site or the CD21 binding site.

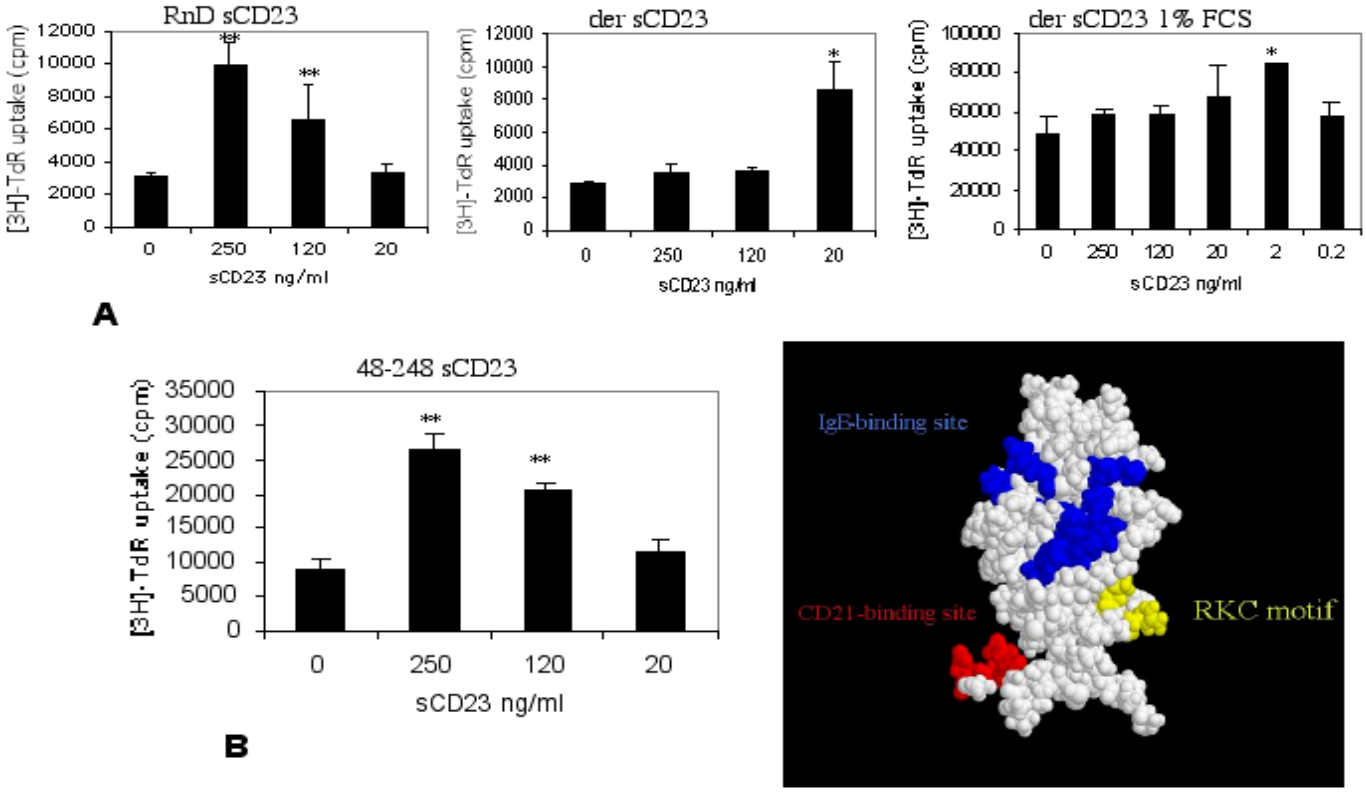

Figure 3.6 SMS-SB cell proliferation in response to a range of recombinant human sCD23.

Low cell density (LCD) cultures of SMS-SB cells were propagated with the indicated concentrations of different types of sCD23. Proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. **(A)** Comparison of proliferation of SMS-SB cells in response to sCD23 from R&D systems, derCD23 (PFHM) or derCD23 (in PFHM with 1%FCS). **(B)** Another recombinant sCD23 from Assay Designs termed sCD23 $^{48\text{-}248}$ was also used to assess proliferation of SMS-SB cells. The IgE binding site (in blue) and CD21 binding site (in red) are illustrated on the structural representation of sCD23. The RKC motif is depicted in yellow. Bars represent the standard deviation of triplicate determinations, with *$p<0.05$ or **$p<0.005$ indicating increase in proliferation in comparison to untreated cells. Data are representative of at least three independent experiments.

Figure 3.7 shows that the three pre-B cell lines SMS-SB, 697 and RS4;11 showed a robust dose-dependent proliferation in response to sCD23 treatment. Both the cell lines SMS-SB (SI at 250ng/ml and 120ng/ml of 3.2 and 2.1, respectively) and 697 (SI at 250ng/ml and 120ng/ml of 3.5 and 3.5, respectively) show an almost identical response while the concentration range for the optimal response is slightly different for RS4;11 cells (SI at 20ng/ml and 2ng/ml of 2.5 and 3.2, respectively) which show a strong response at 2ng-20ng/ml and no response at 250ng/ml. However, the sCD23 used in the case of RS4;11 was derCD23 and that could be the reason for the difference in the effective concentration range. This is verified by the different concentration range at which the 697 cells show optimal effect in cell proliferation in response to derCD23 (Figure 3.7).

The Blin1 cell line showed a slight proliferative response to sCD23 treatment at 2ng/ml and 20 ng/ml (SI of 1.5 and 1.4, respectively). Interestingly, the cell line 1E8 which is more mature than Blin1 also showed a slight increase in cell proliferation at 2ng/ml (SI of 1.9) (Figure 3.7). None of the transitional B cell lines (Daudi, Raji, Ramos) or the mature B cell lines (SKW and IB4) show a robust increase in cell proliferation with derCD23 when compared to early pre-B cell lines (Figure 3.8). However, with the Daudi and SKW cell lines there is a slight but significant increase in cell proliferation observed at 2-20ng/ml (SI of 1.9 for Daudi and SI of 1.6 for SKW). Both of these cell lines express small amounts of αVβ5 integrin (Figure 3.4) and the effect on cell proliferation could be mediated via this integrin. The cell lines Raji, Ramos and IB4 clearly do not show any response in cell proliferation with any of the concentrations of derCD23 used and these were the cell lines that did not express αVβ5 on cell surface (*aVβ5 expression data not shown for Raji cells which were aVβ5-negative and expression of other cell surface were identical to Daudi and Ramos; therefore this cell line was also considered to be a transitional B cell line*).

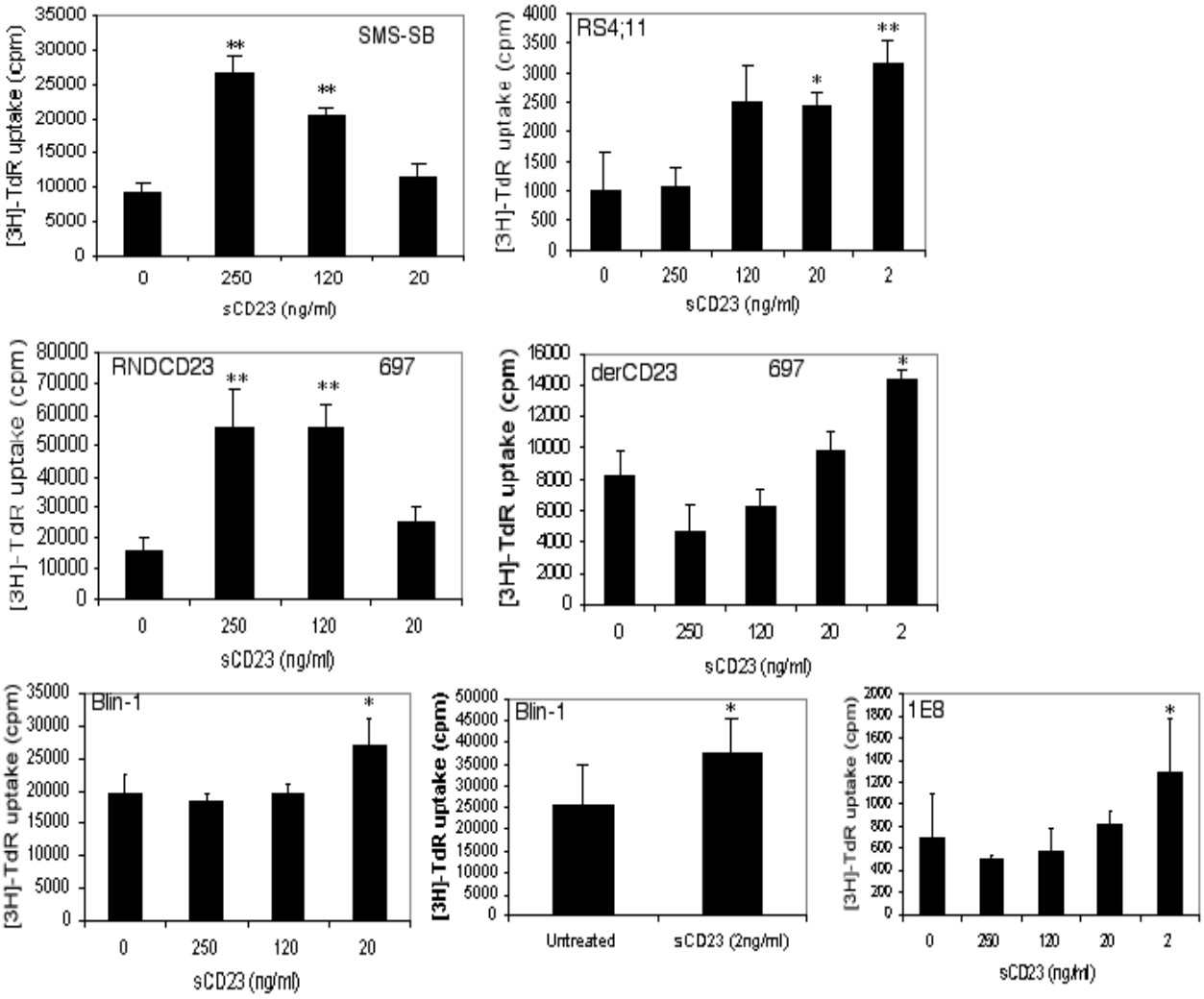

Figure 3.7 Proliferation of different pre-B cell lines in response to human sCD23.

LCD cultures of SMS-SB cells in PFHM or other cell lines in PFHM with 1%(v/v) FCS were propagated with indicated concentration of sCD23 (R&D sCD23 for SMS-SB cells, derCD23 for RS4;11, Blin1 and 1E8 cells and either RND or derCD23 for 697 cells). Proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. Bars represent the standard deviation of triplicate determinations, with *p<0.05 or **p<0.005 indicating increase in proliferation in comparison to untreated cells. Data represent at least three independent experiments.

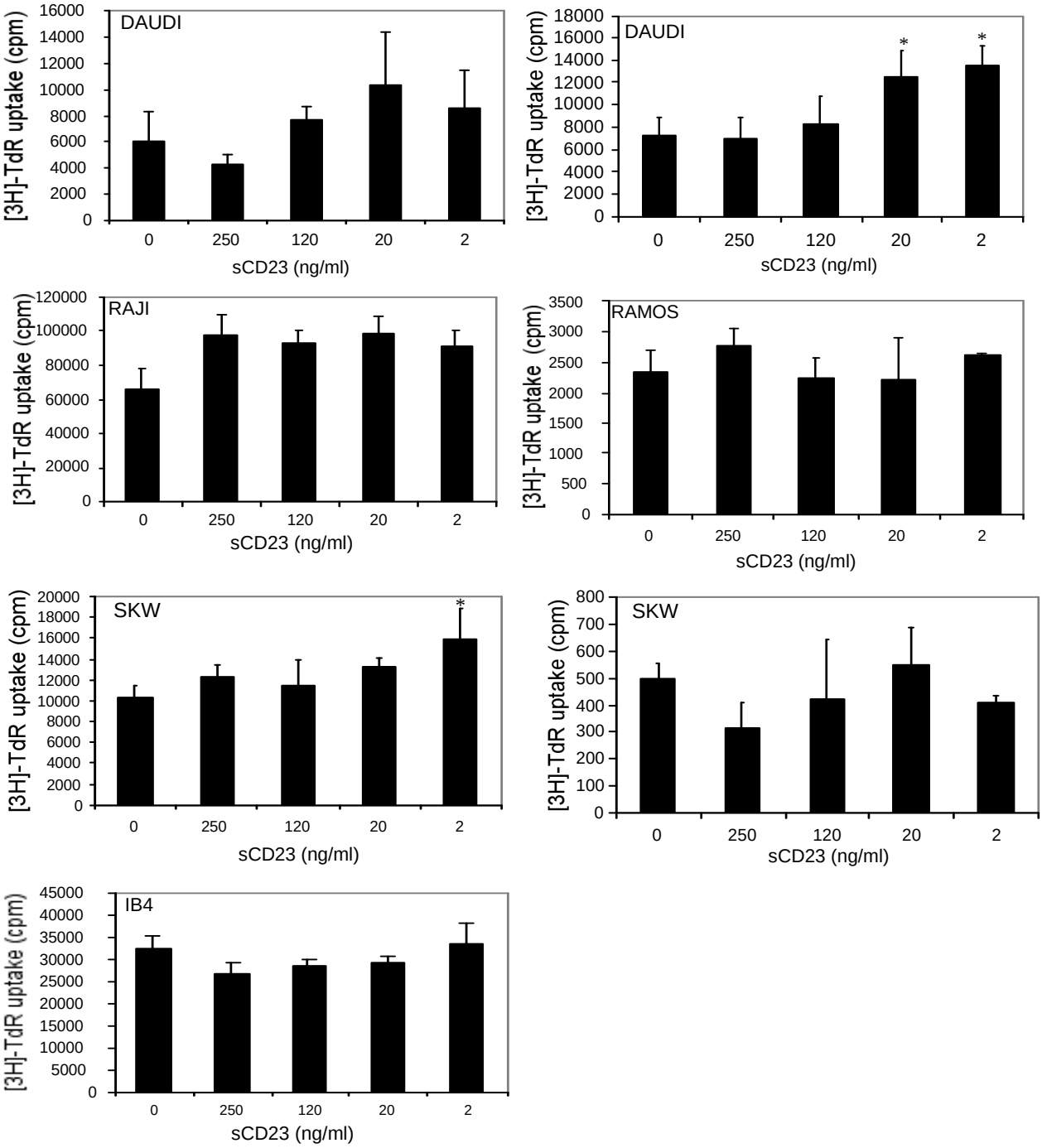

Figure 3.8 Proliferation of different mature B cell lines in response to human sCD23.

LCD cultures (5000 cells/well) of the indicated cell lines in PFHM were propagated with the indicated concentration of sCD23 (derCD23). Proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. Two sets of results are displayed for SKW cell line as in one experiment there was slight proliferative response with sCD23 at 2ng/ml whereas there was no significant response at the same concentration in another experiment. Bars represent the standard deviation of triplicate determinations, with *$p<0.05$ indicating increase in proliferation with sCD23 treatment, in comparison to untreated cells. Data represent at least three independent experiments.

3.2.4.2 Proliferation in response to Long peptide

The change in proliferative response to sCD23 in the different cell lines was verified by using the sCD23-derived long peptide (LP). This peptide contains the RKC motif bound by the integrin αVβ5 and previous surface plasmon resonance (SPR) analysis has confirmed the binding of this peptide to αVβ5. Consistent with the data for sCD23, the pre-B cell lines SMS-SB, RS4;11 and 697 showed a robust dose-dependent proliferative response to LP treatment with stimulation indices (at 10ng/ml) of 7.6, 8 and 17.5 for SMS-SB, RS4;11 and 697 cells, respectively (Figure 3.9). In each case, the negative control peptides that do not contain the RKC motif (peptide 8, 13 or 58) showed no effect on cell proliferation. Proliferation induced by LP is of higher magnitude (when comparing SI) than sCD23, however since the peptides are smaller in structure compared to sCD23 they might bind better to the integrin; the peptides are also used at a higher concentration than sCD23.

The late pre-B cell line Nalm6 did not show any significant increase in cell proliferation with LP but Blin1 cells showed a slight increase in cell proliferation at 5μg/ml. The proliferative response of the 1E8 cell line was more difficult to define because in one assay there was a slight response to LP at 10 and 5μg/ml (SI 2.3 and 2.6; however this proliferative response was not statistically significant in comparison to negative control peptide #8-induced proliferation) but in another assay there was no response at all to LP (Figure 3.9). Again consistent with the data observed for sCD23, the transitional B cell lines and the mature B cell lines did not show a significant increase in cell proliferation with LP treatment apart from a slight increase in proliferation in Daudi and SKW cell lines at 5-10ng/ml LP treatment (Figure 3.10).

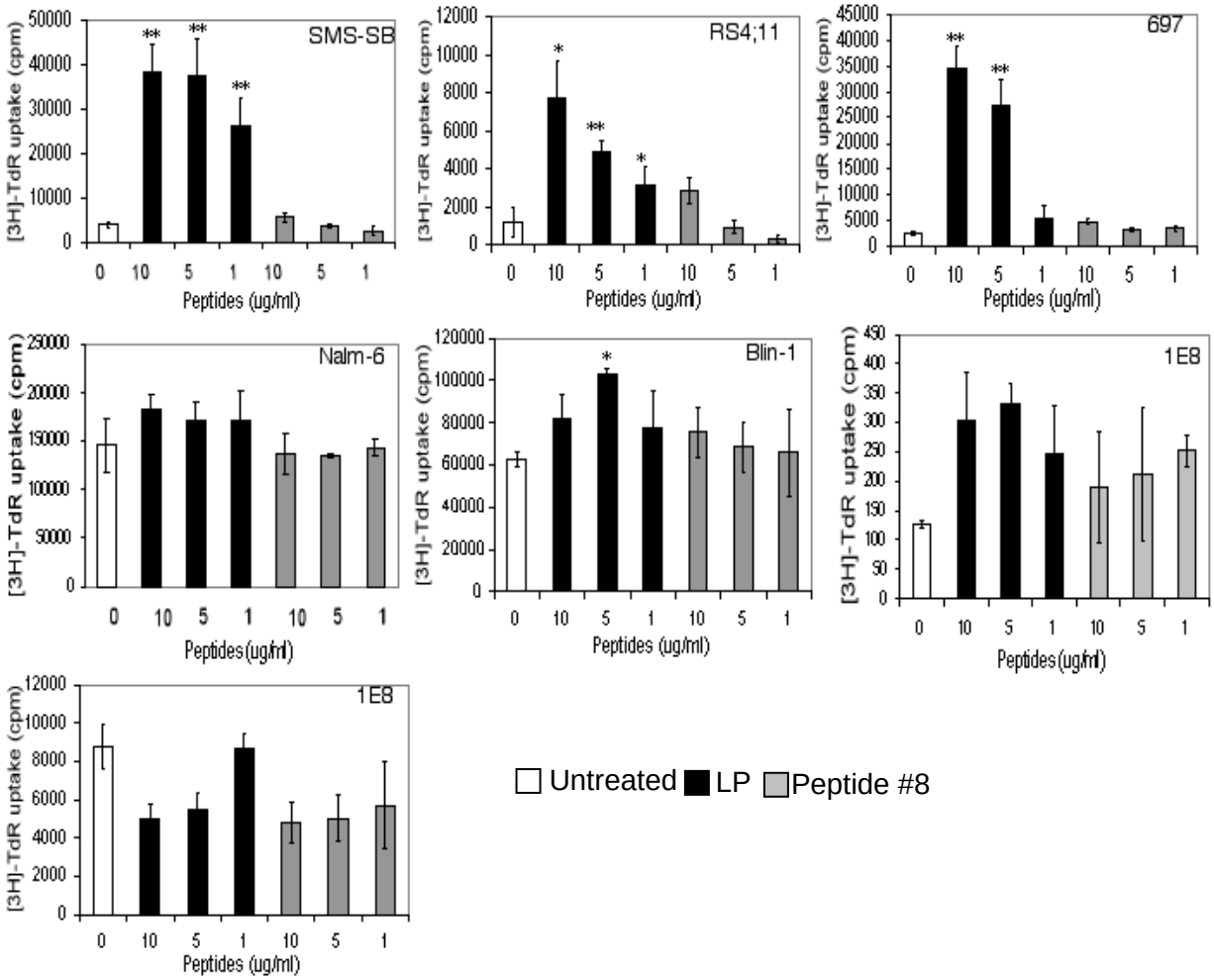

Figure 3.9 Proliferation of different pre-B cell lines in response to the CD23-derived long peptide.

LCD cultures of SMS-SB and Nalm6 cells in PFHM or other cell lines in PFHM with 1%FCS were propagated with indicated concentration of LP (KWINFQRKCYYFGKG) or negative control peptide #8 (PEKWINFQR). Proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. Two sets of results are displayed for 1E8 cell line because different responses were observed in two different experiments. Bars represent the standard deviation of triplicate determinations, with *p<0.05 or **p<0.005 indicating increase in proliferation in response to LP treatment in comparison to peptide #8 treatment at the same concentration. Data represent at least three independent experiments.

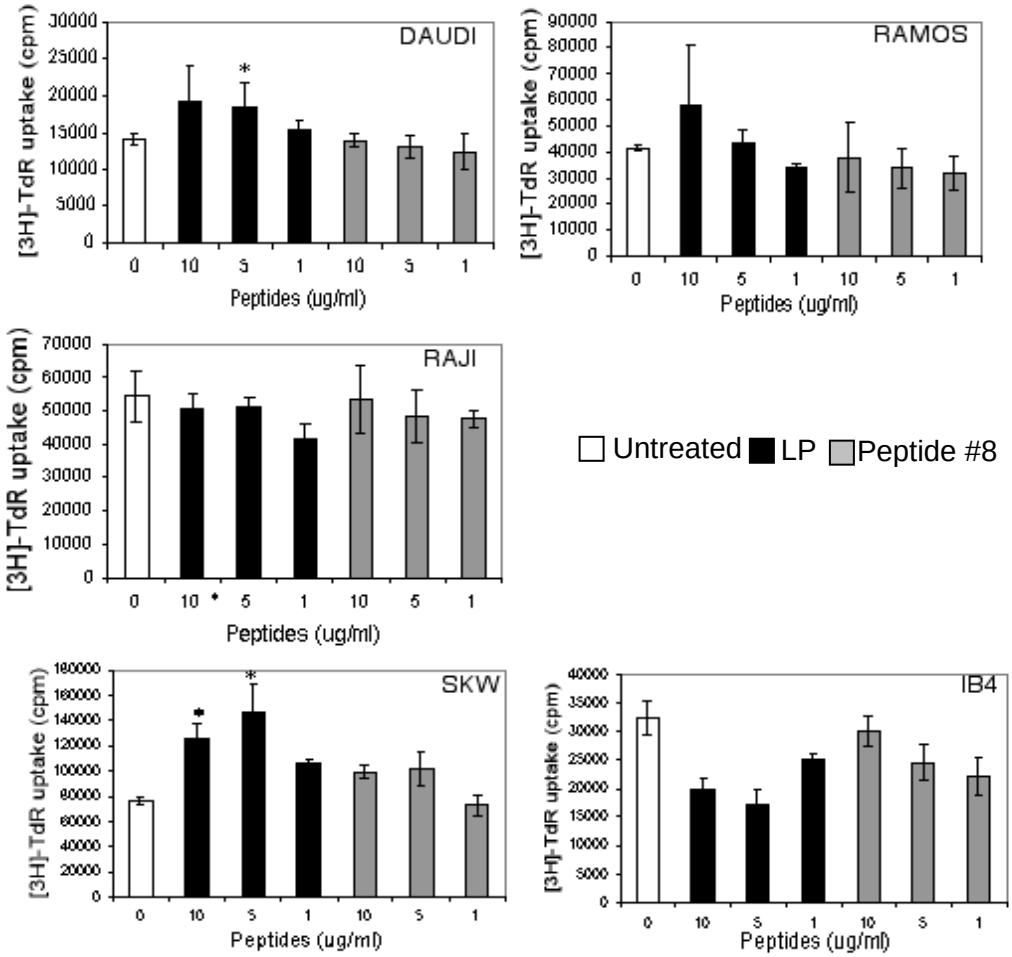

Figure 3.10 Proliferation of different mature B cell lines in response to CD23-derived long peptide.
LCD cultures (5000 cells/well) of the indicated cell lines in PFHM were propagated with indicated concentrations of LP (KWINFQRKCYYFGKG) or negative control peptide #8 (PEKWINFQR). Proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. Bars represent the standard deviation of triplicate determinations, with *p<0.05 indicating increase in proliferation with LP treatment in comparison to peptide #8 treatment at the same concentration. Data represent at least three independent experiments.

3.2.4.3 Proliferation in response to anti-αVβ5 monoclonal antibodies (MAbs)

Figure 3.11 shows that the pre-B cells lines (SMS-SB, RS4;11 and 697) proliferate in response to αVß5 ligation by 15F11 or AMF7 antibody. SMS-SB cells showed a

significant proliferative response when stimulated with either the 15F11 antibody or the AMF7 antibody at 5µg/ml when compared with the respective isotype controls (Figure 3.11). RS4;11 cells showed significant proliferation with 15F11 antibody when compared to the isotype control IgG2a antibody (Figure 3.11), but these cells showed no response with AMF7 antibody (*data not shown*). The 697 cell line was similar to SMS-SB cell line and showed a significant response in cell proliferation with both the AMF7 and the 15F11 antibody. The P1F6 MAb failed to provoke a significant proliferative response in any of the cell lines (Figure 3.11).

None of the other cell lines tested showed any response to any anti-αVß5 MAbs (AMF7, 15F11 or P1F6) (Figure 3.12 and 3.13). The mature B cell lines SKW and IB4 that expressed high levels of αVß3 but little or no αVß5 were used to investigate if αVß3 could regulate cell proliferation in a manner similar to αVß5; both the cell lines showed minimal proliferation in response to the 23C6 antibody challenge compared to the isotype control IgG1 antibody (Figure 3.13). No proliferative response was observed with a second αVß3 antibody LM609 or the αV antibody LM142 (Figure 3.13). However, the case with the transitional cell lines was slightly more complicated. The cell lines Raji and Daudi both demonstrated significant cell proliferation when stimulated with 23C6 although αVß3 expression was very low in these cell lines (Figure 3.12). In contrast, the Ramos cell line showed an inhibition of cell proliferation when treated with the 23C6 antibody (Figure 3.12). No proliferative effect was noted when with the MAbs LM609 or LM142 were used to stimulate these cell lines (Figure 3.12). No proliferative response was seen in the Ramos cell line with the 10D5 antibody (Figure 3.12) although these cells showed some αVß6 expression by FACS analysis (Figure 3.4)

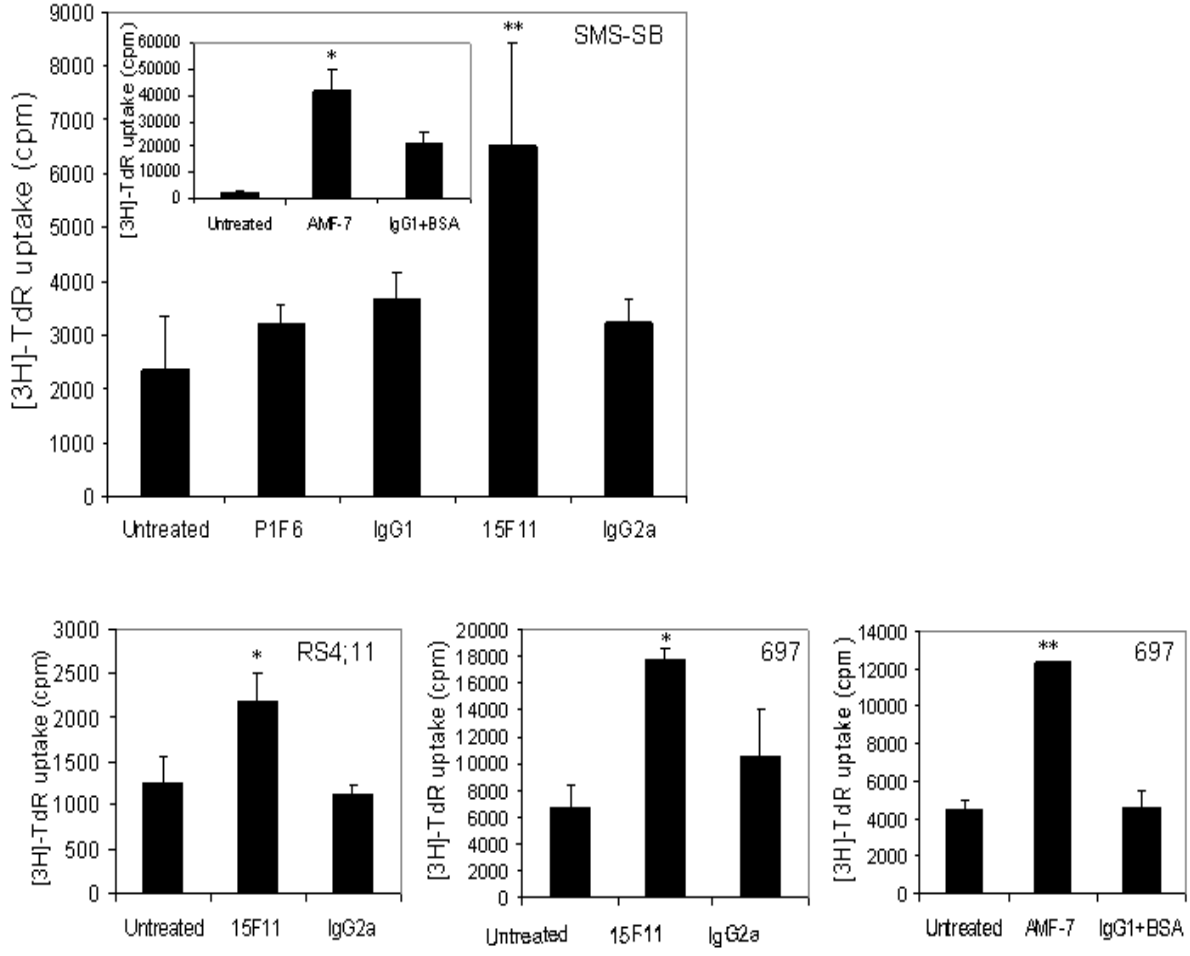

Figure 3.11 Proliferation of pre-B cell lines in response to αVβ5 monoclonal antibodies.

LCD cultures of SMS-SB cells in PFHM or 697 and RS4;11 cell lines in PFHM with 1% FCS were propagated with indicated MAbs. All MAbs and their specific isotype controls were used at 5µg/ml. The AMF7 antibody was dissolved in a buffer containing BSA therefore BSA was used with the IgG isotype control antibody. Proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. The inset on the results for SMS-SB cells displays proliferation in response to AMF7 and antibody and its isotype control. For SMS-SB cells, results are displayed for both the αVβ5 antibodies (P1F6 and 15F11) while for 697 and RS4;11 results are shown only for the antibodies where we observed a proliferative response. Bars represent the standard deviation of triplicate determinations, with *p<0.05 or **p<0.005 indicating increase in proliferation in response to each MAb treatment in comparison with specific isotype control antibody. Data are representative of three independent experiments.

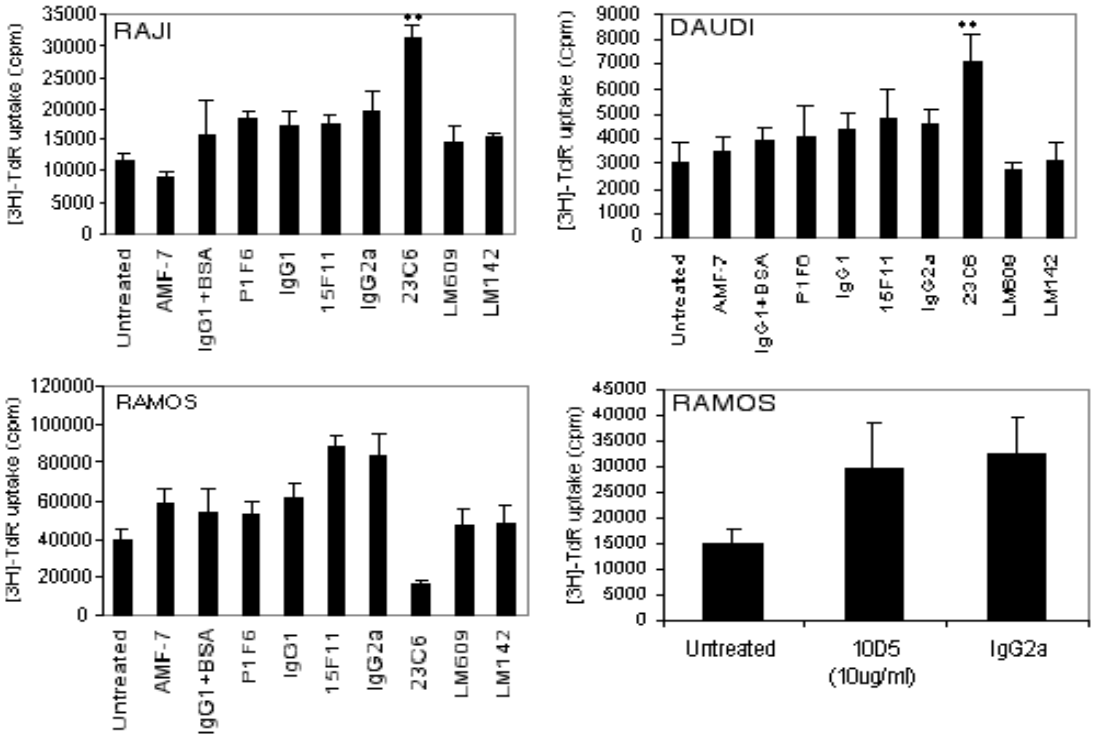

Figure 3.12 Proliferation of mature B cell lines in response to different αV integrin monoclonal antibodies.

LCD cultures (5000cells/well) were propagated in PFM of cell lines Raji, Ramos and Daudi with the indicated MAbs and proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. All the MAbs and there isotype controls were used at 5µg/ml. Bars represent the standard deviation of triplicate determinations, with **p<0.05 indicating increase in proliferation with antiboody treatment in comparison to the isotype control. Data are representative of three independent experiments.

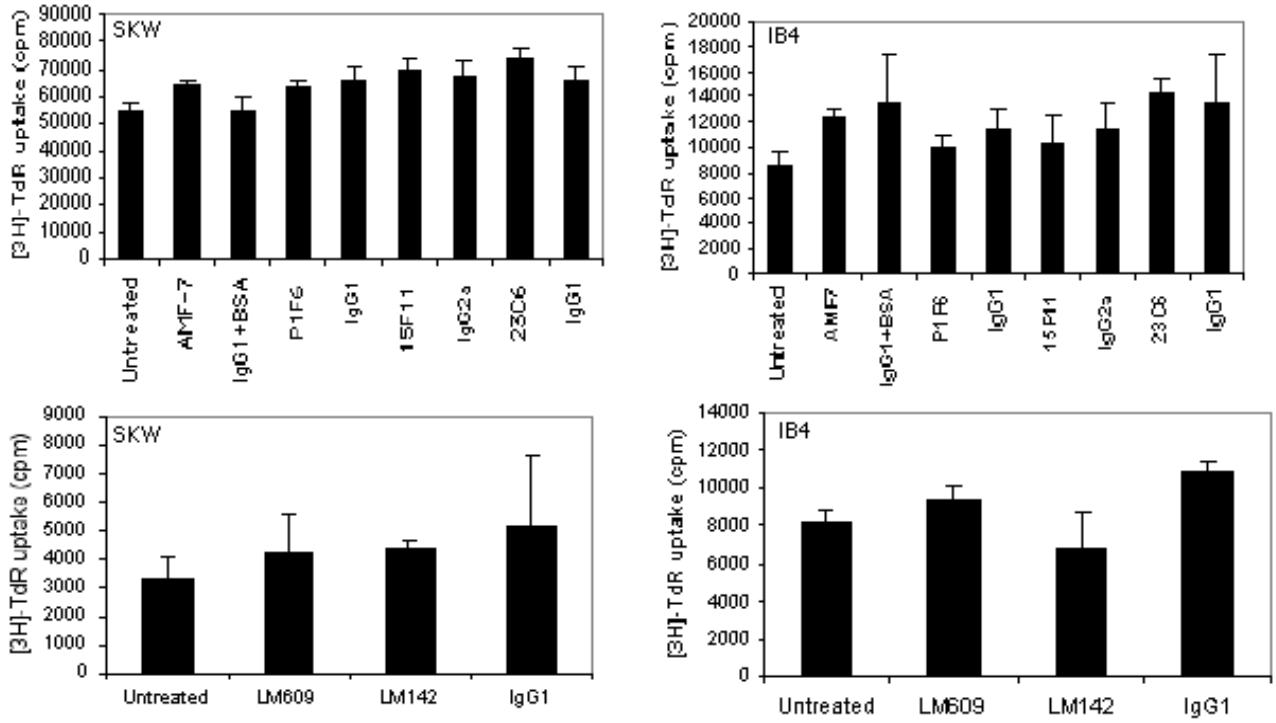

Figure 3.13 Proliferation of mature B cell lines in response to different αV integrin monoclonal antibodies.

LCD cultures (5000cells/well) were propagated in PFM of cell lines SKW and IB4 with the indicated MAbs and proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. All the MAbs and there isotype controls were used at 5µg/ml. Bars represent the standard deviation of triplicate determinations, with **p<0.05 indicating increase in proliferation with antiboody treatment in comparison to the isotype control. Data are representative of three independent experiments.

3.2.5 αV integrin expression on human bone marrow B cell precursors

Frozen human bone marrow mononuclear cells were thawed and used for FACS analysis using a panel of MAbs for CD19, CD10, CD9, μ, κ, αVβ5 and αVβ3 expression. The cell surface markers were chosen to delineate the different stages in B cell development in the bone marrow and to allow for correlation with the cell line data. In all cases lymphocyte populations were first gated from the forward scatter/side scatter dot plots and B cells were identified from the total population by using the anti-CD19 antibody conjugated to PerCP-Cy5.5 and further analysis was only done on the $CD19^+$ B cells.

Figure 3.14 illustrates that bone marrow B cell precursors express both the integrin αVβ5 and αVβ3 as shown by staining with the P1F6 MAb for αVβ5 and 23C6 MAb for αVβ3 expression. However, αVβ5 expression was higher than αVβ3 expression (25.9% of $CD19^+$/$αVβ5^+$ cells vs. 15.3% of $CD19^+$/ $αVβ3^+$ cells on the upper right quadrants of the dot plots). Some of the αVβ5-positive cells were CD10 positive (Figure 3.15) and majority of the αVβ5-positive cells were also positive for CD9 as shown in Figure 3.15. In both cases, positive staining for CD9 or CD10 is represented by the blue histogram. This is in agreement with the cell line data and shows the bone marrow B cell precursors positive for CD9 or CD10 are αVβ5 positive. More importantly, Figure 3.16 depicts that CD10-negative B cells are αVβ5-negative but CD10-positive B cells are αVβ5-positive. αVβ5 staining in CD10-negative population is represented as a red histogram and was derived from the upper left population on the dot plot and αVβ5 staining in CD10-positive cells is shown as a red histogram and these cells represent the population at upper right quadrant. CD10-positive B cells represent the precursor B cells in bone marrow while CD10-negative B cells represent the re-circulating B cells. Therefore these data supports the hypothesis that αVβ5 is expressed on B cell precursors but not on more mature cells.

Figure 3.17 shows the expression of αVβ5 or αVβ3 integrin in three different cell populations based on staining for the μ-chain. B cell populations were gated as either, $CD19^-$ or $CD19^+$/ μ-chain-negative or $CD19^+$/μ-chain-positive and αVβ5 or

αVβ3 staining in each of these population is shown as a histogram overlay. Figure 3.17A illustrates that αVβ5 expression is highest in the $CD19^+$/μ-chain-negative B cell population and is lower in the $CD19^+$/μ-chain-positive B cell population. The μ-chain-negative B cell precursors probably represent pro-B cells, and therefore these data support the hypothesis that αVβ5 expression is highest in the earlier B cell precursors and decreases as the cells mature. However, no staining was observed with the CD34 MAb and, due to the lack of data on CD34-positive pro-B cells, αVβ5 expression on pro-B cell precursors cannot be confirmed. Moreover, αVβ3 integrin showed a very similar expression pattern to αVβ5 (Figure 3.17B). From the cell line data it would be expected that perhaps αVβ3 expression increases as the B cells mature, since more mature cell lines showed more αVβ3 expression. However, the pre-B cell lines also expressed αVβ3 integrin and therefore it is possible that the bone marrow B cell precursors express both these integrins. Analysis of αVβ3 integrins in more mature B cells would be required to further confirm the expression pattern.

Data shown in Figures 3.18A and 3.18B indicate that both μ-chain-positive and κ-chain-positive B cells are also $αVβ5^+$. Since κ-chain-positive B cells represent BCR-positive cells it was surprising to see αVβ5 expression in these cells. However, the kappa-positive immature B cell line 1E8 also expressed αVβ5 and the kappa-positive population encompasses the immature and mature B cells and so the αVβ5 expression could be because of an immature B cell population. Comparing the histograms for αVβ5 expression in μ- chain-positive and κ-chain-positive B cells it is evident that there are more μ-chain-positive cells expressing αVβ5 than the kappa-positive B cells. The kappa antibody showed some non specific binding so it was difficult to analyse different B cell populations with varying κ-chain expression.

Finally, Figures 3.19 A and 3.19 B indicate that both αVβ5 and αVβ3 are expressed on some $CD5^+$ B cells. $CD19^+/αVβ5^+$ cells were analysed for CD5 staining. $CD19^+$ but αVβ5-negative B cells are also $CD5^+$ (red histogram) however there are a proportion of $αVβ5^+$ B cells are $CD5^+$ (blue histogram). A similar result was seen with αVβ3 staining (Figure 3.19B).

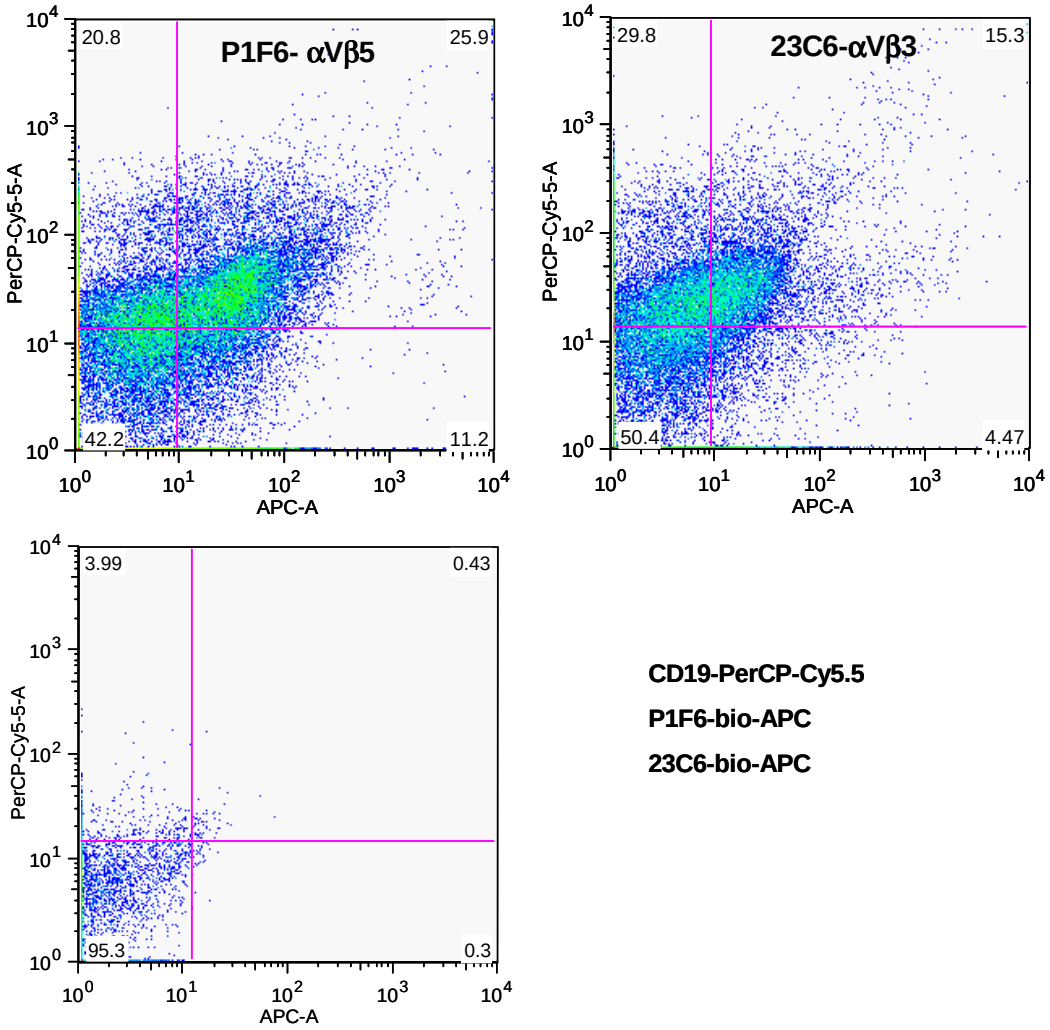

Figure 3.14 Expression of integrins αVβ5 and αVβ3 in bone marrow B cell precursors.

Frozen samples of BM mononuclear cells were thawed and prepared for FACS analysis as described in materials and methods. B cell precursors were identified from the mononuclear cell population by using anti-CD19-PerCpCy5.5 MAb. Compensation gates were based on isotype control mouse IgG1-κ conjugated beads (BD Bioscience, Oxford, UK). Biotinylated P1F6 MAb with secondary streptavidin APC was used to detect αVβ5 integrin and biotinylated 23C6 MAb with secondary streptavidin APC was used to detect αVβ3 integrin.

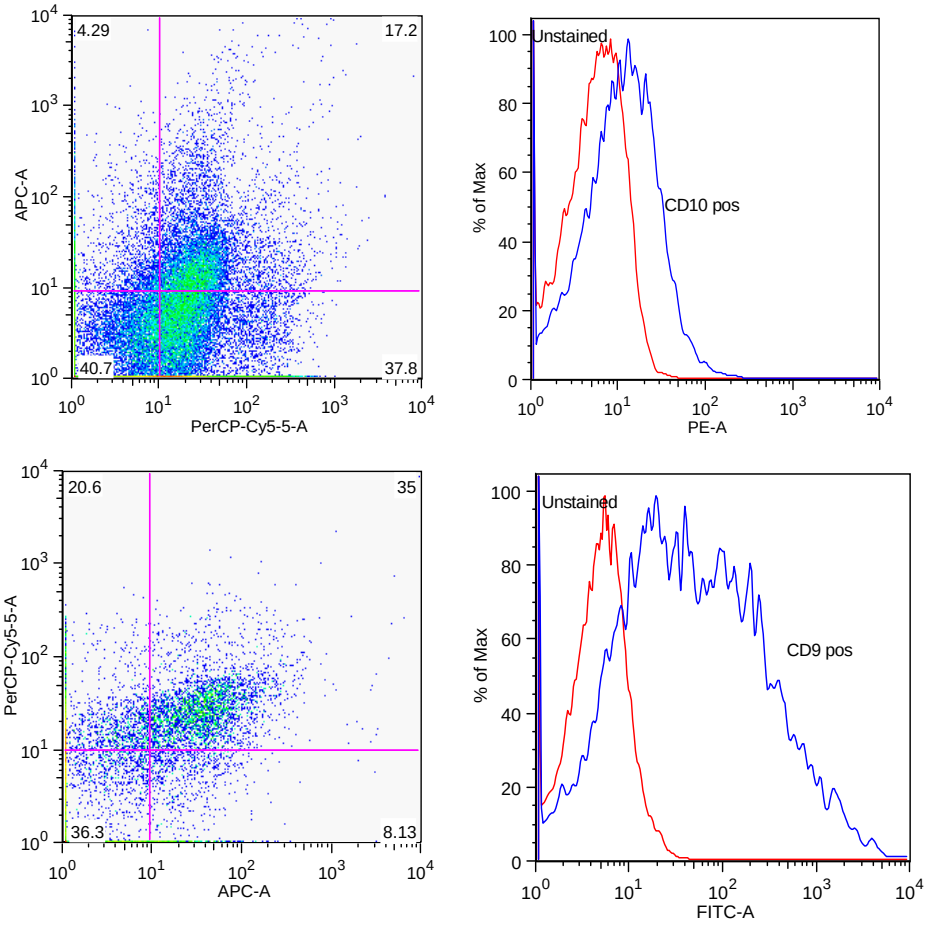

Figure 3.15 Expression of CD9 and CD10 markers on αVβ5 positive bone marrow B cell precursors.

BM mononuclear cells were simultaneously stained with P1F6-biotin (and streptavidin APC), CD9-FITC, CD10-PE and CD19-PerCPCy5.5. The $CD19^{+}$/$\alpha V\beta5^{+}$ population was further analysed for CD10 and CD9 expression as histograms. The twodot plots show the $\alpha V\beta5^{+}$ and $CD19^{+}$ populations on the upper right quadrant and histgrams for CD10 (top) CD9 (bottom) were drawn based on this population. The histogram in red represents background staining for FITC or PE from unstained samples and the histogram in blue represents staining for CD10-PE or CD9-FITC.

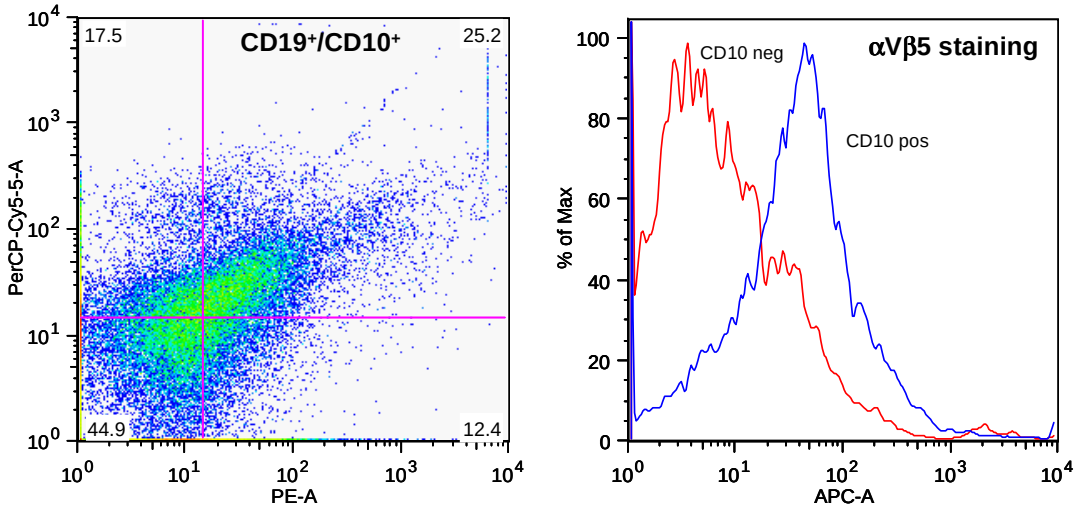

Figure 3.16 Analysis of αVβ5 expression on CD10-negative and CD10-positive bone marrow B cell precursor populations.

BM mononuclear cells were simultaneously stained with P1F6-biotin (and streptavidin APC), CD10-PE and CD19-PerCPCy5.5. The dot plots show $CD19^+$ and $CD10^+$ population in the upper right quadrant and $CD19^+$ and $CD10^-$ population in the upper left quadrant. These two populations were analysed for αVβ5 expression. The histogram shows staining for αVβ5 on $CD19^+$ /$CD10^-$ population in red and αVβ5 staining for $CD19^+$ and $CD10^+$ population in blue.

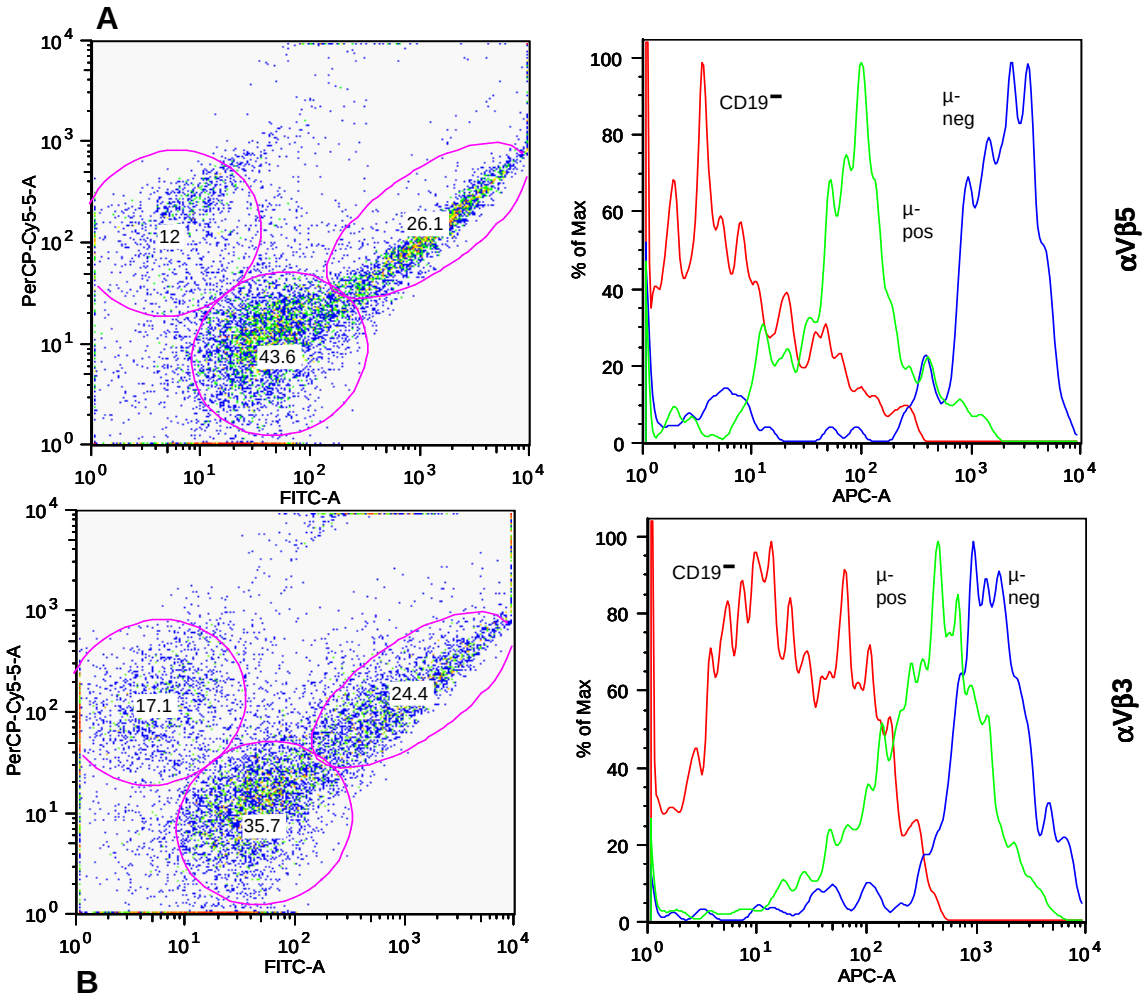

Figure 3.17 Analysis of αVβ5 and αVβ3 integrin in bone marrow B cells at different stages.

BM mononuclear cells were simultaneously stained with either P1F6-biotin or 23C6-biotin (and streptavidin APC), μ-FITC and CD19-PerCPCy5.5. The dot plots show different populations gated on CD19 and μ chain expression. In both dot plots the circular gate on the far left represents the μ-low or negative/CD19^{+} population, the circular gate in the middle represents the CD19-negative population and the elliptical gate on the right represents μ-positive /CD19^{+} B cell precursors. αVβ5 or αVβ3 expression in each of these populations is represented as histograms. The plots on the top **(A)** represent staining for αVβ5 and plots on the bottom **(B)** represent staining for αVβ3.

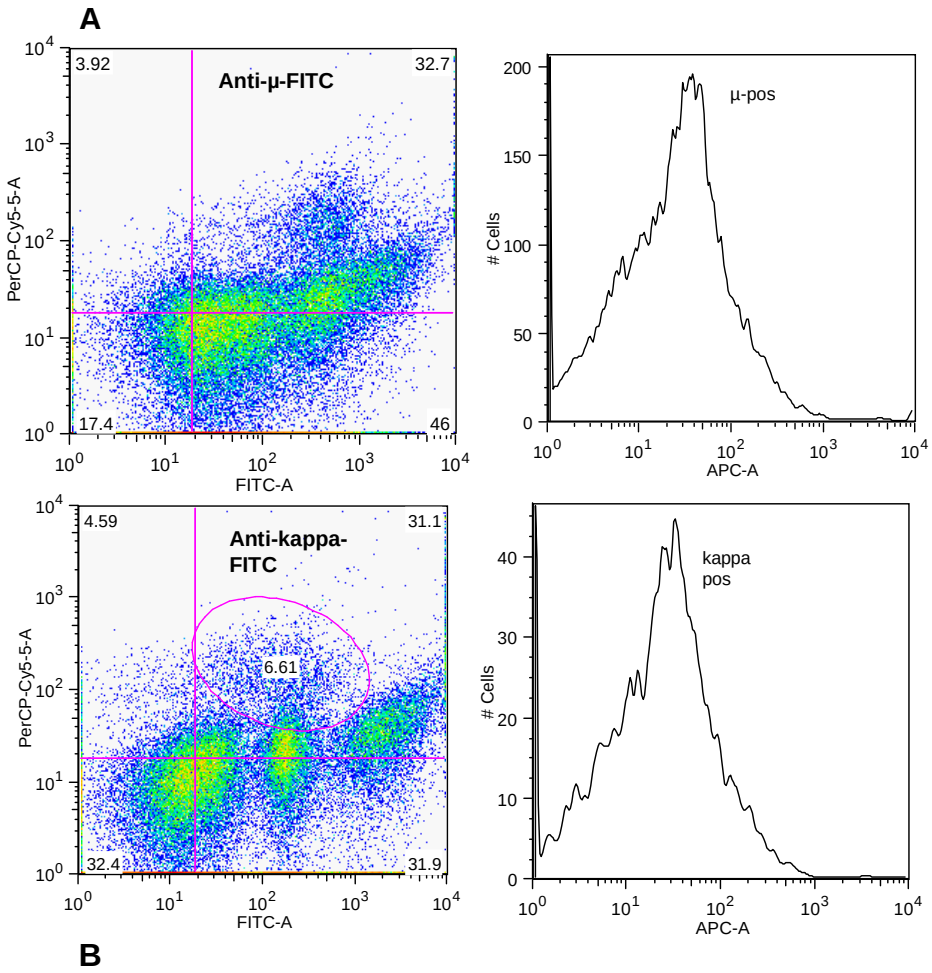

Figure 3.18 Expression of αVβ5 integrin on μ-positive and κ-positive bone marrow B cell precursors.

BM mononuclear cells were simultaneously stained with P1F6-biotin (and streptavidin APC), μ-FITC or κ-FITC and CD19-PerCPCy5.5. The dot plots show the $CD19^+$ and $\alpha V\beta5^+$ B cell population on the upper right quadrants. $\alpha V\beta5^+$ and μ-chain-positive cells are shown as histograms in **(A)** and $\alpha V\beta5^+$ and κ-chain postive cells are shown as histogram in **(B)**

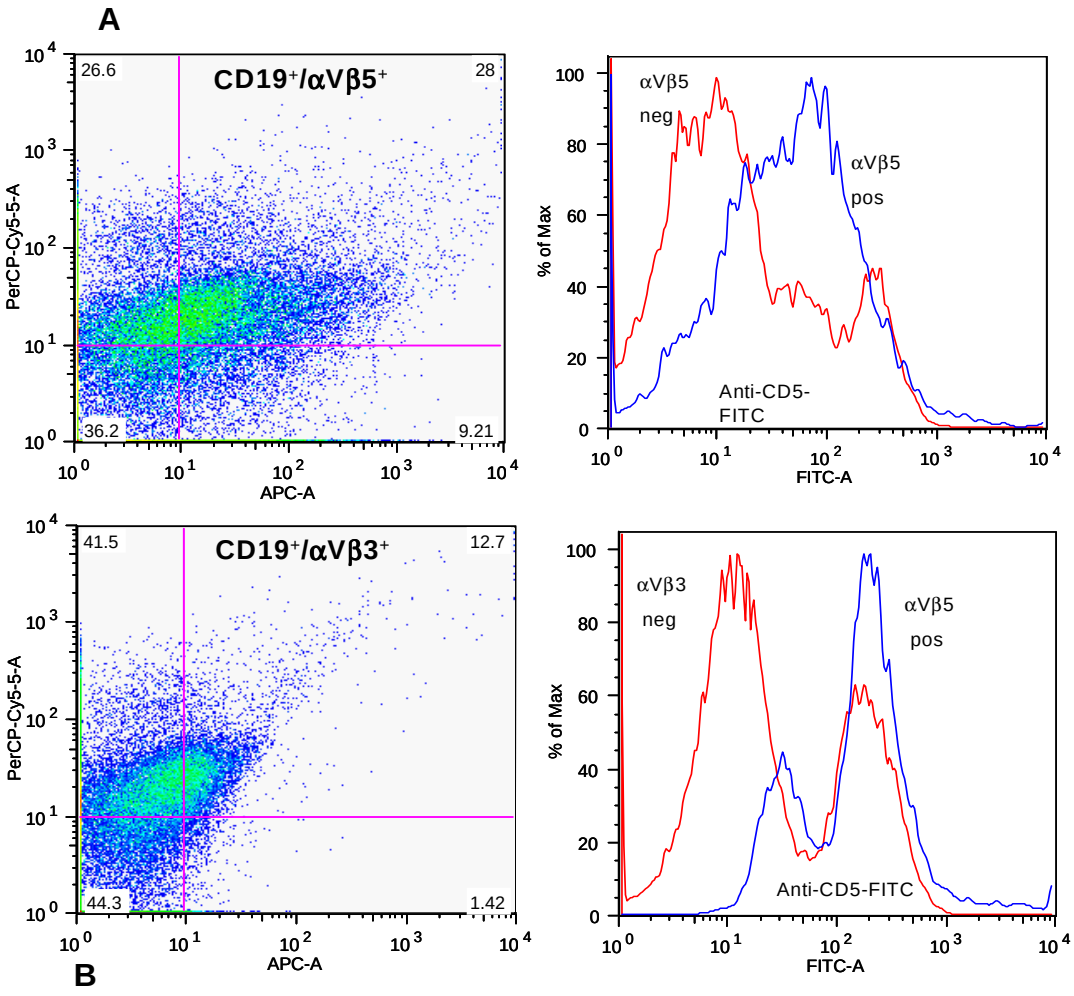

Figure 3.19 Analysis of αVβ5 or αVβ3 integrin expression on CD5$^+$ B cells from the bone marrow.

BM mononuclear cells were simultaneously stained with MAbs P1F6-biotin or 23C6-biotin (and streptavidin APC), CD5-FITC and CD19-PerCPCy5.5. The dot plots identify CD19+ and αVβ5$^+$ B cell population **(A)** or CD19$^+$ αVβ3$^+$ B cell population **(B)** on the upper right quadrants. These cell populations were analysed for expression of CD5 as shown by the histograms. The histogram in blue indicates CD5$^+$ αVβ5$^+$ cells **(A)** or CD5$^+$ αVβ3$^+$ cells **(B)** and the histogram in red shows CD5$^+$/ CD19$^+$ cells that are negative for either integrins; these are derived from the upper left quadrants as shown in the dot plots.

3.2.6 Proliferation response of B cell precursors in the bone marrow

B cell precursors in the human bone marrow mononuclear cells were FACS sorted by using the CD19 and kappa MAbs. The $CD19^+$ population was sorted into kappa positive and kappa-negative populations. The kappa negative population represents precursor B (pro-B to pre-B) cells that have not started to express the kappa light chain while the kappa positive population encompasses immature, transitional and mature B cells all expressing the kappa light chain. Both populations were separately used in proliferation assays to determine their proliferation response to αVß5 ligands. The number of cells sorted were not enough to confirm αVß5 expression in the sorted population. However an aliquot of the bone marrow mononuclear cells was stained for CD19, αVß5 and kappa light chain expression to make sure there were significant numbers of αVß5-positive cells in the sorted populations.

B cell precursors were cultured on irradiated stromal cells (hMSC-Tert) in RPMI with 2% (v/v) FCS with different treatments and proliferation measured as mentioned in materials and methods (Chapter 2). The $CD19^+/\kappa^+$ cells did not show a significant proliferation response with any treatment and did not survive for too many days in the culture. The $CD19^+/\kappa^-$ B cell precursors survived in culture for a few days and as shown in Figure 3.20 they showed a significant proliferative response with sCD23 (250ng/ml) and with the CD23-derived LP (5µg/ml). The negative control peptide #8 did not cause any effect in cell proliferation. Proliferation with the LP was more robust than proliferation with sCD23 and this was seen in proliferation assays with the B cell lines as well. However, we did not see a significant effect in cell proliferation with the 15F11 MAb which binds αVß5 integrin and induces a proliferative response in pre-B cell lines SMS-SB, RS4;11 and 697. Perhaps, a higher concentration of this antibody could be required to see a response in the bone marrow B cell precursors. The other αVß5 antibody, P1F6, or the αVß3 antibody 23C6 also failed to induce a proliferative response. Moreover, these data are representative only of one experiment so this experiment would have to repeated to obtain conclusive data.

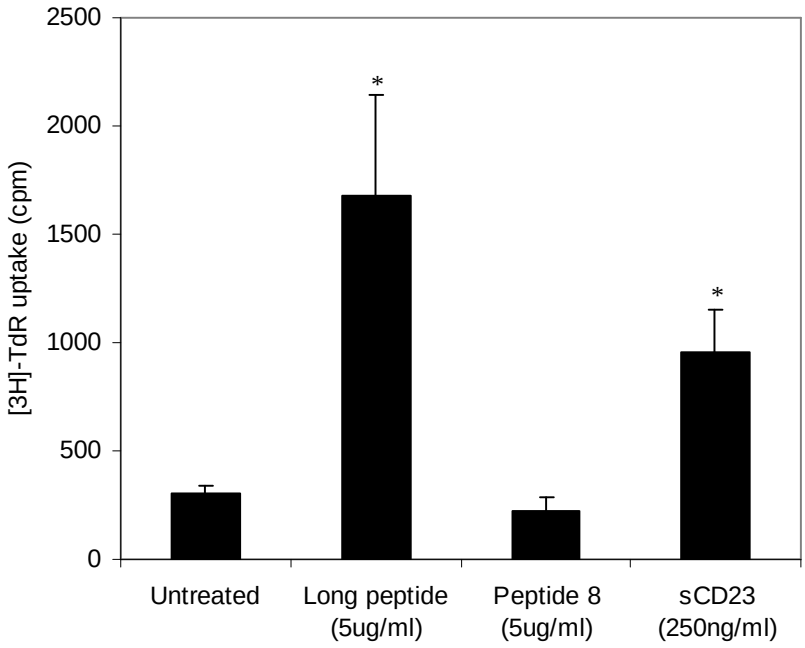

Figure 3.20 Proliferation of human CD19^{+}/ κ^{-} B cells in response to LP and CD23.

CD19^{+}/κ^{-} B cells were FACS sorted from a BM mononuclear cell population and used for proliferation experiments. 4000 sorted B cells/well were plated on γ-irradiated stromal cells in 96-well plates and proliferation was assessed 72 h by ^{3}[H]-TdR incorporation. Bars represent the standard deviation of triplicate determinations, with *$p<0.05$ indicating increase in proliferation with CD23 or LP treatment in comparison to the negative control peptide #8 treatment. Data represent one experiment (n=1).

3.3 Discussion

The data presented in this chapter indicate that αVβ5 expression and its influence on B cell proliferation is regulated in a developmental stage-specific manner.

Different B cell lines were chosen to represent a broad range of developmental stages, but it was not always possible to categorise each cell line into a specific stage definitively. RS4;11 is a well-characterised MLL (mixed lineage leukaemia) bearing an MLL/AF4 rearrangement [283], and known to be at the pro-B stage; in agreement with this, it was surface μ^- , $CD10^-$. However, RS4;11 cells were not $CD34^+$ which is a common marker for pro-B cells. SMS-SB cells were also CD10-negative but expressed low levels of surface μ. Bertrand, *et. al.,* have described another MLL-rearranged Blin-3 cell line with the t(4;11) translocation that was μ^- and became μ^+ *in vitro* [284]. Furthermore, the overall phenotype of the MLL-rearranged pro-B and pre-B ALL is known to be largely identical. Therefore, although we did not know the t(4;11) translocation status of SMS-SB cells, because of the similarities in surface marker expression, SMS-SB cells and RS4;11 cells were categorised together as early pre-B cells.

The $CD10^+$ pre-B cell lines were designated as late pre-B cell lines. However, CD10 expression was lower in the 697 cell line in comparison with Blin1 or Nalm6 cell lines and therefore the 697 cell line could be at a slightly different stage than the Blin1 or Nalm6 cells. CD20 expression at the small pre-B stage is known to coincide with cessation of cell division, exit from the bone marrow and maturation towards a mature B-cell phenotype [285], and this is followed by a concomitant decrease in expression of CD10 and CD24 [286]. Therefore, other pre-B cell markers such as CD20 and CD24 could have been employed to more directly delineate the cell stages. The κ^+ and CD10 negativity showed that 1E8 cells, although derived from the Blin1 cells, were at a more mature stage of development.

CD38, CD10 and CD24 antigens have previously been identified as immature B cell markers in human bone marrow and CD10 expression is characteristic of human transitional (T1) B cells [48] . As the cell lines Daudi, Raji and Ramos were all positive for immunoglobulin light chain expression, they were considered to be more mature than immature B cells and because of their CD10 expression they matched the transitional B cell phenotype. However, human transitional B cells are also known to be IgD positive but none of these cell lines showed IgD expression. These cell lines were also negative for CD21 expression, which is a marker for mature B cells. The mature B cell lines also lacked CD21 expression, therefore it is not clear whether the antibody was not able to detect CD21 expression or the cells were all indeed CD21 negative. The fact that the cell lines SKW and IB4 were CD10-negative indicated that they were a stage further from the transitional B cell lines and therefore were designated as mature B cells.

It is clear from the P1F6 and 15F11 staining data that the pre-B cells express high levels of αVβ5 and the integrin expression decreases as the cell mature. P1F6 staining showed similar levels of αVβ5 expression in the early and late pre-B cells but with the 15F11 MAb early pre-B cell lines and 697 cells showed higher levels of staining for αVβ5 than the late pre-B cells Blin1 or Nalm6. In fact, the three pre-B cell lines (SMS-SB, RS4;11 and 697) showing significant 15F11 staining were the cells lines that showed a robust proliferative response to αVβ5 ligation. More importantly, there was a down-regulation of αVβ5 expression in the 1E8 cell line that had started to express immunoglobulin light chains when compared to the original cell line Blin1 from which they were derived, indicating that B cell maturation may be accompanied by αVβ5 down-regulation.

There was also a clear down-regulation in αVβ5 expression in the transitional and mature B cell lines as shown by FACS analysis. However, this difference was not clear when assessing transcripts for β5 in the different cell lines. The β5 transcript was present in all the cell lines tested except for the Ramos cell line. It is possible that in the cell lines such as IB4 although they have the β5 transcripts they do not express the β5 protein on the cell surface. A western blot

analysis of ß5 would be needed to confirm the presence of ß5 protein in the cells. Nevertheless, the fact that cell lines such as Daudi and SKW still showed slight surface expression of αVß5 indicates that the cell lines such as IB4 may also express small amounts of αVß5 that was not detected by FACS analysis.

In the case of αVß3, it was difficult to conclude exactly at what stage αVß3 expression began and one can only conclusively say that the mature B cell lines like SKW and IB4 expressed high levels of αVß3 in comparison with any of the other cell lines. A further complication in the analysis was the fact that the precise stage of development of the mature B cell lines SKW and IB4 could not be defined. Although they both expressed most of the surface markers tested, IB4 cell lines were not positive for surface light chain expression. These cell lines are both CD23 positive and both are EBV transformed cell lines. Therefore the high αVß3 expression could also be because of EBV transformation as previous studies have shown that EBV transformation induces expression of αVß3 integrins [287].

From the analysis of B cell precursors in the bone marrow, it was noted that the $CD19^+/CD9^+$ and $CD19^+/CD10^+$ cells expressed αVß5 as well as αVß3, but αVß5 expression was higher in comparison to αVß3 expression. αVß3 expression in the bone marrow B cell precursors indicate that low levels of αVß3 might be expressed in pre-B cells, consistent with data for αVß3 expression in Blin1 and Nalm6 cells. The bone marrow data also indicates that $CD10^+$ B cell precursors are $αVß5^+$ but $CD10^-$ re-circulating B cells are αVß5-negative. We also observed in the bone marrow that the $κ^+$ B cells still expressed αVß5 although the expression was lower than the $μ^+$ B cell precursors (Figure 3.18). A more rigorous study of bone marrow B cell precursors is required to obtain conclusive data. In our study we were also unable to detect $CD34^+$ pro-B cells in the bone marrow and this could be because we were using frozen bone marrow samples. Analysis of pro-B cells and pre-B cells is particularly important, since it is in these early stages that αVß5 expression seems to be highest and most functionally important from the cell line data and we could see a similar pattern from the bone marrow expression data (Figure 3.17).

Therefore, it cannot be conclusively said that αVß5 expression is exclusive to the pre-B cells but it can be concluded that αVß5 expression decreases as the B cells mature. This is also in agreement with previous data from the laboratory showing that αVß5 expression is significantly increased in cells from patients with ALL (*Borland, et. al., 2008 manuscript submitted*). With regard to the comparison of αVß5 and αVß3 expression, analysis of the mature B cells in the peripheral blood will also be necessary in order to identify and compare the pattern of expression of these two integrins. Since a similar expression pattern was seen between αVß5 and αVß3 integrins in the bone marrow B cell precursors it would be important to analyse the more mature B cells in order to identify if there is a difference in expression pattern of these two integrins. Another interesting finding from the bone marrow expression study is that the $CD5^+$ B cells are positive for αVß5 and αVß3 integrins (Figure 3.19). $CD5^+$ B cells in humans are considered to be equivalent to the murine B-1a cells. These cells represent a minor B cell population but have been implicated in malignanices such as CLL and in autoimmune diseases. The fact that these cells express αV integrins implies that these integrins could have a role in proliferation/differentiation of these cells which could have an important impact on the development of $CD5^+$ cells associated B cell disorders.

SMS-SB cells proliferate in a similar manner in response to recombinant sCD23 from different sources. The three different sCD23 proteins were slightly different in the amino acids they encompassed and this allowed the comparison of different motifs in inducing a proliferative response. The amino acid sequences of the three different sCD23 are presented in an aligned format in Figure 3.21. R&D sCD23 and $sCD23^{48-248}$ encompassed nearly the same length of amino acids 48-321 and 48-248, respectively, and this could be the reason they both gave nearly identical proliferative responses. The derCD23 contained amino acids 156-298. We also did not assay the specific activities of these sCD23 proteins so they could be inducing proliferative responses at different concentrations because of the difference in their specific activities. The $sCD23^{48-248}$ lacks IgE and CD21 binding motif but contains the RKC motif bound by αVß5.

The proliferative response induced by this sCD23 clearly indicates that proliferation mediated by sCD23 is independent of the need for CD21 and IgE binding.

The three pre-B cell lines SMS-SB, RS4;11 and 697 showed a significant proliferative response when αVβ5 was ligated using sCD23 or the CD23-derived long peptide; a similar response was seen with the αVβ5 antibody, 15F11. The AMF7 MAb which binds to the αV subunit shows a significant response in proliferation in SMS-SB cells and 697 cells but not in RS4;11 cells. The transitional and mature B cell lines that do not express αVβ5 did not show any response in cell proliferation with sCD23, LP or the MAbs. Moreover, the cell lines Daudi and SKW that do express small amounts of αVβ5 showed a slight response in cell proliferation with sCD23 and with LP. The αVβ5 staining in these cell lines was done using P1F6 antibody and it is possible that these cell lines have low expression of the epitope that is recognised by 15F11 antibody and therefore do not show any proliferative response with this antibody. Moreover, both of these cell lines are EBV-transformed cell lines and previous studies have demonstrated that sCD23 induces proliferation of EBV-transformed cell lines [215]. This response is thought to be mediated by the CD23-CD21 interaction. Although we did not see any CD21 expression in these cell lines we cannot be sure they do not express this antigen because we did not see positive CD21 expression in any cell line. Furthermore, the fact that only sCD23 and LP induced a proliferative response in these cell lines, but not the αVβ5 MAbs (AMF7 or 15F11), indicates that perhaps the proliferative response we observed could be delivered via the CD23-CD21 interaction.

Other αV integrin levels might also affect the proliferative response as the levels of different αV integrins change in the cells at different stages of development. The αVβ3 integrin was not associated with a proliferative response in the mature B cell lines that expressed high levels of this integrin and were low or negative for αVβ5. By contrast, in the transitional B cell lines Raji and Daudi, a significant increase in proliferation with the 23C6 MAb that is known to bind to αVβ3 heterodimer was noted; no response with any other anti-αV MAbs was observed. From the expression data it is clear that these cells expressed very low, if any,

αVß3 integrin and therefore it was surprising to see a proliferative response with this antibody on these cells; we did not see such an effect with 23C6 in the early pre-B cell lines. However, it should also be noted that the cell lines that were used for the functional studies had been in culture longer than the cell lines that were used for expression studies and the integrin expression did vary in the cells as they were in culture for an extended period (*data not shown*).

It is possible that as the cells had been in prolonged culture, the αV integrin levels had changed in these cells and therefore their proliferative response to 23C6 was different. It is also equally possible that pattern of expression of different αV integrin varies in the transitional and mature B cell lines and so the proliferative response is different depending on the αV integrins expressed. For example, it is clear from the RT-PCR data that both the SKW and IB4 cell lines express high amounts of transcripts for the ß8 subunit when compared to any of the other cell lines. It is not known whether αVß8 expression has a role in regulating proliferative responses when compared to the ligation of other αV integrins. Similarly, ß1 integrin levels were characteristically different in cell lines at each of the developmental stages so αVß1 integrin expression could also be affecting the function of other αV integrins.

The Ramos cell line that was categorised to be of the same stage as Raji and Daudi cell lines, showed an inhibition in cell proliferation with the 23C6 antibody. Among all the cell lines tested this cell line was the only cell line showing some staining for the αVß6 heterodimer. Therefore, it is possible that the presence of αVß6 negatively regulates the response through αVß3. The αVß6 antibody (10D5) however did not have any effect on this cell line. Indeed a previous study has shown that ß1 integrin regulates αVß3 and αVß5 integrin expression [288] and therefore cross talk between the different αV integrins could potentially have important roles in determining the function of each αV integrin.

Extensive functional studies on bone marrow B cell precursors are required to confirm the effects of αVß5 ligation on cell proliferation of normal B cell precursors. A more detailed study of the expression of all the different αV heterodimers together with the functional studies would also be required to identify exactly the stage(s) of development at which αVß5-mediated cell

proliferation is critical and the role of different αV heterodimers in mediating this proliferative effect. Preliminary results for proliferation of normal human bone marrow B cells precursors, FACS sorted, on the basis of κ light chain expression, showed that the $CD19^{+}/\kappa^{-}$ precursors proliferated well in response to both sCD23 and long peptide. But we did not observe a proliferative response with the αVß5 MAbs. However, the CD $19^{+}/\kappa^{-}$ cells encompass a heterogeneous population of both pre-B, pro-B cells and this could be the reason for not seeing any response with the 15F11 antibody. Therefore this needs to be repeated, and a separate comparison of pro and pre-B cells would be required in order to compare the proliferation data for normal bone marrow B cell precursors with the cell line data. The $CD19^{+}/\kappa^{+}$ cells expressing αVß5 showed no proliferative response with αVß5 ligation.

These findings indicate that the αV integrins, αVß5 and αVß3 are expressed in bone marrow B cell precursors and in B cell lines representing the precursor stages of development in the bone marrow. While the differential pattern of expression of these two integrins could not be identified, the proliferation data indicated that αVß5 integrin regulates proliferation of B cell precursors in a stage-specific manner. It remains to be investigated what is the exact difference in the function of αVß5 and αVß3 integrin and how their expression pattern is regulated. αV integrin expression and function could have important implications for growth and differentiation of normal and neoplastic B cell precursors and therefore it is important to identify mechanisms regulating the expression and function of these integrins in B cell precursors.

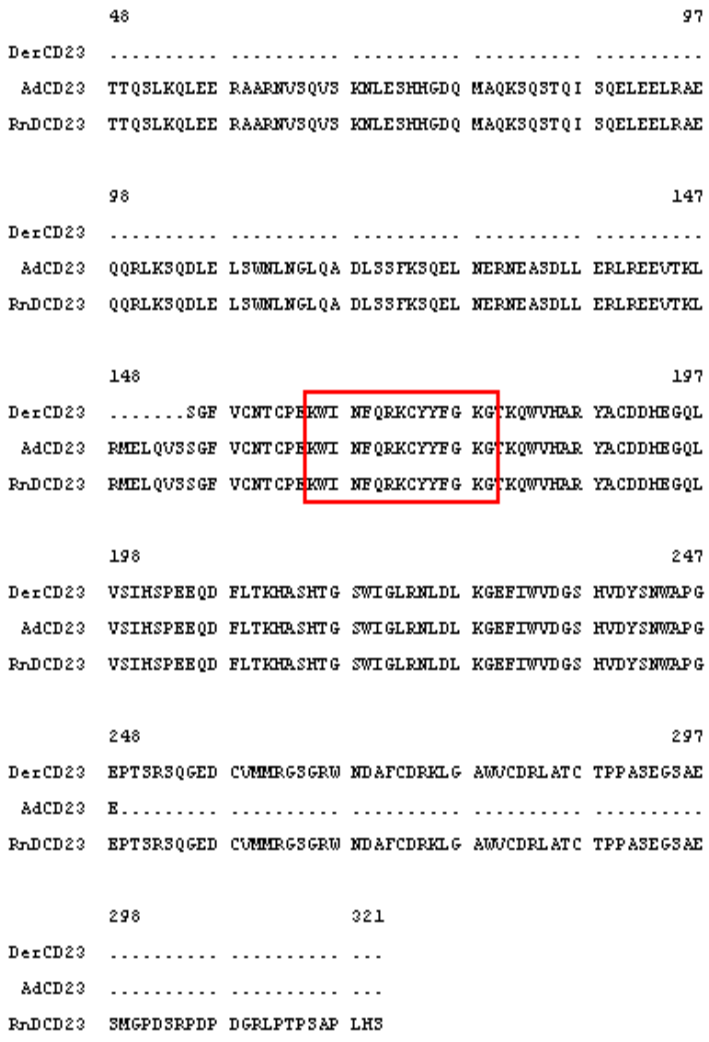

Figure 3.21 Alignment of amino acid sequences for the three different types of sCD23.

The three different types of sCD23 used in the experiments are represented as RnDCD23 (R & D CD23), Assay design CD23 (Ad CD23) and Der CD23. The sequence highlighted in red represents the sequence for the CD23-derived long peptide (LP).

4 Modulation of αVβ5 Integrin Function by Chemokines, Cytokines and Growth Factors.

4.1 Introduction

Signals mediating growth and survival of B cells at any stage of development need to be highly regulated for normal progression through each stage of development. The bone marrow microenvironment comprises a huge variety of cytokines, chemokines, growth factors and adhesion molecules all providing coordinated signals that allow maturation of B cells along the developmental pathway. Therefore, any growth/proliferation signal relating to B cell precursors in the bone marrow will be regulated by a number of positive and negative mechanisms.

The integrin αVβ5 was shown to bind to an RKC motif on CD23 [232] and this interaction sustained proliferation of precursor B cell lines expressing αVβ5 (Chapter 3). Therefore we used sCD23, CD23-derived peptides and MAbs AMF7 and 15F11 to assess the role of chemokines, cytokines and growth factors in modulating αVβ5 function and expression. The chemokine SDF-1 and a combination of cytokines as well as PDGF were used in combination with the αVβ5 ligands to assess SMS-SB cell proliferation.

PDGFs are a family of closely related proteins which exist as disulphide-bonded dimers of approximately 30 kDa and three isoforms of PDGF, αα, αβ, ββ are encoded by two genes termed PDGFA and PDGFB [289]. The PDGF receptor also comprises two isoforms, PDGFR-α and PDGFR-β. The α-receptor binds all three isoforms with high and equal affinity whereas the β receptor preferentially binds ββ homodimers and interacts with αβ heterodimers with reduced affinity. PDGF is a major growth factor in serum and is a potent mitogen for cells of mesenchymal origin. Exaggerated and inappropriate signalling by the PDGF receptor tyrosine kinase has been implicated in a variety of diseases [290].

A role for PDGF in the haematopoietic system has been shown by a number of studies. It was shown that PDGF can alter the phenotype of stromal cells [291] and PDGF has been implicated in myeloid leukaemias [292] and in expansion of $CD34^+$ cord blood cells [293]. As SMS-SB cells were previously shown to express PDGFRß [294] and since PDGF has been extensively studied in the adherenet cell systems for integrin-growth factor cross-talk we assessed the role of PDGF in modulating αVß5 function together with other cytokines and chemokines.

4.2 Results

4.2.1 SDF-1 Enhances Proliferation Induced by Ligation of αVβ5 Integrin

When treated with sCD23, SMS-SB cells show an increase in cell proliferation in a dose-dependent manner (Chapter 3). Other pre-B cell lines cell lines such as RS4;11 and 697 show a similar effect in cell proliferation (Chapter 3). SMS-SB cell proliferation induced by a sub-optimal concentration of sCD23 (158ng/ml) was enhanced by SDF-1 treatment as shown in figure 4.1A. Optimal enhancement of cell proliferation was seen with 250ng/ml SDF-1 and 158ng/ml sCD23. At 250ng/ml sCD23 treatment no enhancement of proliferation was observed with SDF-1 treatment and in fact there was a slight reduction in sCD23-induced cell proliferation of SMS-SB cells (*data not shown*) SDF-1 alone did not show any effect on proliferation of SMS-SB cells at any concentration used. The proliferative effect driven by the antibody 15F11 alone was also enhanced when SDF-1 was used together with 15F11 as shown in figure 4.1B. Peptide #9 (a CD23-derived peptide containing the RKC motif) showed a similar effect in cell proliferation while peptide #34, a negative control peptide (lacking the RKC motif) showed no effect in cell proliferation (Figure 4.1C). Similarly, SDF-1 enhanced proliferation of SMS-SB cells, stimulated with the RKC containing long peptide (LP) (Figure 4.1D). As with sCD23, optimal enhancement of cell proliferation was seen at 250ng/ml SDF-1. Both the peptides and sCD23 showed an enhancement in cell proliferation with SDF-1 at a concentration slightly lower than the optimal concentration at which they themselves induced cell proliferation (Chapter 3).

FACS analysis for surface expression of the receptors showed that in addition to expressing the CXCR4 receptor, SMS-SB cells also expressed CXCR7, another receptor known to bind SDF-1 (*data not shown*). In order to show that the enhancement of αVß5-mediated cell proliferation was indeed mediated via CXCR4 and SDF-1, we used the SDF-1 antagonist AMD3100 that is known to block binding of SDF-1 to CXCR4. Figure 4.2A shows that when SMS-SB cells were treated with AMD3100 (50μM) there was no difference in proliferation induced by long peptide with or without SDF-1 treatment. In the AMD3100-treated cells, basal cell proliferation is slightly increased but LP-induced cell proliferation is still very evident but there is no enhancement of this LP-induced cell proliferation in combination with SDF-1.

A similar result was observed with the antibody 12G5 which is known to bind to CXCR4. There was no difference in proliferation induced by LP with or without SDF-1 in presence of the 12G5 antibody (Figure 4.2B). However, in the MAb 12G5-treated SMS-SB cells, the basal proliferative response was lower than untreated cells or cells treated with IgG2a isotype control antibody. Although the 12G5 treated cells showed a proliferative response with LP treatment the cell count was much lower than in the untreated cells or IgG2a-treated cells.

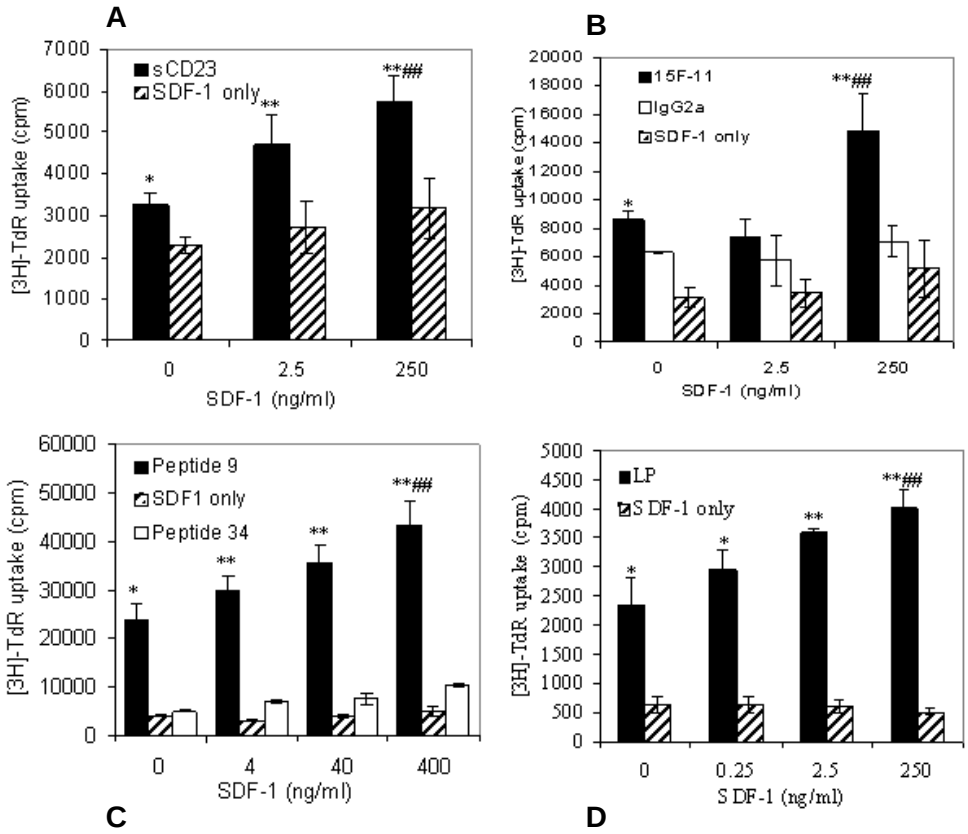

Figure 4.1 SDF-1 enhances proliferation mediated via the αVβ5 integrin in SMS-SB cells.

(A) The proliferation of LCD cultures of SMS-SB cells in response to a sub-optimal concentration of sCD23 (158ng/ml) together with indicated concentrations of SDF-1 (black bars) and SDF-1 alone (hatched bars). **Panels B-D** illustrate the proliferative response of SMS-SB cells to stimulation with 5μg/ml 15F11 MAb **(B),** 5μg/ml peptide #9 **(C),** and 1μg/ml long peptide (LP) **(D),** respectively, in combination with SDF-1. In each panel the treatment with SDF-1 alone is shown as a hatched bar, the combination of SDF-1 and stimulus as a black bar, and the combination of SDF-1 with either isotype control IgG2a **(B)** or irrelevant peptide #34 **(C)** is shown as a white bar. Bars represent the standard deviation of triplicate determinations, with *p<0.05 or **p<0.005 indicating increase in proliferation in cells treated with different concentrations of SDF-1 and αVβ5 stimulants (sCD23, 15F11MAb, peptide #9 or LP) in comparison to SDF-1 treatment alone, and #p<0.05 and ##p<0.005 indicating enhancement in proliferation with 250ng/ml SDF-1 and αVβ5 stimulants when compared with αVβ5 stimulants alone. Data are representative of three independent experiments.

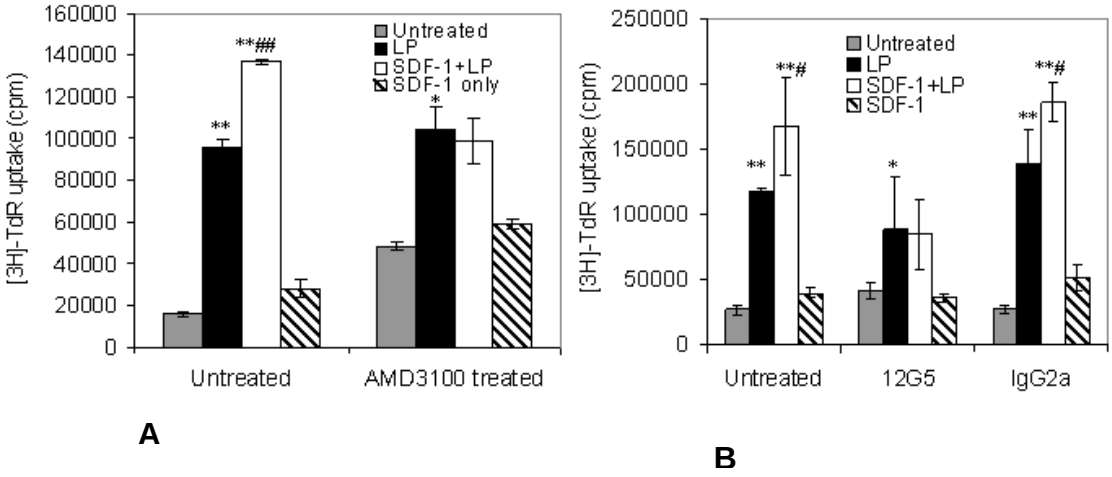

Figure 4.2 SDF-1 enhances proliferation mediated via the αVβ5 integrin in SMS-SB cells via the CXCR4 receptor.

Proliferation response of LCD cultures of SMS-SB cells in presence of LP and CXCR4 antagonist AMD3100 or anti-CXCR4 antibody 12G5. **(A)** represents proliferation with sub-optimal concentration of LP (1µg/ml) and SDF-1 (250ng/ml) in untreated cells and cells treated with 50µM AMD3100 for 30 min prior to initiation of the assay. In both cases no stimulus is represented as grey bars, LP treatment is represented as black bars, the LP and SDF-1 combination as white bars and SDF-1 alone as hatched bars. **(B)** The proliferation response of SMS-SB cells to LP (1µg/ml) alone or with SDF-1(250ng/ml) in untreated cells, cells treated with 12G5 antibody or cells treated with IgG2a isotype control antibody. In all cases no stimulus is represented as grey bars, LP treatment is represented as black bars, the LP and SDF-1 combination as white bars and SDF-1 alone as hatched bars Bars represent the standard deviation of triplicate determinations, with *$p<0.05$ or **$p<0.005$ indicating increase in proliferation in cells treated with different concentrations of SDF-1 and αVβ5 stimulants (sCD23, 15F11MAb, peptide #9 or LP) in comparison to SDF-1 treatment alone, and #$p<0.05$ and ##$p<0.005$ indicating enhancement in proliferation with 250ng/ml SDF-1 and αVβ5 stimulants when compared with αVβ5 stimulants alone. Data are representative of three independent experiments.

4.2.2 SDF-1 treatement does not change αVβ5 expression and αVβ5 ligation does not change CXCR4 expression

In order to verify whether SDF-1 was affecting αVβ5 function by modulating expression levels of the latter, we monitored changes in αVβ5 expression after SDF-1 treatment. SMS-SB cells were treated with 250ng/ml of SDF-1 and αVβ5 surface expression was assessed after 24 and 48 hours. The two antibodies 15F11 and P1F6 are known to recognise different epitopes on αVβ5 [282], therefore we used both the antibodies to assess changes in αVβ5 expression. However, there was no change in αVβ5 expression in cells treated with SDF-1 when compared to untreated cells (Figure 4.3A). SDF-1 is known to modulate the affinity of integrins for their ligands, therefore we also assessed binding of the CD23-derived peptides to SMS-SB cells with and without SDF-1 treatment. A representative result (Figure 4.3B) shows that there was no change in binding of one of the sCD23-derived peptides (peptide #9) to SMS-SB cells regardless of SDF-1 treatment. On the other hand, ligation of αVβ5 did not have an effect on CXCR4 expression either. Figure 4.3C shows that treatment of SMS-SB cells with either the LP (1μg/ml) or sCD23 (160ng/ml) does not cause a change in CXCR4 expression after 24 hours of treatment of SMS-SB cells.

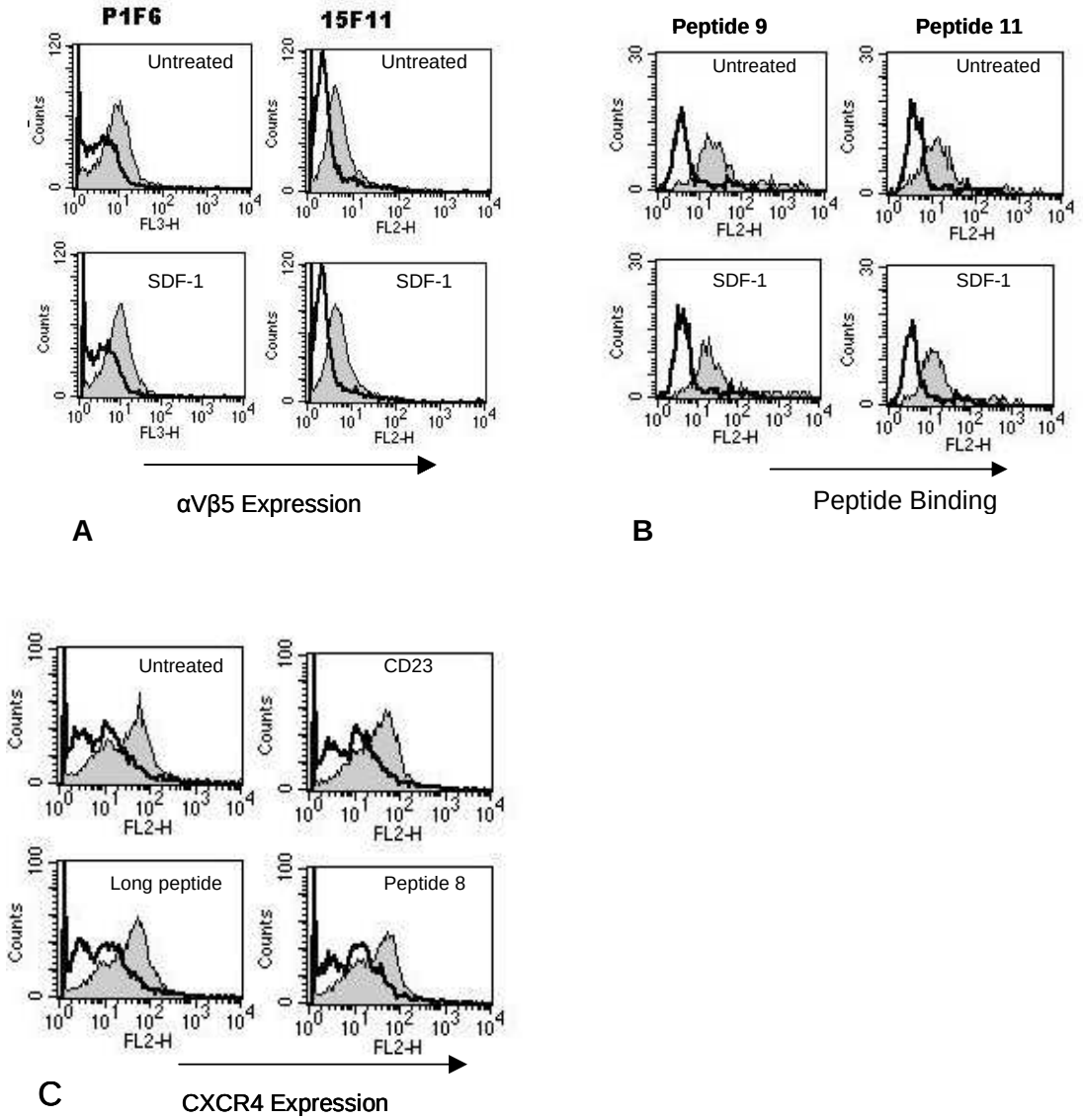

Figure 4.3 Effects of SDF-1 on αVβ5 expression and αVβ5 ligation on CXCR4 expression.

SMS-SB cells cultured in the presence or absence of 250ng/ml SDF-1 (24 h) were stained for αVβ5 expression using the anti-αVβ5 MAbs, P1F6 and 15F11 (**Figure 4.3A**). Fluorescence intensity histograms for the relevant αVβ5 MAb are represented as grey shaded area and isotype control antibody staining is represented by the black line. **Figure 4.3B** illustrates the fluorescence intensity plots for binding of 1μg/ml peptide #9 or #11 (grey shaded area), the negative control peptide #41 (black line) to SMS-SB cells following 24 h treatment with 250ng/ml SDF-1 or no treatment. **Figure 4.3C** represents CXCR4 staining in untreated SMS-SB cells or cells treated for 24 h with sCD23 (160ng/ml), LP (1μg/ml) or the negative control peptide #8 (1μg/ml). CXCR4 was stained using biotinylated-12G5 antibody and detected using streptavidin-PE (grey shaded area); the isotype control IgG2a–biotin is shown as a black line.

4.2.3 The effect of SDF-1 on αVβ5-driven proliferation is specific to early B cell precursors

We assessed CXCR4 expression in different precursor B cell lines and observed that CXCR4 expression varied in the precursor cell lines at different stages of B cell development. The cell lines representing earlier stages of B cell development had lower levels of CXCR4; CXCR4 expression increased in pre-B cell lines such as Blin1 and mature B cell lines like Daudi, and declined again in the more mature B cell lines such as IB4. CXCR4 expression in comparision to αVβ5 expression in the different cell lines is represented in Figure 4.4A and 4.4B. In particular, the pre-B cell lines (SMS-SB, RS4;11) which proliferated well in response to αVβ5 ligation had lower levels of CXCR4 and the expression of CXCR4 increased in the cell lines that were less responsive to αVβ5. Moreover, the enhancement of αVβ5-mediated cell proliferation by SDF-1 was only seen in pre-B cell lines, such as SMS-SB and RS4;11, with low CXCR4 expression (Figure 4.4C). Proliferation was not assessed with SDF-1 for the 697 cell line, but CXCR4 staining in this cell line was also low, similar to SMS-SB or RS4;11, and we would predict that their proliferative response would be similar to SMS-SB or RS4;11 cells. A representative result from the cell line RS4;11 is shown in figure 4.4C. SDF-1 enhances the proliferation induced by LP in a manner similar to that noted in SMS-SB cells. By comparison, SDF-1 had no effect on the cell line Daudi which shows little or no proliferative response to αVβ5 ligation and had higher expression of CXCR4 (Figure 4.4A and C). Furthermore, SDF-1 had no effect on the very modest response to αVβ5 ligation noted in the Blin1 cell line (Figure 4.4C).

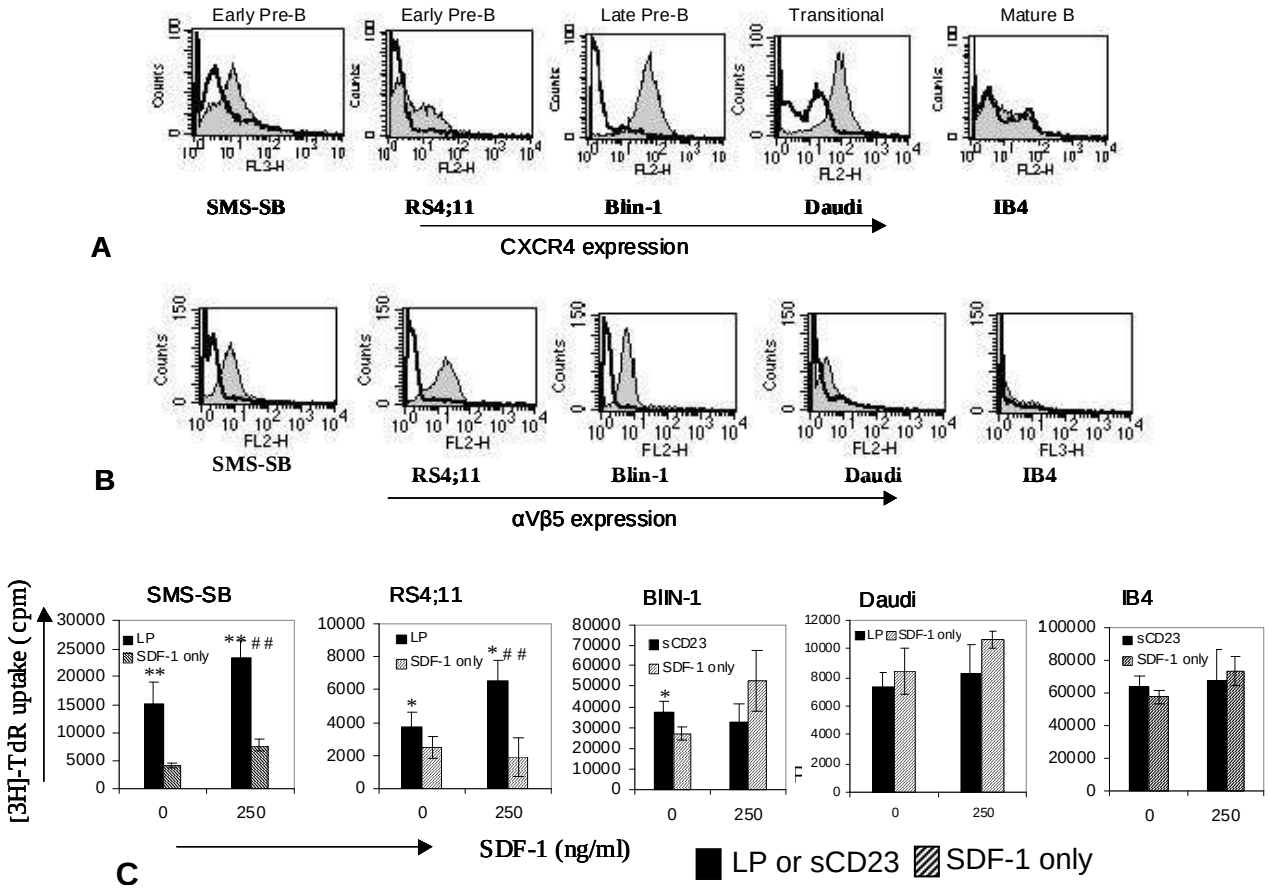

Figure 4.4 CXCR4 expression and cell proliferation in different precursor and mature B cell lines.

Panels **(A)** and **(B)** show the fluorescence intensity histograms for staining of CXCR4 (12G5) and αVβ5 (P1F6) respectively, in five cell lines. In both cases antibody staining is shown in the grey shaded area and the isotype control staining by the black line. The cell lines are represented as early or late pre-B and transitional or mature B, cell stage depending on surface marker expression as described in chapter 3. **(C)** In the different cell lines shown, proliferation driven by αVβ5 ligation (either with 2ng/ml sCD23 or with 1μg/ml LP) and SDF-1 is shown by black bars and proliferation induced with SDF-1 alone is shown as hatched bars. Bars represent the standard deviation of triplicate determinations, with *p<0.05 indicating increase in proliferation in cells treated with different concentration of SDF-1 and αVβ5 stimulants (sCD23, 15F11MAb, peptide #9 or LP) in comparison to SDF-1 treatment alone, and #p<0.05 indicating enhancement in proliferation with 250ng/ml SDF-1 and αVβ5 stimulants when compared with αVβ5 stimulants alone. Data are representative of three independent experiments.

4.2.4 Other cytokines do not mimic SDF-1 but PDGF may have a similar role to SDF-1 in modulating αVβ5 function

Many other cytokines are known to be important during B-cell development, and we therefore examined the effects of the cytokines IL-7, IL-11, IL-3 and IL-4 to determine if they mimicked the effects of SDF-1 on αVβ5-induced cell proliferation. None of the above-mentioned cytokines showed similar effects to SDF-1. IL-7 showed varied effects on cell proliferation induced via αVβ5 but there were no consistent data to show that it enhanced αVβ5-induced cell proliferation (Figure 4.5A). IL-11 itself induced an increase in cell proliferation (Figure 4.5B) but had no effect on αVβ5-induced cell proliferation and neither IL-3 nor IL-4 enhanced αVβ5-induced proliferation (Figure 4.5C and 4.5D).

αVβ5 expression was also analysed in presence of the above-mentioned cytokines. The cytokines were used at the same concentrations as SDF-1 (5nM) and αVβ5 expression was again analysed using P1F6 and 15F11 antibody. IL-7 did not influence the levels of αVβ5 expression as depicted in Figure 4.6A nor did IL-11 (Figure 4.6B). With IL-3 treatment although the biotinylated isotype control staining was lower than the untreated cell, P1F6 staining was of the same intensity as the untreated cells. There was no change in αVβ5 staining in untreated cells and IL-3-treated cells with 15F11 antibody, whereas IL-4 treatment resulted in decreased αVβ5 expression as shown by P1F6 staining (Figure 4.6B). But, when these experiments were repeated IL-4 seemed to increase αVβ5 expression on one instance and no change was seen in another instance, therefore no conclusion could be made from these data. It could be that IL-4 influences αVβ5 expression depending upon the stage the cells are in culture, and therefore we observed different results in different experiments.

However, we noted that the platelet-derived growth factor (PDGFβ) was, like SDF-1, able to enhance SMS-SB cell proliferation driven by αVβ5. PDGF has been studied in connection with the SMS-SB cells previously and this cell line is known to express the PDGF receptor β (PDGFRβ) [294]. Figure 4.7A and 4.7B show that PDGFβ significantly enhanced both LP and sCD23-induced proliferation in SMS-SB to a very similar extent as SDF-1. Similar to SDF-1, PDGF treatment did not result in any changes in αVβ5 expression (Figure 4.7C).

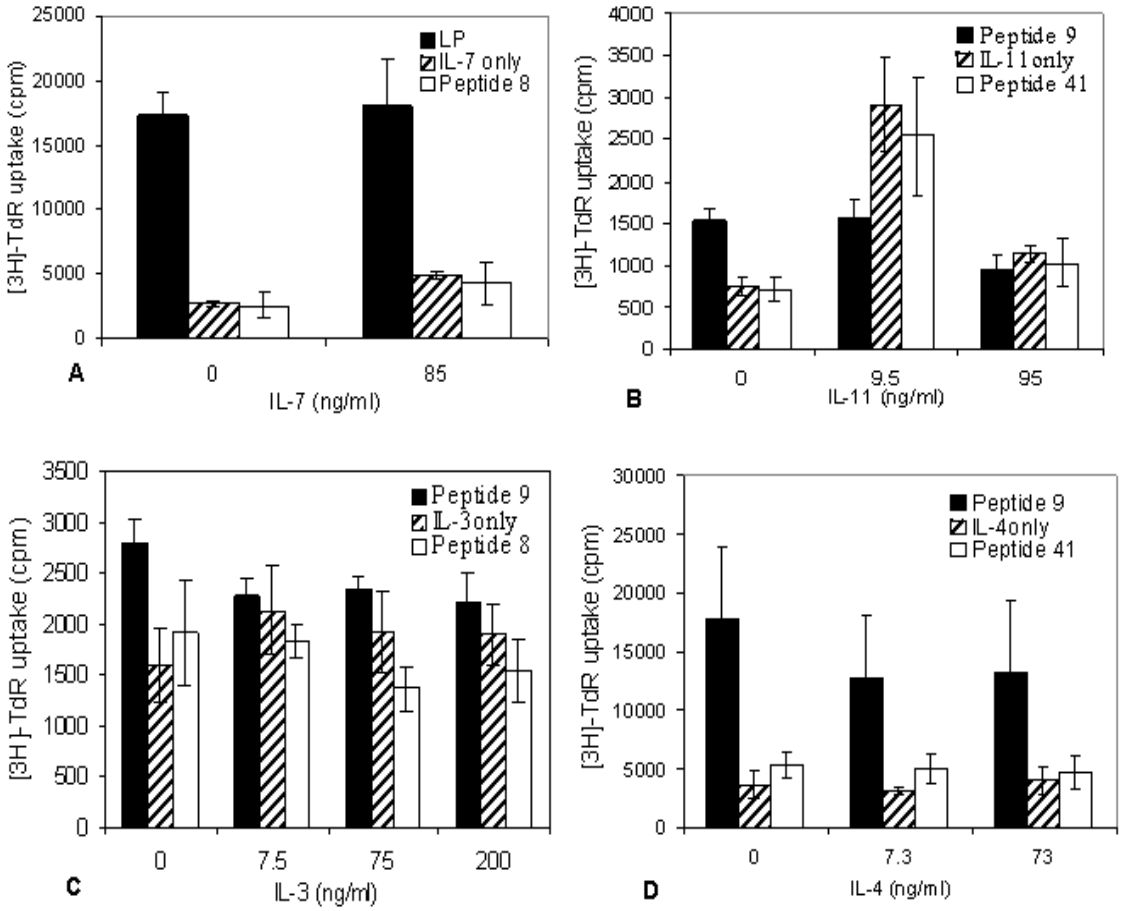

Figure 4.5 αVβ5-mediated cell proliferation in the presence of other cytokines.

Figures 4.4 **(A), (B**), **(C)** and **(D)** represent proliferation of LCD cultures of SMS-SB cells in response to 1µg/ml LP or 5µg/ml peptide #9, together with the indicated concentrations of IL-7, IL-11, IL-3 and IL-4, respectively. In each case, proliferation induced by the peptides and the cytokine is shown in black bars, cytokine alone is shown as hatched bars and cytokine with negative control peptide # 41or #8 is shown as white bars. The concentrations of each cytokine are equivalent molar concentrations. Data are representative of three independent experiments.

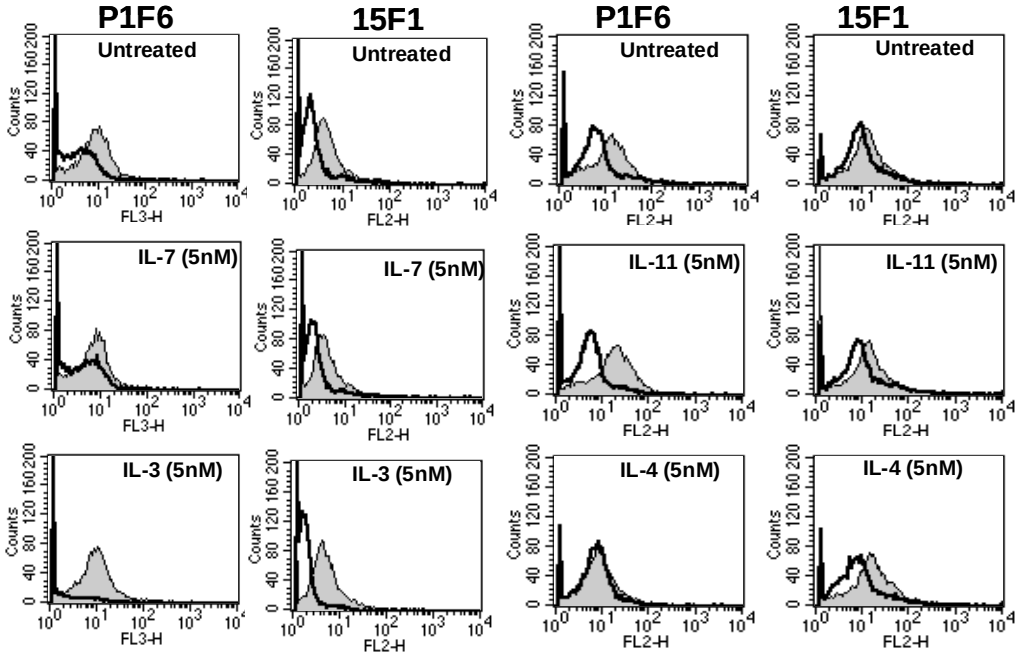

Figure 4.6 αVβ5 expression in presence of the cytokines IL-7, IL-3, IL-11 and IL-4.

SMS-SB cells were treated with 5nM of the indicated cytokines or left untreated and αVβ5 expression was analysed by FACS using P1F6 or 15F11 antibody. **(A)** Represents histograms for staining of αVβ5 in untreated cells or cells treated with IL-7 and IL-3. **(B)** Represents αVβ5 expression in cells treated with IL-11 and IL-4 or untreated cells. In all cases the staining with relevant αVβ5 MAbs is represented as grey shaded area and staining with isotype control antibody is represented as a black line.

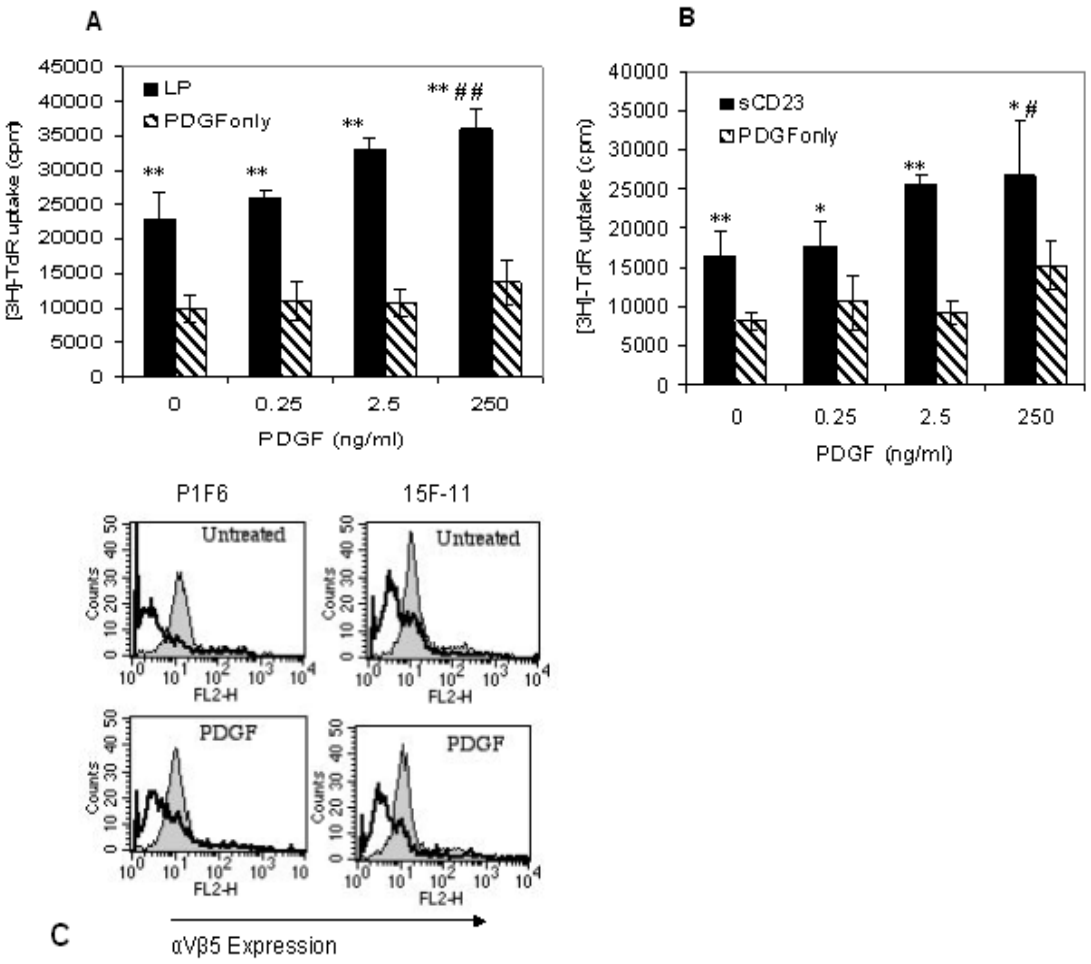

Figure 4.7 αVβ5-mediated cell proliferation in the presence of PDGFβ.

LCD SMS-SB cultures were stimulated with the indicated concentrations of PDGFβ and 1μg/ml LP **(Figure 4.7A)** or 160ng/ml sCD23 **(Figure 4.7B)** for 72 h prior to addition of [^{3}H]-TdR and determination of proliferation. Proliferation induced by PDGF and LP or sCD23 in combination with PDGF is shown as black bars and proliferation driven by PDGF alone is shown as hatched bars. Bars represent the standard deviation of triplicate determinations, with *p<0.05 or **p<0.005 indicating increase in proliferation in cells treated different concentrations of PDGF and αVβ5 stimulants (sCD23, LP) in comparison to PDGF treatment alone, and #p<0.05 and ##p<0.005 indicating enhancement in proliferation with 250ng/ml PDGF and αVβ5 stimulants when compared with αVβ5 stimulants alone. **(C)** SMS-SB cells, untreated or treated with 250ng/ml PDGF (24 h), were stained for αVβ5 expression using the anti-αVβ5 MAbs P1F6 and 15F11. Fluorescence intensity histograms for the relevant αVβ5 MAbs represented by the grey shaded area and isotype control antibody staining is represented by the black line. Data are representative of three independent experiments.

4.2.5 SDF-1 enhances ERK phosphorylation induced via sCD23 and other αVβ5 ligands and PDGF shows a similar effect.

We next examined the signalling pathways that might be involved in synergistic activation of cell proliferation by αVβ5 ligation and SDF-1 treatment, paying particular attention to ERK phosphorylation since SDF-1 is known to target this pathway [121].

Treatment of SMS-SB cells with sCD23 promoted an early (0-2 min) and transient rise in phospho-ERK levels that was followed by a later (30-60 min) more sustained and robust level of ERK phosphorylation (Figure 4.8A). SDF-1 alone provoked an early, modest increase in phospho-ERK levels with no evidence of a later, stronger increase in phosphorylation of ERK (Figure 4.7A). By contrast, combined treatment of SMS-SB cells with SDF-1 and sCD23 stimulated a very robust initial phase of ERK phosphorylation that was sustained until 10 minutes post-stimulation, yielding phospho-ERK levels that were some 15-fold greater than either stimulus alone (Figure 4.8B). The 15F11 MAb induced a modest initial level of phospho-ERK, followed by a more pronounced second phase and in this case co-stimulation with SDF-1 promoted a more sustained wave of ERK phosphorylation over the first 30 minutes of the experiment (Figure 4.8C).

The RKC-containing CD23-derived peptide #9 behaved similarly, inducing a biphasic response by itself and a more robust and sustained response when added together with SDF-1; peptide #8, which lacks the RKC motif, failed to induce robust phosphorylation either alone or in association with SDF-1 (Figure 4.8D). Finally, the RKC-containing pentadecameric LP induced sustained and robust ERK phosphorylation that was enhanced in magnitude and persistence by SDF-1 co-stimulation (Figure 4.8E). LP has an affinity for αVβ5 that is an order of magnitude greater than that of CD23 itself (~ 10^{-7} *M* vs 6 x 10^{-6} *M*) and this greater affinity may explain the higher and more sustained levels of ERK phosphorylation noted with LP.

The data are consistent with the interpretation that ligation of αVβ5 by a soluble ligand alone promotes a biphasic pattern of ERK phosphorylation, with a modest initial peak of phosphorylation followed by a later phase of higher and more sustained phosphorylation. The data also indicate that SDF-1 enhances the initial αVβ5-driven early activation of ERK phosphorylation and maintains this at a robust level for a sustained period, the length of which may reflect the affinity of the ligand used to challenge the αVβ5 integrin. A similar pattern of enhancement of ERK phosphorylation was observed with PDGF treatment. Treatment of SMS-SB cells with 250ng/ml of PDGF resulted in significant ERK phosphorylation (2-10 min) (Figure 4.9A). However this phosphorylation was significantly enhanced when PDGF was used together with 160ng/ml sCD23 (Figure 4.9B) with a sustained increase in ERK phosphorylation from 0-30 min. This enhancement in ERK phosphorylation correlates well with the enhancement in cell proliferation observed with PDGF and sCD23 treatment of SMS-SB cells.

The MEK inhibitor U0126 was used to confirm the role of ERK phosphorylation in SMS-SB cell proliferation. Treatment of SMS-SB cells with U0126 (5μM or 10μM) prior to the initiation of the proliferation assay completely abolished any effect of sCD23 or its peptides on cell proliferation (Figure 4.10A). Moreover, U0126 abolished the basal survival of SMS-SB cells in low-density cultures after few hours of initiation of the culture. U0126 treatment abrogated the ERK phosphorylation driven by SDF-1 or sCD23 individually and also completely prevented the enhancement of ERK phosphorylation seen with SDF-1 and sCD23 (Figure 4.10C).

The PI3K inhibitor LY294002 was employed to confirm that the inhibition of proliferation and survival was specific to U0126. LY294002 also inhibited the proliferation of SMS-SB cells in response to αVβ5 ligation, but the inhibition was less complete than that observed with U0126 (Figure 4.10B). Indeed, after 48 hours of treatment a small increase in cell proliferation was still evident in response to LP treatment in cells pre-treated with LY294002 (Figure 4.10B), whereas proliferation was absent by 12 h following U0126 treatment. Also, unlike U0126, LY294002 treatment does not inhibit the basal survival of SMS-SB cells. We confirmed this difference by measuring survival of SMS-SB cells after

U0126 and LY294002 treatment. Analysis of SMS-SB cell survival using annexinV/ propidium iodide (AV/PI) staining demonstrated that U0126 treatment results in a higher proportion of AV/PI double-positive cells than LY294002 after a 12 h treatment (Figure 4.10D), illustrating that U0126 leads to increased apoptosis in comparison to LY294002 treatment. This indicates that the ERK phosphorylation pathway is important for SMS-SB survival and cell proliferation.

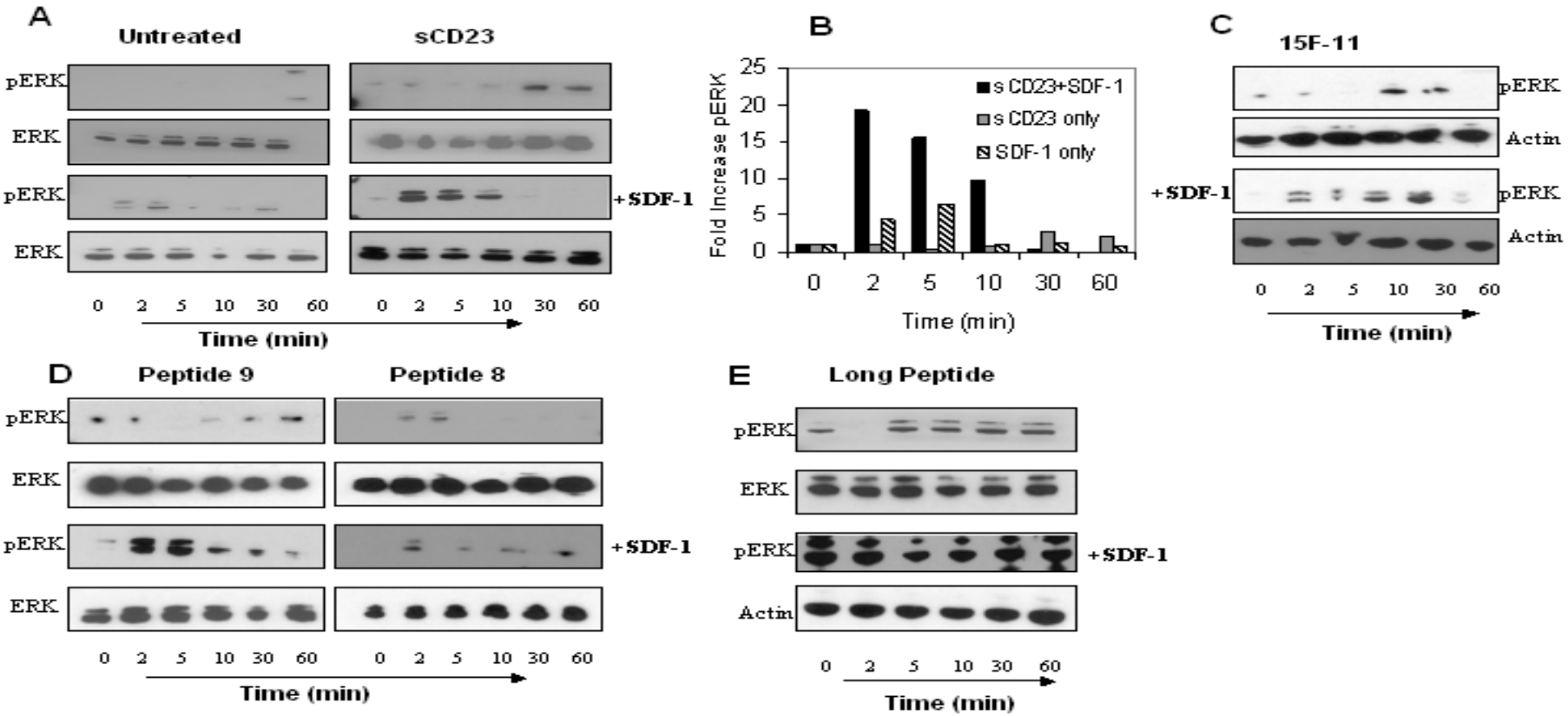

Figure 4.8 ERK phosphorylation in SMS-SB cells treated with SDF-1 alone or in combination with αVβ5 ligands.

Lysates from treated SMS-SB cells were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with antibodies to phosphorylated ERK and subsequently stripped and reprobed with antibodies to total protein (either ERK or actin). **(A)** Western blots of phosphorylated and total ERK in untreated SMS-SB cells and cells treated with sCD23 (160ng/ml), SDF-1 (250ng/ml) or a combination of sCD23 and SDF-1, at the time points shown. The increase in phosphorylation was quantitated by densitometric analysis of the bands **(B)**. Figure **(C**) represents western blots for phosphorylation of ERK and of total actin in SMS-SB cells treated with 15F11 MAb alone or in combination with SDF-1. **(D**) and **(E)** Western blots for phosphorylated and total ERK in SMS-SB cells treated with peptide #9 (5μg/ml), negative control peptide #8 (5μg/ml) or LP (1μg/ml) alone or in combination with SDF-1 (250ng/ml). Data are representative of three independent experiments.

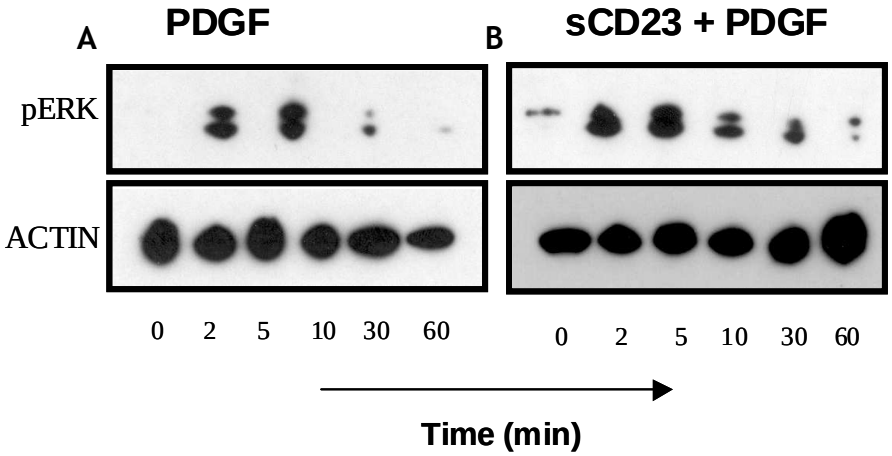

Figure 4.9 ERK phosphorylation in SMS-SB cells treated with PDGF alone or in combination with αVβ5 ligands.

Lysates from treated SMS-SB cells were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with antibodies to phosphorylated ERK and subsequently stripped and reprobed with antibodies to total protein (either ERK or actin). **(A)** Western blots for phosphorylated ERK or actin in SMS-SB cells treated with PDGF (250ng/ml). **(B)** Western blots for phosphorylated ERK and actin in SMS-SB cells PDGF (250ng/ml) with sCD23 (160ng/ml). Data represent three independent experiments.

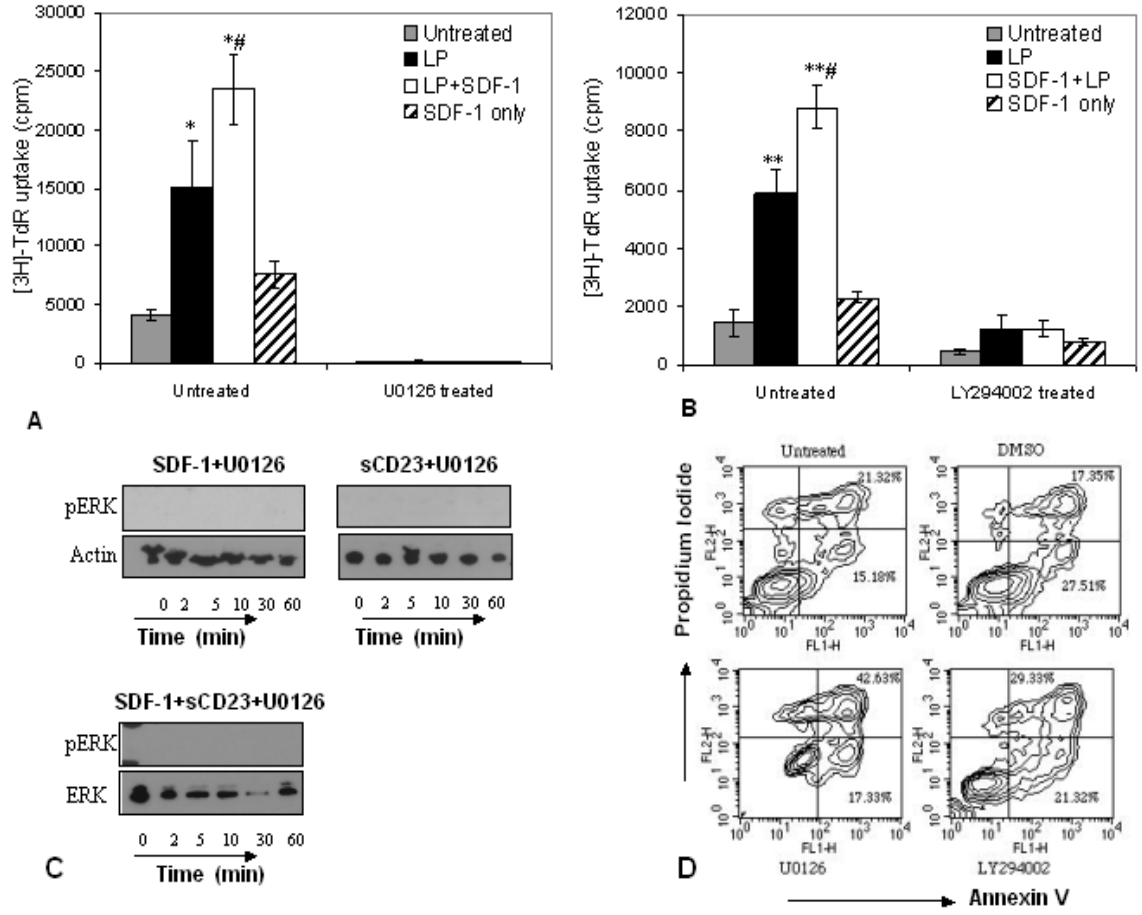

Figure 4.10 Effect of inhibition of ERK and PI-3K signalling on αVβ5-mediated proliferation of SMS-SB cells.

SMS-SB cells were treated with 10μM U0126 or 50μM LY294002 or left untreated for 30 min prior to plating at LCD; proliferation was assessed by ^{3}[H]-TdR incorporation after 72 h of culture. **Panel A** shows cell proliferation with LP (1μg/ml) and/or SDF-1 (250ng/ml) in untreated cells and cells treated with U0126**. Panel B** shows cell proliferation with long peptide and/or SDF-1 in untreated or cells treated with LY294002. In both cases, unstimulated cells are represented as grey bars, LP-treated as black bars, the LP and SDF-1 combination as white bars and SDF-1 alone as hatched bars. Bars represent the standard deviation of triplicate determinations, with *p<0.05 or **p<0.005 indicating increase in proliferation in cells treated with different concentrations of SDF-1 and αVβ5 stimulant (LP) in comparison to SDF-1 treatment alone, and #p<0.05 and ##p<0.005 indicating enhancement in proliferation with 250ng/ml SDF-1 and αVβ5 stimulants when compared with αVβ5 stimulants alone. Data are representative of three independent experiments. **Panel C** Western blot analysis of phosphorylated ERK and total actin in SMS-SB cells pre-treated with U0126 (10μM) 30 min before stimulation with SDF-1 (250ng/ml) or sCD23 (160ng/ml) or a combination of SDF-1 and sCD23. **Panel D** shows contour plots for PI/annexinV staining of cells exposed to U0126 (10μM), LY294002 (50μM), DMSO (vehicle) or no treatment for 12 h. Quadrant gates in the insets are based on unstained, annexinV or PI stains alone. Data are representative of 3 independent experiments.

4.3 Discussion

It is well established that chemokines dynamically regulate the positioning of cells of the immune system by influencing the structure of integrin molecules to ensure cells attracted to a specific niche are retained there via adhesion reactions with tissue matrix proteins. Chemokines can also positively influence signalling via integrins in such adhesion-dependent interactions, as exemplified by the action of SDF-1 on haematopoietic precursors at the level of the VLA-4-ICAM-1 interaction [114,295]. The results presented in this chapter demonstrate that SDF-1 positively influences signalling events delivered to integrins in a soluble, adhesion-independent manner. Thus, ligation of αVβ5 using specific MAbs, sCD23 or RKC-containing peptides derived from sCD23 all provoke ERK phosphorylation and proliferation responses in B cell precursors that are enhanced by SDF-1. Remarkably, PDGF also enhances signalling delivered via soluble ligation of αVβ5, suggesting that both tyrosine kinase and G protein-coupled receptor-linked pathways may modulate adhesion-independent signalling by αVβ5.

Soluble CD23-mediated proliferation of SMS-SB cells was enhanced by addition of SDF-1 and identical effects were seen with sCD23-derived peptides (containing the RKC motif bound by αVβ5) and the anti-αVβ5 MAb 15F11, all of which promote proliferation of these cells. Blocking the binding of SDF-1 to CXCR4 using the previously described SDF-1 antagonist AMD3100 [296,297] abolished this enhancement. However SDF-1 did not influence proliferation induced by the MAb AMF7 which binds to αV subunit and sustains proliferation of SMS-SB cells. In relation to this as mentioned in chapter 3 we did not observe any proliferative response with this antibody in RS4;11 cell line which showed similar proliferation to SMS-SB cells in response to αVβ5 ligation. Moreover, the response to AMF7 might also be dependent on other αV integrins expressed.

Interestingly, SDF-1-mediated enhancement of cell proliferation was seen only in the cell lines SMS-SB and RS4;11, both of which represent the early precursor stage of B-cell development ($CD19^+$, $CD10^-$ and surface μ low or negative). It is in

these early precursors that the most robust proliferative response to ligation of αVβ5 with sCD23, or CD23-derived peptides, was observed (Chapter 3). Blin1 cells, which showed a slight but comparatively modest proliferative response following sCD23 treatment, did not show any enhancement of proliferation with SDF-1. Higher CXCR4 expression did not influence the lack of response to αVβ5 ligation noted in the more mature cell lines. SDF-1-mediated modulation of αVβ5 function seems to be important in early B-cell precursors, which express lower levels of CXCR4. This is consistent with data showing that although B-cell precursors express higher levels of CXCR4 with increasing maturation, the cells chemotactic response to SDF-1 itself is apparently blunted [298].

CXCR4 is continuously expressed on the surface of B-cells. It has been shown that when B lymphocytes mature and start to express IgM at the cell surface they lose their chemotactic response to SDF-1 despite sustained expression of CXCR4 [298-300]. Glodek *et. al.,* showed that SDF-1 triggers a sustained adhesion response specifically in progenitor (pro and pre-B) cells that diminishes during maturation in the bone marrow; circulating mature B cells exhibit only transient SDF-1-induced adhesion [301]. Furthermore, recent studies have linked the expression of CXCR4 with the potentiation of plasma cell long-term survival in the bone marrow [302-304]. This developmental stage-specific function of SDF-1 is interesting in relation to the data showing that αVβ5 function and expression is important in early B cell precursors and decreases as the cell mature (*Borland, et. al., 2008 manuscript submitted*) This supports a developmental stage-specific role for adhesion molecules and growth factors in B lymphopoiesis.

SDF-1 modulation of αVβ5 integrin function does not involve changes in receptor expression. SDF-1 treatment did not change αVβ5 expression (Figure 4.3A) and αVβ5 ligation does not change CXCR4 expression (Figure 4.3C). Although the 15F11 and P1F6 MAbs recognise different epitopes on αVβ5 [282], it is not known what conformational states of the integrin are recognised and so no conclusions can be drawn concerning the effects of SDF-1 on the conformational state of αVβ5. Furthermore, treatment of SMS-SB cells did not influence the CD23-derived peptides binding to SMS-SB cells (Figure 4.3B) indicating that SDF-1 does not alter binding of these peptides to αVβ5.

SMS-SB cells undergo apoptosis in LCD culture conditions [276], therefore we used these culture conditions to study their survival and subsequent proliferation. The inhibition of cell proliferation by U0126 demonstrated that basal ERK phosphorylation is necessary for the survival of SMS-SB cells; in the presence of U0126 the cells rapidly undergo apoptosis. The ERK phosphorylation observed following sCD23, RKC-containing peptides and 15F11 treatment demonstrated that the proliferation induced by ligation of αVß5 with sCD23 is induced via the ERK signalling pathway. The enhancement in proliferation noted with SDF-1 and sCD23 correlated well with the sustained increase in ERK phosphorylation with SDF-1 and sCD23. This was verified by similar enhancement seen with the CD23-derived peptides and 15F11 antibody. Moreover, PDGF showed the same effect as SDF-1 in enhancement of sCD23-driven ERK phosphorylation. As in the case of SDF-1, this enhancement of ERK phosphorylation correlated well with the potentiation of cell proliferation seen with PDGF and sCD23 co-stimulation (Figure 4.7A and B). Other signalling molecules could also be involved in regulating SMS-SB cell proliferation and the observation that LY294002 inhibited the enhancement of αVß5-mediated proliferation by SDF-1 indicates that AKT could be involved in regulation of adhesion-independent signalling by integrins. This would be consistent with other studies that have reported that SDF-1 activates both ERK and AKT [123,124,305,306].

Many other cytokines are known to modulate integrin function and B-cell precursor survival. We noted no similar enhancement in αVß5-mediated proliferation by the cytokines IL-7, IL-3, IL-4 and IL-11. Therefore, we did not assess ERK signalling pathway activation following treatment with these cytokines. However, it is possible that some combination of these cytokines, with or without SDF-1, might influence αVß5-mediated proliferation. For example, recent work by Juarez, *et al.,* shows that interaction of SDF-1, IL-3 and IL-7 induce proliferation of ALL cells co-cultured with stromal cells. This study shows that IL-7 alone does not induce ERK activation but there is synergistic ERK activation following IL-7 and SDF-1 co-stimulation [306]. However, the ALL cells were propagated on stromal cells and the effects on ERK and other signalling pathways would have been affected by multiple adhesion contacts between the ALL cells and stromal cells. Therefore, the Juarez data illustrate

convincingly that ERK phosphorylation can occur in an adhesion-dependent manner, while our data demonstrate the synergistic activation of ERK signalling can also occur when αVβ5 is stimulated using soluble ligands in an adhesion-independent assay.

With regard to the influence of other cytokines it was particularly interesting to observe that IL-3 and IL-4 potentially could influence αVβ5 expression although these cytokines did not influence αVβ5-mediated proliferation. IL-4 has previously been shown to inhibit pre-B cell proliferation [307] and to induce differentiation of murine precursor B cells [308] and therefore it would be interesting to assess further the role of IL-4 on αVβ5 expression. Although these cytokines tested differ from SDF-1 which is a chemokine, because of the well established role of these cytokines in precursor B cell growth and development we assessed their role in the same manner as SDF-1 instead of investigating other chemokines.

Finally, PDGF enhanced the proliferation induced via αVβ5 in a manner similar to that observed with SDF-1. Interestingly, a previous study showed that early pre-B cells, but not mature B cells, express the PDGFRβ [309]. PDGF was shown to promote proliferation of these cells and not of more mature or even immature pre-B cells [309]. PDGF has previously been shown to interact with the integrin αVβ3 and to stimulate proliferation of oligodendrocytes by activation of this integrin in adhesion-dependent manner [310]. Therefore it is plausible that PDGF and αVβ5 also interact and are involved in mediating adhesion-independent proliferation. Further studies would be needed to investigate interaction of PDGF and αVβ5 integrin. Moreover, further experiments are also required to assess αVβ5-mediated proliferation after blocking the PDGF receptor in order to confirm the role of PDGF in enhancing αVβ5-mediated proliferation.

The data indicate that αVβ5 synergises with two distinct signalling pathways, via a tyrosine kinase receptor (PDGFR-β) and G protein-coupled receptor (CXCR4), to promote ERK phosphorylation and pre-B cell growth. It will be important to determine the common molecules downstream of both these pathways leading to ERK phosphorylation to understand further the mechanisms involved in αVβ5-mediated cell proliferation. Both PDGFR-β and CXCR4, as well as the ERK

signalling pathway are targets for drug therapies, and the interaction of these receptors with the αVβ5 integrin need to be explored in order to evaluate whether they have potential as a new therapeutic strategy for ALL since αVβ5 was shown to be highly expressed in ALL cells (*Borland, et. al., 2008 manuscript submitted*).

B cell development in different compartments of the haematopoietic microenvironment is accompanied by various stage-specific changes that are critical for proliferation, differentiation and maturation of the cells into later developmental stages. A range of adhesion and signalling molecules controlling each stage are not only key to understanding the progression into the developmental pathway but also in developing therapies for neoplasias that arise in different stages of B-cell development.

5 CD23-αVβ5 Interactions in Murine B Cells

5.1 Introduction

Cytokine-like activity is known to be unique to human CD23 [231]. The RKC motif on human CD23 that is bound by the integrin αVβ5 is QKC (gln-lys-cys) in the murine CD23 sequence. This suggested the hypothesis that the arginine 172 (R172) on the RKC motif is critical for the cytokine-like activity of human CD23. We investigated the importance of the RKC motif for CD23 and integrin interaction by assessing the activity of human sCD23 and CD23-derived peptides on murine B-cell precursors.

5.2 Results

5.2.1 Human sCD23-derived peptides bind to murine B cells

Figure 5.1 shows that the human CD23-derived, biotinylated peptides (#9-12), all containing the RKC motif, bind significantly to murine bone marrow B cells when compared with the negative control peptide #17 that lacks the RKC motif. The B cell population in the bone marrow was identified using the B220 antibody and similar binding to peptide #9 is seen in $B220^+$ B cells from murine spleen. In both the bone marrow and spleen there are $B220^-$ populations that also bind the peptides (Figure 5.1), indicating other cell types apart from B cells also bind the peptides. In the $B220^+$ compartment from the bone marrow, the majority of the cells bind to the peptides, but there is a small percentage of cells in the bone marrow that are $B220^+$ but do not bind the peptides (especially in the cases of peptide #10 and #11). As the bone marrow cells comprise a heterogenous population of cells at different stages in development, this could indicate a difference in αVβ5 expression in cells of different stages. Binding of the CD23-derived peptides was also shown in a murine pro-B cell line, BAF03. As shown in figure 5.2A the CD23-derived peptides (#9-#12) bind significantly to BAF03 cells (shown in grey) in comparison the negative control peptide #58 (shown as a black line). BAF03 cells displayed a characteristic feature of two populations, one with strong peptide staining and the other negative for peptide binding. This

feature has also been observed in some human cell lines previously and could reflect the cell cycle status of the cells. We also assessed the binding of biotinylated sCD23 to the BAF03 cells. Figure 5.2B illustrates the binding of sCD23 to BAF03 cells in comparison to binding of the peptides. At 1µg/ml sCD23 showed significant binding to the BAF03 cells, although the binding was less than that observed for the peptides. Furthermore, no binding of sCD23 was observed at 250ng/ml, which was the concentration of sCD23 used for functional experiments in human cell lines. Therefore, the higher concentration of sCD23 was used for functional studies in the BAF03 cell line.

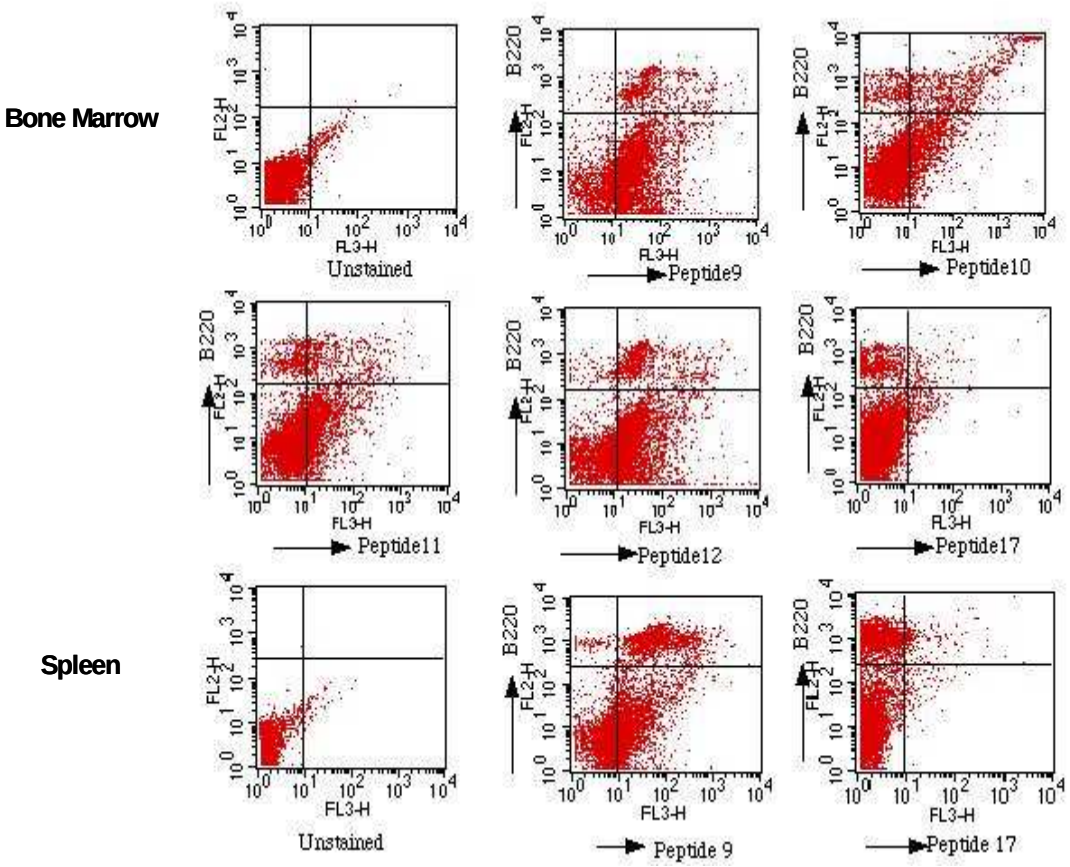

Figure 5.1 Binding of CD23-derived peptides to murine bone marrow and splenic B cells.

Primary murine bone marrow cells and spleen cells were stained simultaneously with B220-PE antibody and biotinylated peptides {visualised with Streptavidin-QR (SA-QR)}. Peptide #17 lacking the RKC motif was used as a negative control peptide and quadrant gates on the insets are based on the unstained sample and staining for the negative control peptide.The upper right quadrant represents $B220^{+}$ cells that bind the peptides. Data represent three independent staining procedures.

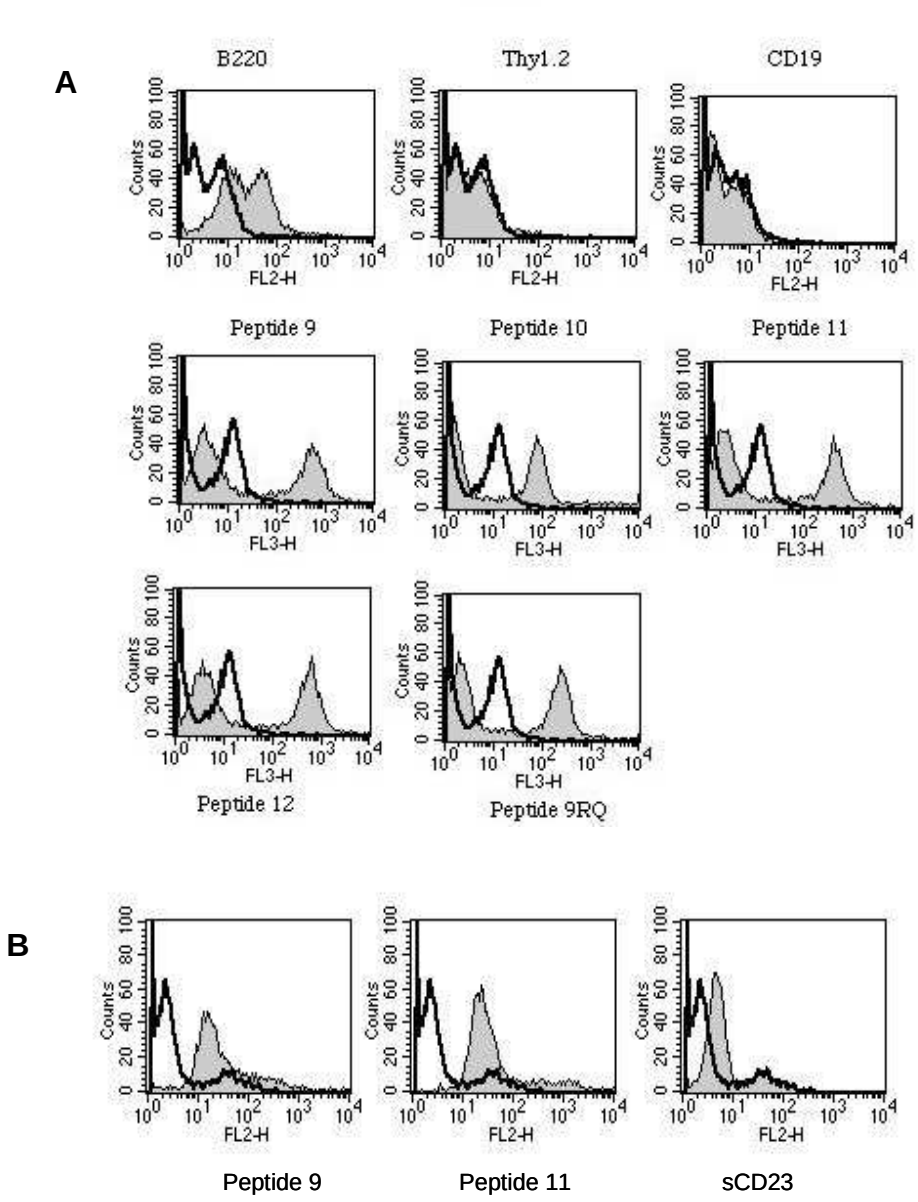

Figure 5.2 Binding of CD23-derived peptides and sCD23 to BAF03 murine pro-B cell line.

(A) Fluorescence intensity histograms for binding of biotinylated peptides #9-#12 to BAF03 cells was detected using SA-QR. The top row in panel A represents antibody staining for BAF03 cells with CD19 and B220 antibodies as B cell specific markers and Thy 1.2 as T- cell marker. Binding of peptides with the RKC motif is shown as grey shaded area and binding of the negative control peptide #58 is shown as black line. **(B)** Binding of sCD23 (1µg/ml) in comparison to binding of peptide #9 and #11 (1µg/ml) to BAF03 cells. Peptide binding was detected using streptavidin-PE. In each case binding of the peptides or sCD23 in grey shaded area is compared with binding to the negative control peptide shown as black line.

5.2.2 Arginine 172 (R172) influences, but is not mandatory for, peptide binding

In order to assess further the importance of the RKC motif on human CD23, the effect upon binding of various substitutions in the RKC motif of peptide #9, #11 or #12 was assessed. As shown by the mean fluorescence index (MFI) for binding, peptides with scrambled sequences (9SCR, 11SCR) and peptides without the arginine in the RKC motif (peptides with 9R-Q, 11R-Q and 11R-K mutations) all bind to BAF03 cells, indicating that the arginine 172 itself is not critical for binding (Figure 5.3A). However, binding of these peptides is significantly reduced when compared to the MFI for binding of wild type peptides #9-#12. Peptides #9 and #12 with replacements (R-A, RK-AA, K-A and C-S) were also assessed for binding to BAF03 cells. The peptides with R-A, RK-AA and K-A mutations bind to the cells but peptides with RK-AA and C-S mutations showed significantly reduced binding (Figure 5.3B).

Peptide 9R-Q, representing the sequence at the αVβ5 binding motif in murine CD23 also binds to the cells, but the binding is lower compared to peptide #9 (Figure 5.3A). Peptide 11R-Q representing the same substitution shows a greater reduction in binding when compared to wild type peptide #11. These peptides have different affinities of binding to the integrin αVβ5 (*A. Edkins, personal communication*) and [232] and therefore the replacements might affect the binding of each peptide in a different manner. Furthermore, the peptide 9C-S substitution almost completely inhibits the binding ability of both peptide #9 or #12 (Figure 5.3B) suggesting that perhaps the peptides work best as disulphide-bonded dimers. Previous SPR analysis have also demonstrated that peptide 9C-S does not bind to the to αVβ5 integrin (*A. Edkins, personal communication*)

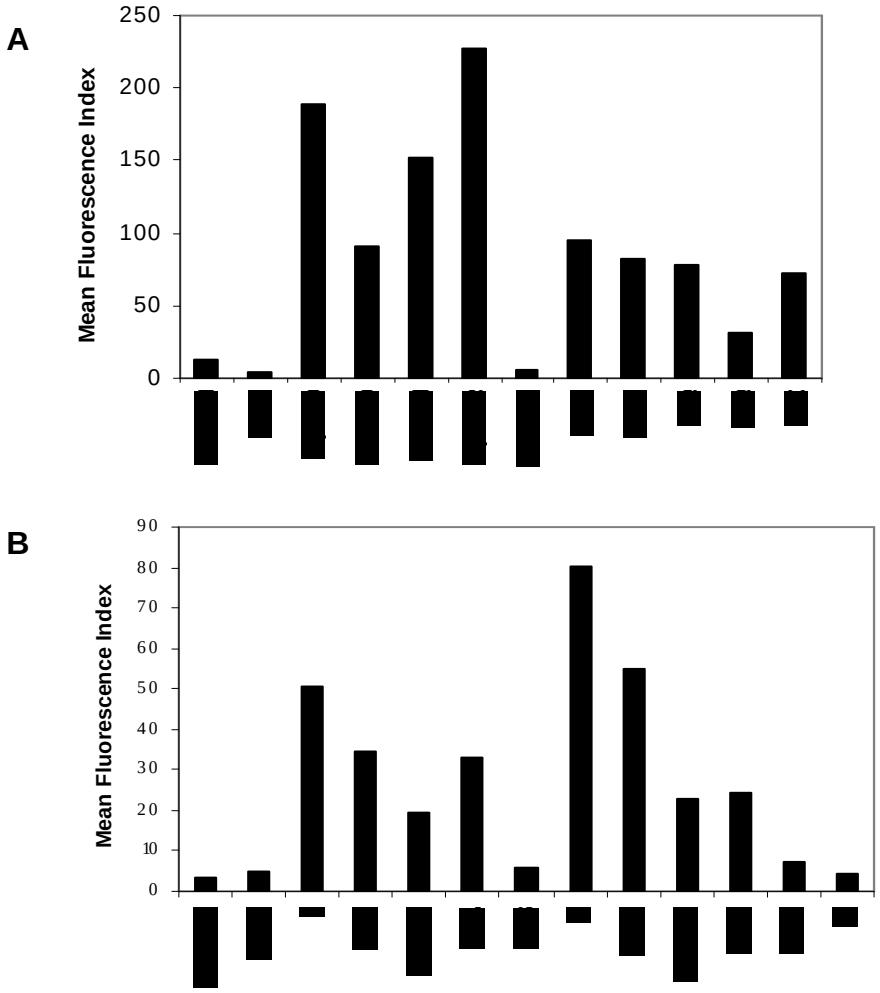

Figure 5.3 Importance of the RKC motif in CD23-derived peptides for binding to BAF03 cells.

Mean fluorescence Index (MFI) data derived from FACS analysis for binding of the peptides #9-#12 to BAF03 cells was compared with the MFI for binding of peptides with different substitutions in the RKC motif. Peptide #58 was used as a negative control peptide to assess binding. **(A)** A comparison of binding of wild type peptides #9-#12 and peptides with the original sequences scrambled as well as peptides with R-Q and R-K substitution. **(B**) Depicts comparison of peptide #9 or #12 wild-type with peptides with indicated substitutions in the RKC motif. Data are representative of 3 independent experiments.

5.2.3 BAF03 cells express the integrin αVβ5 but not αVβ3

Well-characterised antibodies for the murine αVß5 heterodimer are not commercially available, therefore we used a rabbit anti-human ß5 antibody to confirm the expression of this subunit on BAF03 cells. Surface expression of the ß5 subunit was confirmed by cell surface biotinylation of BAF03 membrane proteins followed by western blot using the anti-human ß5 antibody (Figure 5.4B). The murine ß5 subunit is known to be of approximately 95 kDa and western blot with the human anti-ß5 resulted in a product of slightly less than 97 kDa. Since the ß5 subunit cannot reach cell surface membrane without pairing with the αV subunit [311] expression of the ß5 subunit was used as an indication of expression of the αVß5 heterodimer. Expression of the ß3 subunit was determined by FACS analysis using a commercially available anti-mouse ß3 antibody. As shown in figure 5.4A there was no staining for the ß3 antibody when compared to the isotype control antibody in BAF03 cells.

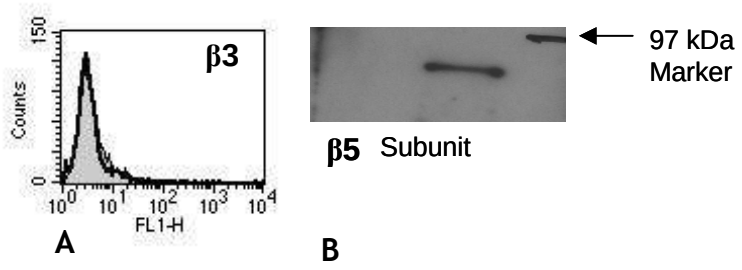

Figure 5.4 Expression of the β3 and β5 integrin subunits in murine pro-B cell line BAF03.

(A) FACS staining for the murine anti-β3 antibody is shown as grey shaded area and the isotype control antibody staining is shown as black line. **(B**) Lysates from surface biotinylated BAF03 cells were incubated with streptavidin-agarose beads and separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with either anti- β5 or β3 antibodies. A band of less than 97 kDa was detected in western blot using anti- β5 antibody and no band was detected with the anti-β3 antibody (data not shown).

5.2.4 BAF03 cells proliferate in response to human sCD23-derived peptides or sCD23

Data from the human cell line work (Chapters 3 & 4) indicated that the αVß5-CD23 interaction sustains proliferation of some of the cell lines expressing the αVß5 integrin. We also assessed proliferation of the murine BAF03 cell line in response to sCD23 or peptide treatment

BAF03 cells are IL-3-dependent for survival [273] and the cells did not survive in culture without IL-3 in the presence of the CD23-derived peptides alone. In the presence of IL-3 alone, the BAF03 cells proliferated effectively in a dose-dependent manner without any addition of serum (Figure 5.5A). However, when the peptides were used with a sub-optimal concentration of IL-3 (1ng/ml), cell proliferation was greatly enhanced in the presence of 10 or 5μg/ml peptides (Figure 5.5A and 5.5 B). Figure 5.5B depicts the proliferative response of BAF03 cells in response to the human sCD23-derived peptides #9, #12 and long peptide (LP). In the human cell line system, peptide #9 and LP induced the best proliferative response, and the proliferation response of the BAF03 cell line is similar to this. The negative control peptide #8 showed no effect in cell proliferation either alone or in combination with sub-optimal IL-3. At 1ng/ml IL-3 there is a very slight proliferative response in comparison to untreated cells and this response is significantly enhanced in the presence of the peptides. Stimulation of BAF03 cells with 1ng/ml IL-3 supplemented with 5μg/ml or 10μg/ml peptide #9 induced a sustained increase in proliferation compared to cells exposed to 1ng/ml IL-3 only (SI of 4 and 2 respectively for peptide #9 induced cell proliferation).

Figure 5.6A illustrates that proliferation of BAF03 cells induced by peptide #9 is reduced when the RKC motif is replaced by QKC in the 9R-Q peptide. This is important because the 9R-Q peptide represents the QKC motif present in murine CD23. The decrease in proliferation is obvious at 10μg/ml but less so at 5μg/ml of peptide. At 5μg/ml peptide #9R-Q also has some significant effect on cell proliferation but nevertheless it is slightly less than peptide #9 ($P<0.05$). Figure 5.3B demonstrated that the C-S substitution in the RKC completely inhibited binding of peptide #9 or #12 to BAF03 cells. This inhibition of binding correlates well with the inability of the peptide 9C-S to induce proliferation compared to peptide 9 as illustrated in figure 5.6B. This indicates that the cysteine residue in the RKC motif influences the binding and activity of these peptides. The peptides would be expected to form intermolecular disulphide bonds and the fact that the substitution of cysteine with serine inhibits the proliferative capapcity of peptide #9 is consistent with the interpretation that the formation of disulphide bonded peptide dimers is necessary for biological activity. The existence of the peptide as disulphide bonded dimers was also tested by Ellman's test for free sulphydryl groups, (A . Edkins, *personal communication*). This notion was further tested by using 2-Mercaptoethanol (2-ME) to reduce the inter-peptide disulphide bonds as BAF03 cells grow in culture with 2-ME and it does not interfere with their growth capacity. Reduction with 12mM 2-ME greatly reduced peptide #9 bioactivity but 6mM 2-ME did not. The data strengthen the idea that the disulphide bonded peptide dimers may be the optimally active form.

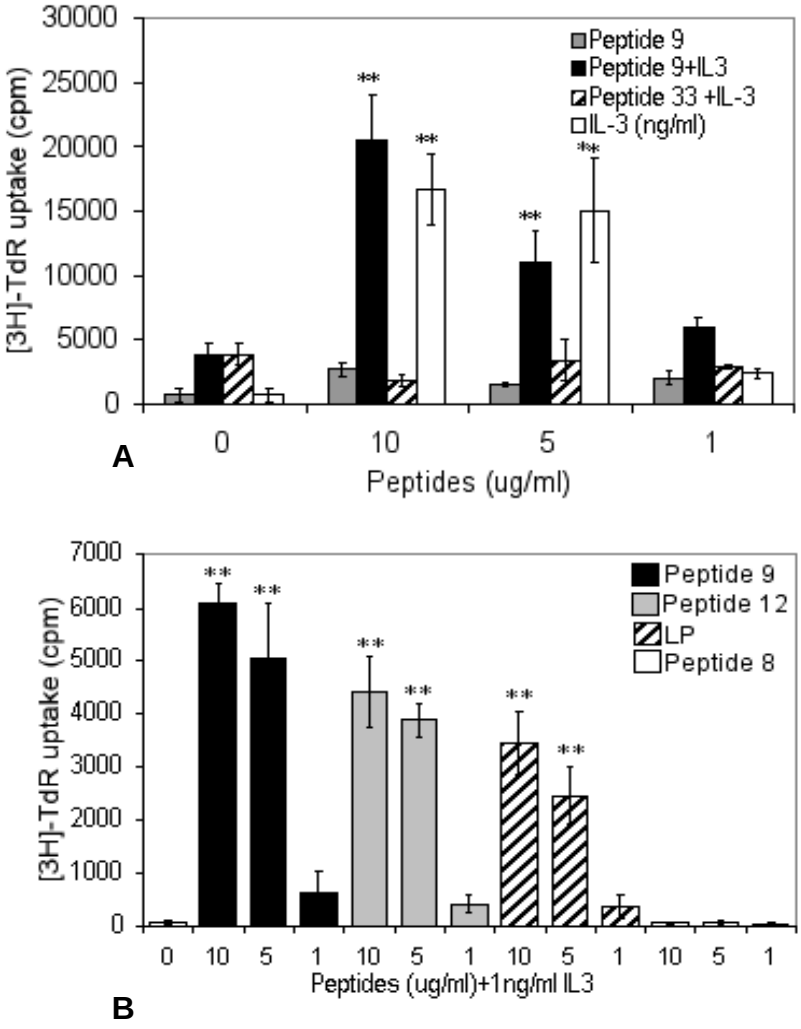

Figure 5.5 Proliferation of BAF03 cells in response to CD23-derived peptides.

(A) Cultures of BAF03 cells (5000 cells/well) were propagated with the indicated concentration of peptides #9 and IL-3 alone or in combination with 1ng/ml IL-3. Proliferation with peptide #9 alone is shown as grey bars, peptide #9 with 1ng/ml IL3 as black bars, peptide #33 with 1ng/ml IL3 as hatched bars and proliferation with different concentrations of IL3 alone is shown as white bars. Proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation **(B)** Proliferation of BAF03 cells in response to indicated concentrations of peptide #9 #12, LP or peptide #8 with sub-optimal concentrations of IL-3 (1ng/ml). 1ng/ml IL3 was used together with the different peptides and proliferation with peptide #9 is shown as black bars, peptide #12 as grey bars, LP as hatched bars and peptide #8 as white bars. Bars represent the standard deviation of triplicate determinations, with **p<0.005 indicating increase in proliferation in comparison to the negative control peptide at the same concentration or in comparison to untreated cells (in case of IL-3 treatment alone). Data are representative of at least three independent experiments.

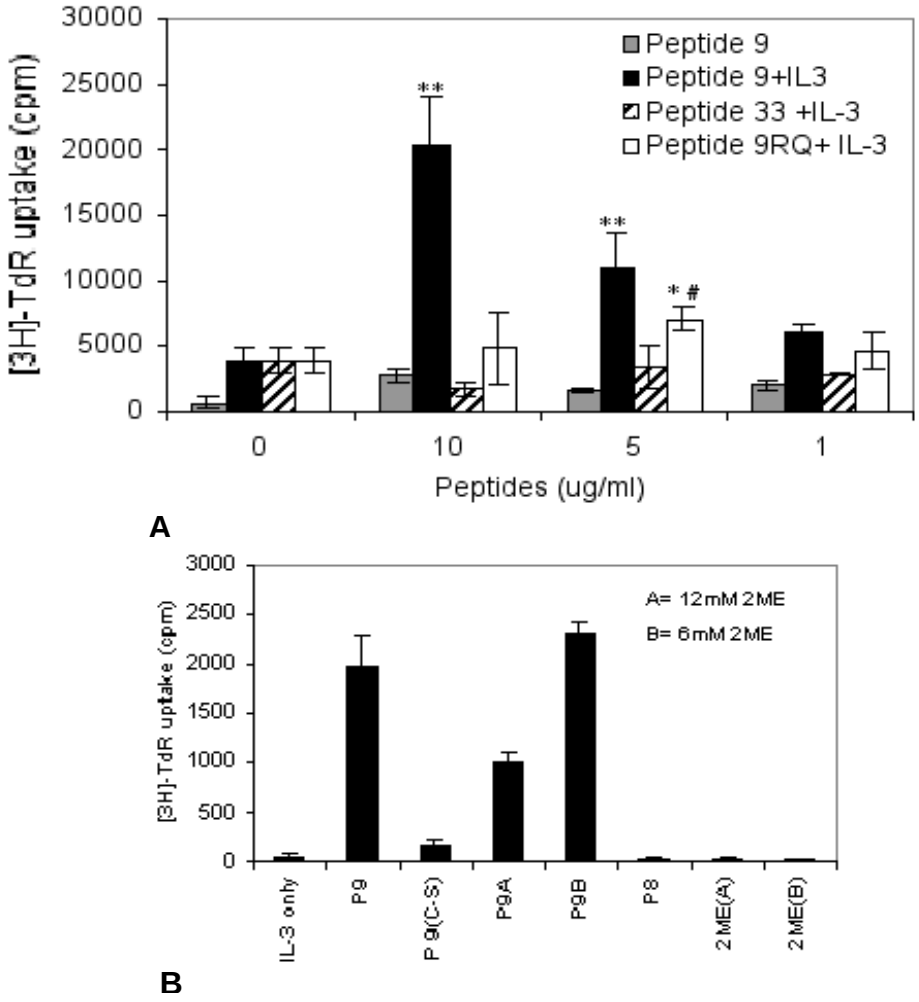

Figure 5.6 Importance of the RKC motif on peptide #9 in mediating proliferation of BAF03 cell line.

Peptide #9 and 9R-Q and 9C-S were used to assess the difference in proliferation resulting from these replacements. **(A)** BAF03 cell proliferation in response to the indicated concentrations of peptide #9, 9R-Q and negative control peptide #33 in presence of 1ng/ml IL-3. Proliferation with peptide #9 alone is shown as grey bars, peptide #9 with 1ng/ml IL3 as black bars, peptide #33 with 1ng/ml IL3 as hatched bars and peptide 9R-Q with 1ng/ml IL3 as white bars **(B)** BAF03 cell proliferation in response to 5µg/ml peptide #9, 9C-S or 9A and B (peptide #9 dissolved in buffer supplement with 12mM (A) or 6mM (B) 2-ME); all in presence of IL-3 1ng/ml. Bars represent the standard deviation of triplicate determinations, with *$p<0.05$ or **$p<0.005$ indicating increase in proliferation in comparison to the negative control peptide at the same concentration and $^{\#}p<0.05$ indicating a significant difference between proliferation induced by peptide #9 and peptide 9R-Q at 5µg/ml. Data are representative of at least 3 independent experiments.

The effect on proliferation of BAF03 cells, mediated by the CD23-derived peptides was verified by assessing proliferation induced by recombinant human sCD23. As shown in Figure 5.7, human sCD23 induces a robust increase in cell proliferation at 5µg/ml. We observed no significant effect on cell proliferation at lower concentrations of 1µg/ml or 0.5µg/ml. The proliferation data clearly indicate that human sCD23-derived peptides containing the RKC motif and sCD23 itself are able to sustain proliferation of murine B cell line BAF03.

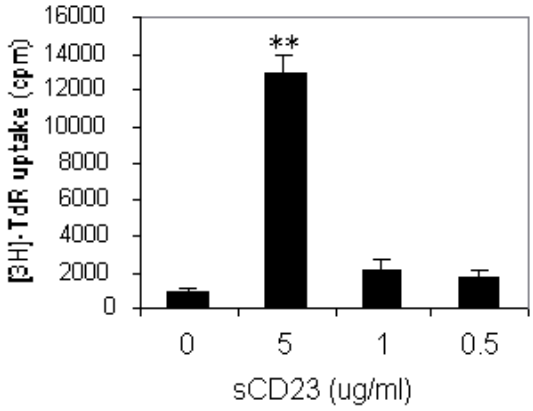

Figure 5.7 Proliferation of BAF03 cells in response to human sCD23.

Cultures of BAF03 cells (5000cells/well) were propagated in PFHM with the indicated concentration of recombinant sCD23 (R&D Systems). Proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. Bars represent the standard deviation of triplicate determinations, with *p<0.05 or **p<0.005 indicating increase in proliferation in comparision to the untreated cells. Data are representative of 3 independent experiments.

Proliferation assays with murine bone marrow B cells isolated from mice bearing a B cell-specific knockout of the αV gene (CD19-cre) were used to investigate the role of sCD23 in proliferation of murine bone marrow B cell precursors. $CD19^+$ B cells from bone marrow of control or αV knockout mice were harvested and grown on OP9 stromal cells with 10% FCS and 1ng/ml IL-7 and used to assess proliferation in the presence of human sCD23. As shown in figure 5.8 there was a significant increase in cell proliferation following sCD23 treatment (5μg/ml) in B cells from control mice but there was no change in proliferation of B cells from αV knockout mice with or without sCD23 treatment. Surprisingly, the untreated B cells from αV knockout mice had a slight increase in proliferation when compared to the untreated B cells from control mice but sCD23 induced no change in their survival.

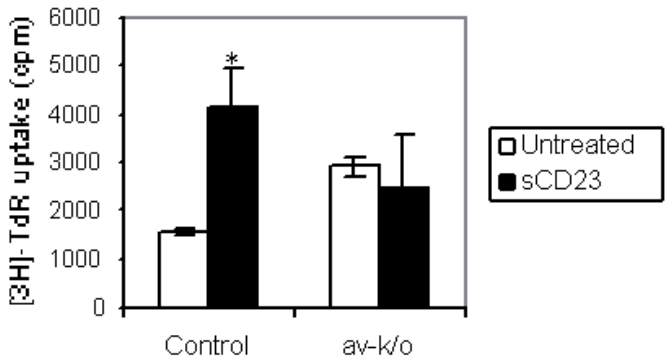

Figure 5.8 Proliferation of control and αV knockout murine bone marrow B cells in presence of human sCD23.

Bone marrow B cells purified from either the control or the knockout mice bone marrow were propagated in the proliferation assay culture on γ-irradiated stromal cell line OP9 and stimulated with 5μg/ml sCD23 or left untreated. Proliferation was assessed after 72 h by ^{3}[H]-TdR incorporation. Bars represent the standard deviation of triplicate determinations, with *p<0.05 or **p<0.005 indicating increase in proliferation in comparision to untreated cells. Data represent one experiment (n=1).

5.2.5 ERK phosphorylation in response to sCD23-derived peptides in BAF03 cells

In the human cell line SMS-SB, sCD23-mediated cell proliferation was shown to be associated with the activation of ERK phosphorylation pathway (Chapter 4). We therefore investigated whether there was a similar role for ERK phosphorylation in BAF03 cells.

Figure 5.9A shows that peptide #9 alone induces a very slight ERK phosphorylation. However, IL-3 alone induces a significant increase in ERK phosphorylation from 5-30 min. When peptide #9 is used together with IL-3 there is a significant increase in ERK phosphorylation from 2-30 min, but this pattern of phosphorylation is not very different from ERK phosphorylation stimulated by IL-3 alone. Although an earlier onset of ERK phosphorylation is noted (at 2 min) with peptide #9 and IL-3, when compared to IL-3 alone, this difference in ERK phosphorylation does not clearly explain the enhancement in cell proliferation seen with peptide #9 and IL-3 together.

In the proliferation assays, an obvious difference in survival of BAF03 cells treated with peptide and IL-3 compared to cells exposed to IL-3 treatment alone, was observed only after approximately 12 h of initiation of the assay. Therefore, we investigated if there was a difference in ERK phosphorylation in a longer time course experiment (24 h). Figure 5.9B illustrates that with IL-3 alone or with IL-3 plus peptide #9 there is a significant increase in ERK phosphorylation from 30-60 min. However, in the cells treated with IL-3 and peptide #9, ERK phosphorylation was observed at 4 h and 12 hr that was absent in cells treated with IL-3 alone. This could be the reason we observe an enhancement in proliferation with addition of IL-3 and peptide #9 in comparison to proliferation with IL-3 alone.

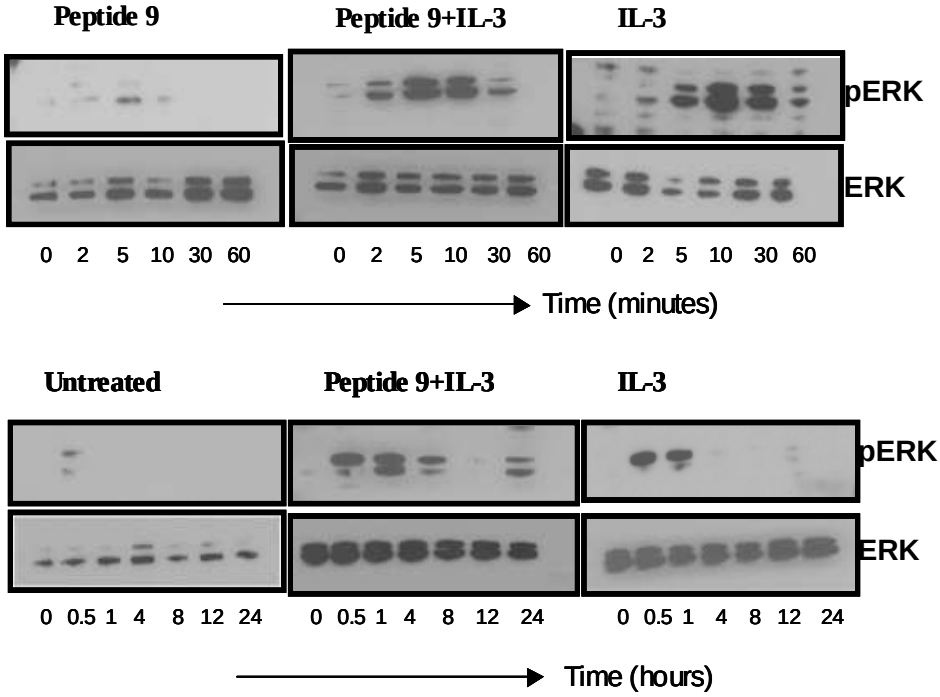

Figure 5.9 ERK phosphorylation in BAF03 cells in response to treatment with CD23-derived peptide #9 or IL-3 alone or in combination.

Lysates from treated BAF03 cells were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with antibodies to phosphorylated ERK and subsequently stripped and reprobed with antibodies to ERK. **(A)** Western blots for phosphorylated and total ERK in BAF03 cells treated with peptide # 9 (5µg/ml) alone, IL-3 (1µg/ml) alone or combination of both, at the time points shown. Figure 5.8 **(B)** illustrates western blots for phosphorylated and total ERK in BAF03 cells unstimulated, stimulated with peptide #9 (5µg/ml) and IL-3 (1ng/ml) or IL-3 (1µg/ml) alone, for a longer time course up to 24 hr. Blots represent three independent experiments.

We also assessed cell proliferation in the presence of the MEK inhibitor U0126 in order to elucidate further the role of the ERK phosphorylation pathway in mediating proliferation and/or survival of BAF03 cells. As shown in figure 5.10, LP induced a significant increase in cell proliferation in comparison to untreated cells (cultured with 1ng/ml IL-3 alone) or cells exposed peptide #8 treatment. This proliferative effect was abrogated by treatment with the MEK inhibitor U0126. DMSO was used as a vehicle control as it was the solvent for the inhibitors and was without any effect. Although the MEK inhibitor abolished proliferation we also observed total inhibition of proliferation of BAF03 cells with the PI3K inhibitor LY294002. Therefore, the enhancement in cell proliferation observed via IL-3 and the CD23-derived peptides or CD23 could also be potentially mediated via the PI3K signalling pathway.

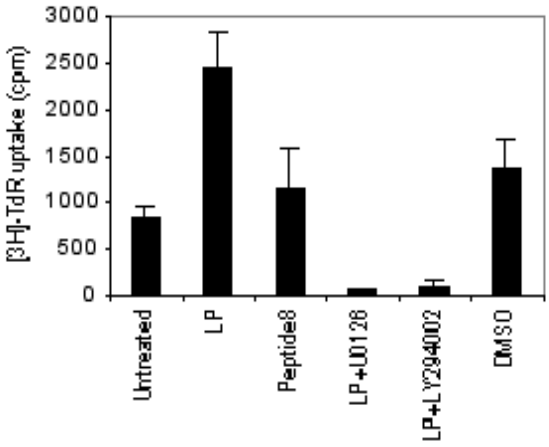

Figure 5.10 10 Effect of inhibition of ERK and PI-3K signalling on LP-induced proliferation of BAF03 cells.

BAF03 cells were treated with (10µM) U0126, 50µM LY294002, equivalent volume of DMSO or left untreated for 30 min prior to plating at 5000 cells/well; proliferation was assessed by ^{3}[H]-TdR incorporation after 72 h of culture. Proliferation induced by the long peptide in presence of 1ng/ml IL-3 was compared with the cells treated with the two different inhibitors or vehicle control DMSO. Peptide #8 was used as a negative control peptide.

5.2.6 Discussion

Human sCD23 displays pleiotropic cytokine-like activities and in pre-B cell lines the sCD23-αVß5 interaction was shown to sustain proliferation (Chapter 3). However, the cytokine-like activities of sCD23 *in vitro* have not been shown in murine cells. Murine CD23 is a 49 kDa sialoglycoprotein and shares only 57% amino acid homology with the human CD23 molecule. $CD23^{-/-}$ mice display normal lymphocyte development, normal B cell proliferation and germinal centre formation but have impaired IgE-dependent functions [312].

Murine CD23 lacks the inverse RGD motif present on human sCD23, as a natural truncation and there are other known structural differences between human and murine CD23; these differences could provide more understanding into why human CD23 and not murine CD23, possesses cytokine-like activity. In relation to human sCD23-αVß5 interaction it was interesting to observe that the RKC motif bound by αVß5 is present as QKC on murine CD23. We investigated whether murine B cell precursors could bind and respond to human CD23-derived peptides and how important arginine 172 on the RKC motif of the peptides was in regulating their activity.

We first compared the binding of various CD23-derived peptides and sCD23 to murine B cells and then assessed proliferation mediated by the peptides and sCD23 itself. Binding of the CD23-derived peptides and sCD23 to αVß5 has already been studied in human cell lines and also the interactions of the peptides and sCD23 to αVß5 has been extensively characterised using SPR analysis (*A. Edkins, personal communication*) and [232]. Therefore, we used the different peptides available to assess their binding to murine B cells.

CD23-derived peptides #9-#12 containing the RKC motif bound to murine bone marrow B cells, splenic B cells and to BAF03 cells, a murine pro-B cell line. In all the cell populations, peptides with the RKC motif showed strong binding and peptides with substitutions in the RKC motif showed reduced binding. As the substitutions (R-Q and R-K) and scrambled sequences did not abolish binding, peptides with further replacements in the RKC sequence were also assessed for binding. In figure 5.3B peptides with 9RK-AA mutation show greatly reduced binding compared to peptides with 9K-A mutations. In comparison to binding of peptide #9, binding of peptide #9R-K is reduced by about 20-30% while binding of peptide 9RK-AA is further reduced by about 50%. Peptides with K-A substitution retain slightly more basic characteristic compared to peptides with RK-AA substitutions where both the basic amino acids (arginine and lysine) are converted to alanine. This indicates that the integrins require the basic domains on the peptides for binding. This could also explain the binding seen in peptides with R-Q and R-K mutations as these peptides still retain some basic residues and peptides with the scrambled RKC sequences also retain the basic characteristics of the RKC motif. Similar results have also previously shown the importance of the basic residues for binding in human B cell line [232].

CD23-derived peptides #9, #12 and the LP all induced a strong proliferative response in the BAF03 cells when used in combination with sub-optimal concentrations of IL-3. The data indicate that the peptides synergise with IL-3 to induce a proliferative response. A strong effect in cell proliferation was also confirmed with the commercially-available recombinant human sCD23. These cells express the αVβ5 integrin as shown in figure 5.4B and the proliferation results indicate that the αVβ5-CD23 interaction is important for proliferation of murine B cells. Peptide #9R-Q, representing the sequence in murine sCD23 showed reduced binding to the cells in comparison to peptide #9 and in functional experiments proliferation induced by peptide #9 is significantly reduced by substitution of the glutamine for arginine in the RKC motif; although the proliferative effect is not completely inhibited with peptide 9R-Q. This implies that the arginine on the RKC motif is important for the binding and inducing cell proliferation but not critical since replacement of the arginine still resulted in some binding and a modest effect on cell proliferation. Furthermore

the lack of ability to induce proliferation by peptide 9C-S and peptide #9 treated with 2-ME indicated dimers of these peptides may be necessary for their activity.

Interestingly, a higher concentration of human sCD23 was required to produce a response in the BAF03 cells in comparison to human cells (5μg/ml vs. 250ng/ml). The binding affinity of human sCD23 to the murine integrin could be much lower than the equivalent affinity for human integrin and therefore a higher concentration of ligand might be required. These results establish the importance of human sCD23 and the CD23-derived peptides with the RKC motif in mediating cell proliferation; however we did not have access to any murine sCD23 protein and proliferation data with murine sCD23 would be important to verify the unique role for human sCD23.

In order to confirm that the CD23-mediated proliferation was via αVβ5 integrin we performed preliminary experiments with B cell precursors from the B cell-specific αV conditional knockout mice. The data showed that sCD23-mediated proliferation was not seen in B cell precursors from bone marrow of αV knockout mice, but B cell precursors from the control mice showed an enhancement in cell proliferation after sCD23 treatment. These experiments will have to be repeated to confirm the effect of the αVβ5-sCD23 interaction. Due to the lack of an effective RNAi system in human pre-B cell lines, the B cells from the αV knockout mice provide an important model to confirm αVβ5-CD23 interactions.

The ERK phosphorylation data indicated that perhaps ERK phosphorylation at later time points (4 h and 12 h) could be responsible for the enhancement in proliferation noted with peptide #9 and IL3 together. However, inhibition of peptide #9 mediated cell proliferation by both U0126 and LY294002 indicated that both the ERK and the PI3K/AKT pathway could be responsible for enhancement of cell proliferation. Therefore, it will also be important to investigate AKT phosphorylation in the same way as ERK phosphorylation.

CD23 is reported to interact with the β_2 integrins on murine monocytes[204] and $\alpha v\beta 3$ expression in murine B-cell precursors has been reported [313] but there are no data to suggest that murine CD23 interacts with the αv family of integrins. Previous studies have shown that CD23 or its soluble fragments have potential regulatory effects on hematopoiesis. It was shown to promote differentiation of T cell precursors in synergy with IL-1α [207] and also to induce proliferation of myeloid stem cells and down-regulate proliferation of myeloid leukemic cells [210]. It is important to understand the differences in the role of human and murine sCD23 not only because sCD23-αVβ5 interactions can have profound implications in the growth of human B cells but also because human sCD23 and the sCD23-derived peptides can be used in a mouse model system, to study the effects of $\alpha v\beta 5$ on B-cell precursors.

6 Discussion

The data presented in this thesis demonstrate that the αVβ5 integrin is expressed on pre-B cell lines and B cell precursors in the bone marrow and that the αVβ5-CD23 interaction sustains proliferation of some pre-B cell lines. Previous work in the laboratory demonstrated that αVβ5 is a receptor for sCD23 and that this interaction sustains proliferation of the pre-B cell line SMS-SB. The main aim of this project was to identify exactly at what stage of B cell development αVβ5 is expressed and is functional.

Results in Chapter 3 demonstrated that αVβ5 is expressed on and sustains proliferation of early pre-B cell lines. Transitional and more mature B cell lines down-regulated αVβ5 expression and did not proliferate in response to αVβ5 ligation. Data in Chapter 4 indicated that this proliferative function of αVβ5 was modulated by the chemokine SDF-1 and PDGF, both important molecules in early B cell development. This regulation of αVβ5-mediated proliferation was shown to be via modulation of the signalling pathways activated downstream of integrin and the growth factor or chemokine receptors. SDF-1 and PDGF treatment both enhanced ERK phosphorylation induced via αVβ5 ligation. The role of αVβ5-CD23 interaction in mediating proliferation of B cell precursors was further confirmed in a murine pro-B cell line BAF03 as shown by the results in Chapter 5.

6.1 αVβ5 Expression

Different pre-B cell lines were characterised into distinct stages of B cell development based on cell surface marker expression, and αVβ5 expression was assessed at each stage of development (Chapter 3). All pre-B cell lines showed significant levels of αVβ5 expression and transitional B cell lines such as Raji, Daudi and Ramos showed low levels or no αVβ5 expression (Figure 3.3 and 3.4). Mature B cell lines like SKW and IB4 also showed low or no αVβ5 expression (Figure 3.4). These data indicate that αVβ5 expression is down-regulated as the pre-B cells differentiate into transitional B cells and more mature B cells. This was supported by the finding that αVβ5 expression decreases in the immature B cell line 1E8 in comparison to the pre-B cell line Blin1, which was the original

cell line from which 1E8 was derived (Figure 3.3). The expression of integrin αVβ3, which is closely related to αVβ5 and is also known to bind sCD23, was more difficult to define. Although it was obvious that the levels of this integrin were higher in the mature B cell lines SKW and IB4, in comparison to other cell lines, most of the cell lines seemed to express low levels of this integrin.

In order to correlate the data from the cell lines to bone marrow B cell precursors, αVβ5/αVβ3 expression was analysed in bone marrow B cell precursors, using a similar panel of cell surface markers used to characterise the pre-B cell lines. The data indicated that αVβ5 was expressed in a percentage of pre-B cells in the bone marrow ($CD19^+/CD9^+$ and $CD19^+/CD10^+$) (Figure 3.15). Furthermore the data also indicated that αVβ3 integrin was expressed in similar pattern to αVβ5 (Figure 3.14, 3.17). Although αVβ5 expression was higher than αVβ3 expression, the general pattern of expression was similar with relation to most of the markers. A clear down-regulation of αVβ5 expression could not be detected in the bone marrow B cell precursor population as αVβ5 expression was noted in $CD19^+/\kappa^+$ cells (Figure 3.18), which are more mature than the pre-B cells owing to the expression of the kappa light chains. Therefore we could not conclude that αVβ5 expression is exclusive to pre-B cells. We could not stain any $CD34^+$ pro-B cells or any mature B cells from the blood, and so a more extensive staining of B cell precursors from the bone marrow and B cell populations from peripheral blood is required to confirm αVβ5 expression and down-regulation of this expression in mature B cells.

6.2 αVβ5 Function

The sCD23-αVβ5 interaction was previously shown to sustain proliferation of the SMS-SB cell line. Therefore, the proliferative response to sCD23 treatment was used to assess αVβ5 function in the different cell lines (Chapter 3). The pre-B cell lines SMS-SB, RS4;11 and 697 proliferated robustly in response to sCD23 and sCD23-derived LP treatment (Figure 3.6, 3.7 and 3.9), and this response was mimicked by the 15F11 MAb which binds to αVβ5 integrin (Figure 3.11), indicating that the sCD23-dependent proliferation is mediated via this integrin. The MAb P1F6 which is also known to bind αVβ5 did not induce a proliferative

response and the MAb AMF7 which binds the αV subunit induced proliferation in SMS-SB cells but failed to induce a proliferative response in the RS4;11 cell line. A slight proliferative response to sCD23 treatment was observed in the pre-B cell line Blin1 and in the cell lines Daudi and SKW (Figure 3.7 and 3.8). However, all of these responses were much lower in comparison to the response of SMS-SB, RS4;11 and 697 cells.

The SMS-SB and RS4;11 cell lines had very low levels or lacked surface μ expression (Figure 3.1), suggesting that perhaps very early B cell precursors (either pro-B cells or pre-BI) respond well to αVβ5 stimulation. The $CD10^-$ status of both these cell lines (Figure 3.1) also indicated that the αVβ5-mediated growth response was restricted to cells at a specific stage of development. However, B cell precursors in the normal bone marrow, including the pro-B cells are $CD10^+$ and the cell line 697 which was $CD10^+$ also responded well to αVβ5 stimulation, indicating that it is not only the $CD10^-$ cells that respond to αVβ5 stimulation. However, a common feature of these three cell lines was that they all stained well with the 15F11 antibody that recognises the αVβ5 heterodimer (Figure 3.3). While the αVβ5 MAb P1F6 indicated similar levels of staining in all the pre-B cell lines tested, 15F11 staining for αVβ5 was higher in SMS-SB, 697 and RS4;11 and lower in all other pre-B cell lines (Figure 3.3).

Another common characteristic of these three responding cell lines was that they all expressed very low levels or none of the integrin αVβ3 in comparison to the other pre-B cell lines (Figure 3.3). These data indicate that αVβ5-mediated proliferation is important in early B cell precursors at a specific stage in B cell development. However, a confirmation of this proliferative response from bone marrow B cell precursors would be needed to define the exact window of B cell development where αVβ5-mediated proliferation is important. The proliferation data presented for bone marrow B cell precursors was on $CD19^+/\kappa^-$ B cells (Figure 3.20). These data indicated that B cell precursors in the bone marrow that have not begun to express the κ light chain but express αVβ5 integrin, can proliferate in response to sCD23 stimulation but the κ-positive B cell precursors do not. This is in agreement with the hypothesis that αVβ5-mediated proliferation is important for early B cell precursors and not for the more mature ones. A more

detailed analysis of the proliferation of bone marrow B cell precursors in the pro-B and pre-B stage would be required to make specific conclusions relating to the stage of B cell development where αVβ5-mediated proliferation could have important implications. Together with this, a study of proliferation of B cells from patients with ALL would also be needed to establish the importance of αVβ5-mediated cell proliferation in neoplastic cells. Previous results from the lab demonstrated that αVβ5 is highly expressed in B cells from patients with ALL. As ALL is malignancy of early B cell precursors in the bone marrow, αVβ5 could be delivering an important proliferative signal to these cells depending on their maturation stage.

The cell line data clearly indicate that some pre-B cells proliferate in response to αVβ5 stimulation and this could have important implications for growth and proliferation not only of bone marrow B cell precursors expressing αVβ5 but more importantly, of leukemic B cell precursors. Moreover, the cell line RS4;11 is a well-known MLL-rearranged leukemic cell line and the SMS-SB cells have a similar phenotype to RS4;11. The fact that both of these cell lines show a significant proliferative response to αVβ5 indicates that this stage-specific proliferative response to αVβ5 could be very important in survival of some specific types of leukemic cells. Human ALL frequently represents clonal expansion of a $CD19^+$ B-lineage cells at one of several stages of B-cell development and the t(4;11)(q21;q23) cytogenetic abnormality, characterized by expression of the MLL/AF4 fusion protein is a frequent subtype of infant acute leukaemia and some adult leukaemia. Leukemic blasts expressing MLL/AF4 are arrested at an early progenitor stage with lymphoid or monocytoid characteristics. MLL-rearranged B cell precursor ALL represents a more aggressive leukaemia, particularly in infants, and is associated with a poor prognosis [272,314]. Therefore, the fact that αVβ5 stimulates proliferation of these cell types indicates that this integrin may have a very important therapeutic potential in the treatment of these leukaemias.

The proliferative function mediated via αVβ5 was confirmed in the mouse cell line BAF03 which expressed the integrin αVβ5 but not αVβ3 (Chapter 5). BAF03 cells which represent a murine pro-B stage cell line are dependent on IL-3 for

survival [273] and offer a good model to test the proliferative function of αVß5. Due to the lack of murine αVß5 antibody, αVß5 expression was confirmed via surface biotinylation and αVß3 expression was confirmed by flow cytometry for the expression of the ß3 subunit (Figure 5.4). In the presence of sub-optimal IL-3 concentrations, BAF03 cells showed significant proliferation in response to human sCD23 treatment (Figure 5.7) or stimulation with sCD23-derived peptides (Figure 5.5), thus indicating that the proliferative function of human sCD23 can be detected in murine B cell studies. In support of this, murine bone marrow B cell precursors also proliferated well in response to sCD23 treatment and murine B cells from αV knockout mice did not show similar enhancement in proliferation with sCD23 treatment when compared to the B cells from the bone marrow of wild-type mice (Figure 5.8). These results indicate the importance of the αV integrin and sCD23 in mediating this proliferative response.

6.3 Modulation of αVβ5-mediated proliferative effects by molecules important in early B cell development.

A variety of soluble factors as well as contact interactions between the precursor cells and the stromal microenvironment dictate the fate of B cell progenitors. A number of growth factors, chemokines, cytokines and adhesion molecules have been described that regulate the survival, differentiation and migration of B cell progenitors. In order to assess the importance of the αVß5-mediated proliferation during B cell development, the role of cytokines, chemokines and growth factors in modulating αVß5 function was investigated.

SDF-1, a chemokine with established roles during B lymphopoiesis, enhanced the proliferative effect mediated via αVß5 (Figure 4.1). Furthermore, PDGF had a similar effect to SDF-1 in enhancing αVß5-mediated cell proliferation in SMS-SB cells (Figure 4.7). As both PDGF and SDF-1 have been implicated in early B cell development these data further support a role for αVß5-mediated cell proliferation in early B cell developmental stages. However, surprisingly, the cytokine most implicated in early B-cell proliferation, IL-7, did not show a similar effect to SDF-1 (Figure 4.5). Although the requirement for IL-7 in human B lymphopoiesis is not as absolute as in murine B lymphopoiesis, many studies

have shown that IL-7 induces proliferation of early human B cell precursors [1,12]. It could be that the requirement for IL-7 and requirement for αVß5 are at slightly different stages of development and they do not overlap, or it could also be that concentrations of IL-7 used in the experiments here were different from the concentrations of IL-7 required for enhancing the proliferative effect. The cytokines were all used in the same concentration range as SDF-1 and it is possible that these cytokines produce a response at different concentration ranges. Similarly, IL-3 and IL-4 have both been implicated in having different roles in B cell development but neither affected the function of αVß5 in the experimental context used (Figure 4.5). Expression data indicate that perhaps IL-4 could modulate αVß5 expression, which might have important implications, and none of the other treatments used affected αVß5 expression (Figure 4.6).

A number of other molecules, such as Flt3 ligand [90], are known to be important in early B cell development and are also known to influence integrin function and therefore could be regulating αVß5 function. Similarly, the role of αVß5 integrins cannot be studied in isolation from other integrins. Not only could other αV integrins be modulating αVß5 function but also other integrins such as the ß1 and ß2 families have been implicated in hematopoiesis in an adhesion-dependent manner. Therefore, these integrins could be having an important role in modulating αVß5 function in an adhesion-dependent or independent manner. So, it would also be important to assess whether ligation of the ß1 and ß2 integrins influences αVß5-mediated cell proliferation. Moreover, the role of the αVß5 integrin function also cannot be assessed without considering the matrix proteins and in particular, vitronectin since αVß5 is known to bind primarily to vitronectin.

Previous work regarding the αVß5-CD23 interaction indicated that αVß5 binds to the RKC motif on sCD23 and that RGD peptides do not influence the αVß5-mediated proliferation of SMS-SB cells. Furthermore, the αVß5 MAb P1F6 that blocks binding to vitronectin did not elicit a proliferative response and did not influence sCD23-mediated proliferation either (*data not shown*). Vitronectin coated on to 96-well plates and soluble vitronectin were both used to assess their influence on αVß5-mediated cell proliferation; however, there were no

conclusive data to suggest either increase or decrease in αVβ5-mediated cell proliferation in the presence of vitronectin, and vitronectin itself did not influence the proliferation of SMS-SB cell (*data not shown*) [232]. Fibronectin alone also did not influence SMS-SB cell proliferation or αVβ5-mediated cell proliferation *(data not shown*).

A key molecule guiding B cell development is the pre-BCR on precursor B cells and BCR on mature B cells. Pre-BCR signalling has not only been implicated in B cell differentiation but cross-linking the pre-BCR in the presence of other soluble factors such as IL-7 is known to induce B cell proliferation [315,316]. Therefore, it will be important to determine whether pre-BCR ligation influences αVβ5-mediated cell proliferation.

Proliferation and differentiation are interlinked and interdependent events in many ways. Therefore, it was hypothesised that αVβ5 expression was linked to B cell differentiation. This seemed particularly plausible as αVβ5 function seemed to be important in early B cell precursors. The influence of pre-BCR on αVβ5 expression was assessed in the pre-B cell lines SMS-SB and Blin1. However, pre-BCR cross-linking by anti-μ MAb did not influence αVβ5 expression and αVβ5 stimulation did not influence surface μ expression *(data not shown)*. A signalling complex, termed pro-BCR, competent in transducing signals has been recently reported and it was demonstrated that cross-linking of pro-BCR on pro-B cells isolated from bone marrow induced phosphorylation of various signalling molecules including Syk, PI3K and ERK and that the pro-B cells could be induced to differentiate to the pre-B stage of development [15]. Since αVβ5 seems to regulate proliferation of early B cell precursors it is possible that it regulates differentiation of the pro-B cells and therefore it would be interesting to investigate if ligation of the pro-BCR and the subsequent differentiation induced, influences αVβ5 function or expression.

Some preliminary results indicated that expression of κ light chains on Blin1 cells resulted in down-regulation of αVβ5 expression (*data not shown*) but cultures of Blin1 cells expressing κ light chains could not be established on a long-term basis to assess this down-regulation conclusively. Evidence for the role of αV integrins in differentiation is provided by studies showing a switch between the αV-

associated ß integrin subunit during development in different cell systems. It has previously been shown that oligodendrocyte progenitors sequentially express αVß1, αVß3 and αVß5 integrins during differentiation *in vitro* with up-regulation of αVß5 and down-regulation of αVß3 [317]. Furthermore it was also demonstrated that, in these cell types, αVß3 may regulate cell proliferation and that both down-regulation of αVß3 expression and subsequent signalling through αVß5 may be critical for continued differentiation *in vitro* [318]. A similar developmental switch, with up-regulation of αVß3 and down-regulation of αVß5 expression, has also been established during osteoclast differentiation [319]. Therefore, it would be very interesting to characterise the role of both αVß5 and αVß3 and the other αV integrins during B cell development with regard to proliferation and differentiation. This information would be valuable in understanding B cell malignancies that can arise at specific stages of development due to disruption in proliferation and differentiation mechanisms.

6.4 Signalling via the αVβ5 integrin

Although integrins and growth factor receptors can independently propagate intracellular signals, many of the signalling pathways and effectors which are activated by integrin ligation are also activated by growth factor stimulation. There are two major families of growth factor receptors that signal via distinct pathways and cooperate to regulate cell growth: the G-protein-coupled receptors (GPCRs) and the receptor tyrosine kinases (RTKs). The GPCRs signal by inducing dissociation of heterotrimeric G-proteins into Gα and Gßγ subunits, each of which initiate a specific response [320,321], while RTKs are activated following binding of polypeptide growth factors which induces RTK dimerization [239,322]. A number of studies, particularly in the adherent cell system, have demonstrated that the function of growth factors can be positively modulated by integrin-mediated cell adhesion. Both integrins and growth factors are known to activate Rho family GTPases, the Ras-Raf-MAPK pathway, PI3K, ribosomal S6 (RSK) and Jun amino-terminal kinase (JNK) [323,324]. Synergy between growth factors and cell adhesion has been observed in the activation of the MAPK pathway [325,326] and PI3K/AKT pathway [327]. Coimmunoprecipitation of growth factor receptors with integrins has also been shown in various studies; for

example, αVβ3 has been found to associate with PDGFR or VEGFR (vascular endothelial growth factor receptor) [328-331]. Studies on cross-talk between the integrins and growth factor signalling has also given important insights into specific functions of the integrins. For example, cell adhesion and migration studies have identified a critical role for cross-talk between growth factor receptors with the integrin αVβ5 but not with αVβ3 during adhesion and migration on vitronectin [332]. Similarly, it was demonstrated in both chick and mouse models that αVβ5-bearing melanoma cells depend on *ex-vivo* pre-stimulation with IGF (insulin-like growth factor) for metastasis, whereas αVβ3 bearing melanoma cells metastasize in the absence of growth factor treatment [333]. In the haematopoietic cell system, a number of studies have shown that SDF-1 modulates signals for adhesion and migration via the β1 integrins VLA-4 and VLA-5 and the β2 integrin LFA-1 [295]. Signalling data from the studies investigating modulation of αVβ5-mediated proliferation by SDF-1 and PDGF (Chapter 4) indicated a role for growth factor-integrin cross talk in an adhesion-independent manner leading to enhanced proliferation of SMS-SB cells.

Stimulation of αVβ5 integrin using sCD23, LP or 15F11 MAb induced significant ERK phosphorylation in SMS-SB cells showing that the αVβ5-mediated proliferative response was probably regulated by the ERK, MAPK pathway (Figure 4.8). A transient ERK phosphorylation induced by SDF-1 treatment in these cells was markedly enhanced when SDF-1 was added together with sCD23, LP or 15F11 MAb. These results indicated that the synergy between SDF-1 signalling and αVβ5 signalling could be via the ERK signalling pathway. Furthermore, the synergy between PDGF and sCD23 in mediating proliferation and ERK phosphorylation in a manner similar to SDF-1 indicated that αVβ5-mediated signalling mechanisms synergise with both GPCR-mediated and RTK-mediated signalling to produce proliferative responses. Further support for synergy of αVβ5-mediated signalling with growth factor signalling came from the mouse cell line data. The IL-3-dependent cell line BAF03 proliferates in a dose-dependent manner in response to IL-3 treatment. When sCD23 or CD23-derived peptides were added together with a sub-optimal concentration of IL-3 there was a robust enhancement in cell proliferation indicating a synergy between IL-3-mediated and αVβ5-mediated proliferative responses.

However, in the BAF03 cells it was not clear whether this proliferation was mediated via the ERK signalling pathway. Although there was an enhancement in ERK signalling with IL-3 and sCD23-derived peptides on a longer time course experiment there was no difference in ERK phosphorylation induced by CD23-derived peptide alone or in combination with IL-3 on a shorter time course (Figure 5.9). These data indicated that there could also be other signalling pathways involved in regulating the synergistic effect on cell proliferation. The PI3K/AKT-mediated pathway could potentially be involved in mediating this synergy and it needs to be investigated. In the SMS-SB cell line, the PI3K inhibitor LY294002 inhibited the proliferation induced by stimulation of αVβ5 alone or in combination with SDF-1 (Figure 4.10). Although this inhibition was not as absolute as the inhibition seen with the MEK inhibitor, these results indicate that PI3K/AKT pathway could also be mediating this enhancement of αVβ5-mediated signalling by growth factor receptors.

With regards to signalling mechanisms, it would be very useful to characterise the signalling pathways or patterns induced by αVβ5 ligation versus αVβ3 ligation. Particularly in some transitional cell lines where a proliferative response was seen with 23C6 (anti-αVβ3 MAb) ligation, it would be interesting to investigate whether there is a similar ERK phosphorylation pattern to that seen with αVβ5-mediated proliferation. A characteristic biphasic pattern of ERK phosphorylation was seen in SMS-SB cells with ligation of αVβ5 and this pattern of phosphorylation, when compared with ERK phosphorylation due to αVβ3 ligation, could give important information regarding why αVβ5 but not the αVβ3 integrin induces proliferation in some pre-B cell lines.

Similarly, a comparison of ERK phosphorylation due to αVβ5 ligation in the different pre-B cell lines would further establish why a proliferative response is observed in some cell lines and not in others. αVβ5 and αVβ3 integrins may also regulate different signalling pathways leading to different outcomes in cell proliferation, therefore comparison of other signalling pathways, particularly PI3K/AKT pathway, could provide important information regarding the distinct function of these two integrins in B cells. Furthermore, signalling mechanisms could also give more information on the role of vitronectin in influencing αVβ5

function. A comparison of ERK phosphorylation induced by soluble vitronectin, vitronectin coated on plates and sCD23 could give important information in distinguishing the role of stimulation of αVβ5 by sCD23 and vitronectin.

These data support a model in which signals from αV integrins together with signals from other soluble molecules regulate important proliferation and differentiation decisions during B cell development and can impact on B cell disorders that arise due to disruption of normal proliferation and differentiation pathways (Figure 6.1). As indicated by the proliferation data, the model in Figure 6.1 suggests that the αVβ5-CD23 interaction probably regulates proliferation of B cell precursors at early pre-B stage. The exact stage, whether it is at the pro-B stage or just after this stage still needs to be verified. The expression of αVβ5 integrin in ALL cells and the role of the αVβ5-CD23 interaction in regulating pre-B cell proliferation indicates that the αVβ5-CD23 interaction might be important in the growth of ALL B cells. Although there are no data indicating a role for αVβ5 in B cell differentiation, the fact that αVβ5 integrin regulates proliferation and is expressed in stage-specific manner indicates that αVβ5 could be directly or indirectly linked with B cell differentiation. Coordinated signals from the different αV integrins probably regulate the B cell proliferation/differentiation balance. The role of αVβ5 in proliferation and differentiation could also implicate this integrin in the mature B cell malignancy CLL. As the $CD5^+$ B cell population is expanded in B-CLL, and since the data suggested the presence of αVβ5 and αVβ3 integrins on $CD5^+$ B cells from bone marrow, there could potentially be a link between αV integrin expression and CLL (Figure 6.1).

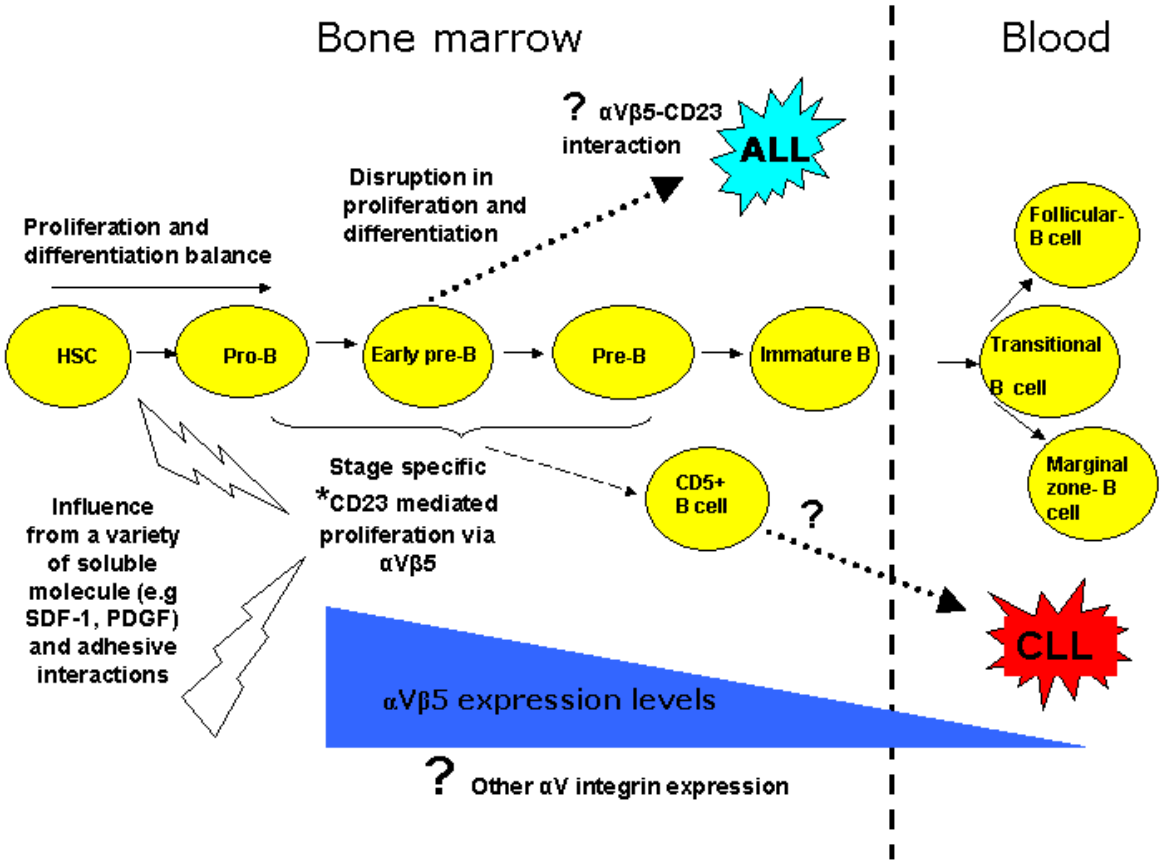

Figure 6.1 Model for the role of αVβ5 integrin during B cell development and implications for B cell leukaemias.

This model represents the potential role of αVβ5 integrin in B cell proliferation and differentiation based on the data available. Different stages in B cell development in the bone marrow and blood are depicted in yellow. Changes in αVβ5 integrin expression levels are presented in blue. B cell development is regulated by a balance between cell proliferation and differentiation which determines the number and maturation stages of the cells in each compartment. ALL is a B cell malignancy that arises during early B cell stages in the bone marrow due to disruption in this balance and results in clonal proliferation of cells arrested at a certain stage. While CLL is a B cell malignancy that arises at a more mature stage of B cell development. The αVβ5-CD23 interaction regulates proliferation of B cell precursors at early stages of B cell development. The * indicates that the CD23 could either be found in the bone marrow stromal cells or could be soluble CD23 in blood. This stage specific role of αVβ5-mediated proliferation could be influenced by a variety of other molecules important in B cell development. Deregulation of αVβ5 or αV integrin expression and function might also be implicated in pathogenesis of CLL.

The data from this thesis illustrate that stimulation of αVβ5 integrin via sCD23 or antibodies such as 15F11 activates signalling pathways leading to cell proliferation. Furthermore, this activation is probably developmental stage-specific and is regulated by cross talk with signals from other soluble molecules important during B cell development. Further work on αVβ5-mediated cell proliferation could establish αVβ5 integrin as an important molecule in proliferation of normal B cell precursors and in proliferation of leukemic B cells in ALL. Data from αV conditional knockout mice which have αV integrin deleted in the B cells (CD19-cre) indicate that αV integrin could regulate differentiation into the $CD5^+$ (B1) B cell development, as these subsets are increased in the knockout mice (*Dr. Adam Lacy-Hulbert personal communication*). Therefore, αV integrins could not only have important roles in ALL but could be involved in autoimmune disease and malignancies such as chronic lymphocytic leukaemia, both of which involve a clonal proliferation of $CD5^+$ B1 B cells. Although the role of αV integrins could probably be slightly different in the mouse models and in humans, as indicated by the difference in activities of human and mouse CD23, a detailed study of αV integrins in mouse models relating them to human disease context is required. These studies can provide important information not only for B cell disorders but will place αV integrins as important receptors in regulating B cell development.

7 References

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