SDF-1 and PDGF enhance αvβ5-mediated ERK activation and adhesion-independent growth of human pre-B cell lines.

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Abbreviations

ALL	Acute lymphoblastic leukaemia
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
GPCR	G protein-coupled receptor
ICAM-1	Intracellular adhesion molecule-1
LCD	Low cell density
LFA-1	Lymphocyte function-associated antigen-1
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinase
SDF-1	Stromal cell-derived factor-1
SLE	Systemic lupus erythmatosus
[^{3}H]	Tritiated thymidine
VCAM-1	Vascular cell adhesion moleucle-1
VLA-4	Very late antigen-4
VLA-5	Very late antigen-5
VnR	Vitronectin receptor

Abstract

CD23 acts via the αvβ5 integrin to promote growth of human pre-B cell lines in an adhesion-independent manner. αvβ5 is expressed on normal B cell precursors in the bone marrow. Soluble CD23 (sCD23), short CD23-derived peptides containing the arg-lys-cys (RKC) motif recognised by αvβ5, and anti- αvβ5 monoclonal antibodies all sustain growth of pre-B cell lines. The chemokine SDF-1 regulates key processes during B-cell development. SDF-1 enhanced the growth sustaining effect driven by ligation of αvβ5 with anti- αvβ5 MAb 15F-11, sCD23 or CD23-derived RKC containing peptides. This effect was restricted to B-cell precursors and was specific to SDF-1. The enhancement in growth was associated with activation of ERK and both these responses were attenuated by the MEK inhibitor U0126. Finally, PDGF also enhanced both αvβ5-mediated cell growth and ERK activation. The data suggest that adhesion-independent growth-promoting signals delivered to B cell precursors via the αvβ5 integrin can be modulated by crosstalk with receptors linked to both G-protein and tyrosine kinase-coupled signalling pathways.

Introduction

B lymphopoiesis is a tightly-regulated multi-stage process. The bone marrow microenvironment provides a variety of cytokines, chemokines, growth factors and adhesion molecules that co-ordinately regulate B cell development. Perturbation of B cell differentiation in the bone marrow leads to malignancies including acute lymphoblastic leukemia (ALL) (1).

The interaction between soluble CD23 (sCD23) and the integrin αvβ5 sustains growth of human pre-B cell lines derived from ALL patients (2, 3). Human CD23, a 45kDa type II transmembrane glycoprotein can be cleaved from the cell surface to release a range of sCD23 proteins with cytokine-like activities. The cytokine-like activities include promoting the release of cytokines from monocytic cells, mediated via the αMβ2, αXβ2 (4, 5) and αvβ3 integrins (6), and influencing the survival and differentiation of centrocytes via binding to CD21 (7-9). Plasma sCD23 levels are low in normal subjects, but high levels are reported in inflammatory disorders such as rheumatoid arthritis and SLE (10). Particularly high levels of sCD23 with prognostic value are a hallmark of B cell chronic lymphoblastic leukaemia (11). The αvβ5 integrin, a member of the vitronectin receptor (VnR) family, is expressed by bone marrow B cell precursors (2). The VnR family members comprise the αv chain in non-covalent association with one of five β subunits (β1, β3, β5, β6 and β8) and bind a broad spectrum of ligands, including fibrinogen, fibronectin and vitronectin, typically by recognition of arg-gly-asp (RGD) motifs (12-14). VnRs have diverse roles in cell adhesion, migration and survival and are implicated in growth of number of cancer cells. The VnRs αvβ5 and αvβ3 are recognised CD23 receptors (2, 6) and αvβ5 recognises an arg-lys-cys (RKC) motif on sCD23 in an adhesion-independent interaction (2).

The growth and survival of normal and leukaemic B cell progenitors is dependent on intimate contact with bone marrow stromal layers (15-17) and the factors responsible for proliferation of progenitor cells include both cellular adhesion molecules and soluble

proteins (15). The αvβ5-CD23 interaction could represent an important mechanism regulating the growth of B cell precursors, in combination with other factors present in the bone marrow microenvironment.

Stromal cell-derived factor-1 (SDF-1 or CXCL12) is a member of the CXC chemokine family (18) that binds to CXCR4 and CXCR7 receptors (19, 20). Gene inactivation of SDF-1 or CXCR4 *in vivo* causes embryonic lethality due to abnormal cerebral and gastrointestinal vasculatures and haematopoietic development (21-24). SDF-1 is the most powerful chemoattractant for undifferentiated human $CD34^+$ haematopoietic progenitors (25, 26). Several studies have shown that SDF-1 activates the integrins LFA-1, VLA-4 and VLA-5 (27-31), and facilitates transendothelial migration, homing and engraftment of precursor B cells in the bone marrow (31, 32). In addition to its established role in regulating cell motility, SDF-1 also plays a role in cell survival (33, 34) and proliferation (35), often in synergy with other cytokines (36). CXCR4 ligation by SDF-1 induces receptor internalisation, elevation of cytoplasmic Ca^{2+} levels, activation of phosphatidylinositol 3-kinase (PI3K), and phosphorylation of MEK, ERK and components of focal adhesion complexes in many cell types including B cell precursors (37-43).

The well-established role of SDF-1 in B-lymphopoiesis and in influencing integrin function, make it an attractive candidate for modulating αvβ5 function. The data in this report indicate that SDF-1 modulates the growth-sustaining function of αvβ5, by influencing ERK signalling downstream of both CXCR4 and αvβ5; platelet-derived growth factor (PDGF) also enhances αvβ5-mediated signalling. PDGF has been extensively studied in the context of integrin-growth factor receptor cross-talk (44) and we therefore assessed the role of PDGF together with other cytokines, in modulating αvβ5 function. The data demonstrate that biological responses activated via the αvβ5 - CD23 interactions in early B cell precursors are modulated by inputs from both G-protein-coupled and tyrosine kinase-linked receptors.

Materials and Methods

Antibodies and Reagents

Recombinant cytokines (IL-7, IL-3, IL-11, IL-4), anti-CXCR7 (clone 358426, IgG2a) biotinylated anti-CXCR4 (12G5, IgG2a) antibody, and recombinant human 25kDa sCD23, encompassing residues Met^{151}-Ser^{231} with an N-terminal His_6 tag, were purchased from R&D Systems, Abingdon, UK. Anti- αvβ5 (P1F6, IgG1; 15F11, IgG2a), biotinyl-murine IgG1 and recombinant SDF-1β were from Chemicon, Hampshire, UK, and anti-phospho-p44/42 MAP kinase, anti-p44/42 MAP kinase, PI3K inhibitor LY294002 and platelet-derived growth factor (PDGF-B) were supplied by Cell Signalling, Technologies, Beverly, MA, USA. Streptavidin-PE, annexinV-FITC, anti-CD19-PE, anti-kappa-FITC and anti-CXCR4-PE were from BD Bioscience, Oxford, UK. Isotype control IgG2a antibody, anti-actin antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulins, AMD3100, propidium iodide (PI) and DMEM (Dulbecco's Modified Eagle Medium) were from the Sigma Chemicals Co, Poole, UK. Biotinylated isotype control IgG2a antibody was from Serotec, Oxford, UK. The MEK inhibitor U0126 was purchased from Promega, UK. Tritiated thymidine ($[^3H]$-TdR) was obtained from Amersham International plc, Amersham, Buckinghamshire, England. RPMI-1640 medium, AIMV serum free medium and protein-free hybridoma medium-II (PFHM) were from GIBCO-BRL (Paisley, Scotland). Anti-ERK/2 antibody was purchased from Santa Cruz Biotechnology, Inc, California, USA.

Cell lines and cell culture

The SMS-SB cell line was derived from a female patient presenting with acute lymphoblastic leukaemia (45); SMS-SB cells were cultured in PFHM supplemented with 2 mM fresh glutamine and 1% (w/v) penicillin and streptomycin at 37°C in 5% CO_2 in air in a humidified atmosphere, at 2.5-5 x 10^5 cells/ml. RS4;11 (46), Blin-1, RAJI , Daudi and IB4 B cell lines were from laboratory stocks. Cell lines were maintained in RPMI -1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM fresh glutamine, and penicillin and streptomycin, at 37 °C in a 5% CO_2 in air in a humidified

atmosphere. hMSC-tert telomerised human stromal cell line was a gift from Dr. M Kassem (Denmark) (47) and was maintained in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM fresh glutamine, and penicillin and streptomycin. Normal human bone marrow was obtained from Lonza Biologics, Slough, UK. Aliquots were subjected to sterile sorting on a FACSAria instrument (BD Bioscience) using FITC anti-κ chain and PE-anti-CD19 MAbs to obtain a fraction of cells enriched for B cell precursors. For cultures with sCD23, peptides and anti-αvβ5 MAbs, the cells were propagated in medium supplemented with 2% (v/v) FCS and hMSC-tert stromal cells. Mononuclear cells were isolated from samples from ALL patients and were cultured in AIMV serum free medium, hMSC-tert stromal cells and stimulants.

Cell Growth Assays

SMS-SB cells were washed then plated at a density of 2500 cells/100µl culture (low cell density (LCD), a seeding density at which the cells are prone to apoptose (3), in 96-well microtitre assay plates in PFHM. All other cell lines were washed extensively in PFHM prior to culture at 5000 cells/100µl cultures. Cultures were propagated in the presence or absence of cytokines, MAbs or peptides, at 37 °C for 72 h followed by addition of 0.3 µCi/well [^{3}H]-TdR for 18 h prior to harvest; incorporation was determined by liquid scintillation spectrometry. For assays with bone marrow B cells or ALL cells, γ-irradiated (30 Gy [3000 rad]) hMSC-tert stromal cells were plated at a density of 2 X 10^5 cells/ well at least 7 days prior to addition of mononuclear cells. FACS sorted human bone marrow B cells were plated at a density of 3000 cells/ well on the stromal cells in RPMI - 1640 medium supplemented with 2% (v/v) heat-inactivated fetal calf serum, 2 mM fresh glutamine, and penicillin and streptomycin. ALL cells were plated at the same cell density in AIMV serum-free medium. All cultures were established in triplicate. Cytokines were used at 0.5-32nM 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.

Peptide Biochemistry

CD23-derived peptides containing the RKC motif recognised by the 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 (2). The sequences of the peptides used in this study are #9, KWINFQRKC; #11, FQRKCYYFG; LP long peptide, KWINFQRKCYYFGKG. Peptides #8, PEKWINFQR; #41, SGSGGEFIWVDGS; and #34, SGSGHTGSWIGLR that lack the RKC motif and do not bind to αvβ5 were used as negative control peptides.

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; unlabeled primary antibody was visualized using a secondary PE-conjugated anti-mouse IgG or, in the case of biotinylated antibody or peptides, streptavidin-PE. 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-KOH, pH 7.4,150mM NaCl, 5mM $CaCl_2$, 0.5μg 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 wash buffer (10mM HEPES-KOH, pH 7.4**,**150mM NaCl, 5mM $CaCl_2$). Cells were analyzed on a *FACScan* flow cytometer, using *CellQuest* software.

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 #8 or #9 (5μg/ml), 15F-11 MAb (5μg/ml) alone or in combination for specified time periods at 37ºC. 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 containing detergents and protease inhibitors (RIPA buffer as above plus 1% (w/v) NP40, 1mM Na deoxycholate, 1mM PMSF and 2μg/ml Leupeptin) and clarified by centrifugation at 13,000 x ***g*** for 15 min. Protein concentrations were determined using the Bradford

reagent (BioRad Laboratories Ltd, Hampstead, UK) with BSA as a standard. Equal amounts of protein (30-40μg) were loaded per lane on 4-12% NuPAGE Novex Bis-Tris polyacrylamide gradient gels (Invitrogen, Paisely, UK) and transferred onto nitrocellulose. Phosphorylated and total protein profiles were detected sequentially on the same membrane using specific primary antibodies, secondary antibodies conjugated to HRP and enhanced chemiluminescence (Pierce, Rockford, USA). Bands were quantified by densitometry using *ImageJ* software.

Results

SDF-1 enhances growth of Pre-B cell lines induced by ligation of αvβ5 integrin

The αvβ5-sCD23 interaction sustains growth of human precursor B cell lines (2). CD23-derived peptides containing the RKC motif recognised by the αvβ5 integrin and the 15F-11 MAb, which binds the assembled αvβ5 heterodimer, all sustain growth in a manner similar to sCD23 (2). SMS-SB cells treated with sCD23 show a dose-dependent increase in [^{3}H]-TdR incorporation (Figure 1A). Other pre-B cell lines such as RS4;11 and 697 show a similar response to sCD23 stimulation (*data not shown*). SMS-SB cell growth induced by a sub-optimal concentration of sCD23 is enhanced by SDF-1 treatment (Figure 1B) in a dose-dependent manner; SDF-1 alone has a minimal effect on growth of SMS-SB cells at any concentration used. Growth of SMS-SB cells stimulated by the 15F-11 MAb is also enhanced by SDF-1 (Figure 1C) confirming that the growth-promoting effect is specific to αvβ5 ligation. SDF-1 enhances growth of SMS-SB cells stimulated with CD23-derived peptide #9, a nonameric peptide containing the RKC motif, but has no effect on the ability of peptide #34, which lacks the RKC tripeptide, to promote cell growth (Figure 1D). SDF-1 also enhances cell growth stimulated by the long peptide (LP), a particularly potent CD23-derived peptide containing the RKC motif (Figure 1E). The CXCR4-specific antagonist AMD3100 was used to inhibit binding of SDF-1 to CXCR4. Figure 1F shows that after AMD3100 treatment there is no difference in cell growth induced by LP in the presence or absence of SDF-1 in SMS-SB cells,

indicating that the enhancement in LP-induced cell growth is via SDF-1 and CXCR4. There was no change is αvβ5 expression in cells treated with SDF-1 compared to untreated cells (Figure 1G), nor was there any increase in peptide binding to treated cells (Supplementary Figure 1A). Furthermore, stimulation of αvβ5 with either LP or sCD23 did not influence CXCR4 expression (Supplementary Figure 1B). While the data demonstrate that SDF-1 can cooperate with soluble αvβ5-directed ligands to enhance growth of SMS-SB cells, it remains to be determined whether this reflects either inhibition of apoptosis, stimulation of cell division or a combination of both processes.

Enhancement of αvβ5-driven cell growth by SDF-1 is specific to B cell precursors

CXCR4 expression varies in cell lines representing distinct stages of B cell development (Figure 2A), and a similar assessment of αvβ5 expression (Figure 2B) illustrates that expression of the integrin is highest in cell lines representative of B cell precursors and decreases in mature B cell lines (Figure 2B). Cell lines representing earlier stages of B cell development (RS4;11 and SMS-SB) have low levels of CXCR4 expression and show the most robust growth response following αvβ5 ligation; SDF-1 enhances αvβ5-mediated cell growth induced by LP in these cells (Figure 2C). Despite higher CXCR4 expression, SDF-1 does not affect the growth of either Blin-1 cells, which show a modest increase in cell growth after αvβ5 ligation (by sCD23), or Daudi cells, which show little or no growth response via αvβ5 (as shown by LP treatment). Finally, the cell line IB4 which is low/negative for both CXCR4 and αvβ5 expression shows no increase in cell growth following αvβ5 ligation with LP in the presence or absence SDF-1 (Figure 2C).

The cytokines IL-7, IL-11, IL-3 and IL-4 were also assessed to determine if any of them could enhance αvβ5-induced cell growth; none of these cytokines mimicked the ability of SDF-1 to enhance αvβ5-mediated cell growth (Figure 3). IL-7 has no effect on LP-induced SMS-SB cell growth (Figure 3A), and while IL-11 alone induces an increase in [^{3}H]-TdR incorporation (Figure 3B) it has no effect on αvβ5-regulated cell growth; neither IL-3 nor IL-4 enhances αvβ5-induced growth (Figure 3C and 3D). Growth

responses to the different cytokines were tested using all of the different αvβ5-directed stimuli and none of the cytokines tested modulated αvβ5 expression (*data not shown*).

SDF-1 enhances ERK phosphorylation induced by sCD23 and other αvβ5 ligands

The signalling pathways that might regulate synergistic activation of cell growth by αvβ5 ligation and SDF-1 treatment were evaluated, with a focus on ERK phosphorylation since SDF-1 is known to target this pathway (41). Treatment of SMS-SB cells with sCD23 promotes robust phosphorylation of ERK at 30-60min (Figure 4A). SDF-1 alone provokes an early, modest increase in phospho-ERK levels (Figure 4A). By contrast, co-stimulation of SMS-SB cells with SDF-1 and sCD23 stimulates a very robust ERK phosphorylation that is sustained until 10 minutes post-stimulation, yielding phospho-ERK levels that are some 15-fold greater than either stimulus alone (Figure 4A and 4B). The 15F11 MAb induces ERK phosphorylation at 10-30 min post-stimulation, while co-stimulation with SDF-1 and 15F11 promotes an earlier onset of ERK phosphorylation that is sustained to 30 min (Figure 4C). The RKC-containing CD23-derived peptide #9 behaves similarly, inducing a modest response by itself and a more rapid and sustained response when added together with SDF-1; peptide #8, which lacks the RKC motif, fails to induce significant phosphorylation either alone or in association with SDF-1 (Figure 4D). Finally, the RKC-containing pentadecameric LP induces sustained and robust ERK phosphorylation that is enhanced by SDF-1 co-stimulation (Figure 4E). 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*) (2) 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 modest level of ERK phosphorylation that is enhanced by SDF-1; SDF-1 also drives a more rapid elevation in ERK phosphorylation when added together with a soluble αvβ5 ligand.

We used the MEK inhibitor U0126 and PI3K inhibitor LY294002 to further investigate the specificity of the ERK signalling pathway in αvβ5-mediated functions. Pre-treatment

of SMS-SB cells with U0126 completely abolishes the effect of sCD23 and CD23-derived peptides on cell growth (Figure 5A). Moreover, U0126 inhibits basal survival of SMS-SB cells in low-density cultures within a few hours of initiation of the culture. U0126 treatment also abrogates the ERK phosphorylation driven by SDF-1 or sCD23 individually and also completely prevents the enhancement of ERK phosphorylation observed with SDF-1 and sCD23 co-stimulation (Figure 5C). The PI3K inhibitor LY294002 also attenuates growth of SMS-SB cells in response to αvβ5 ligation, but this inhibition is less complete than that observed with U0126 (Figure 5B). Thus, after 48 hours of LP stimulation a small increase in [^{3}H]-TdR incorporation remains evident in cells pre-treated with LY294002, whereas no growth is detectable after 12 h of U0126 treatment (Figure 5A and 5B). 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 treatment after a 12 h period (Figure 5D), illustrating that U0126 leads to increased apoptosis in SMS-SB cells relative to LY294002 treatment. This underscores the importance of the ERK signalling pathway in the survival of SMS-SB cells.

PDGF enhances αvβ5-driven growth of pre-B cell lines

PDGF stimulates SMS-SB cell growth and the cells express PDGF receptor β (PDGFRβ) (48). Figure 6A and 6B, respectively, show that PDGF-β significantly enhances both LP- and sCD23-induced growth of SMS-SB cells. As observed with SDF-1, PDGF treatment does not alter surface expression of αvβ5 (Figure 6C) and, furthermore, the increase in [^{3}H]-TdR incorporation promoted by sCD23 and PDGF treatment correlates well with enhancement in ERK phosphorylation. Stimulation of SMS-SB cells with PDGF alone results in significant ERK phosphorylation, and co-stimulation with PDGF and sCD23 strikingly enhances this ERK phosphorylation (Figures 6D).

Response of normal bone marrow B cells and ALL cells to soluble αvβ5-specific stimulation

B cell precursors from the bone marrow that are positive for αvβ5 integrin also express CXCR4 and cells from patients with ALL also express the αvβ5 integrin and CXCR4. Growth of BM-derived B cell precursors and ALL cells was investigated in order to relate the data from pre-B cell lines with their normal and malignant counterparts in the bone marrow. The ability of $CD19^+$ / κ^- FACS-sorted B cell precursors isolated from bone marrow aspirates to respond to stimulation with soluble αvβ5-directed ligands was assessed. A robust incorporation of thymidine was stimulated by both LP (5μg/ml) and by sCD23 (250ng/ml) (Figure 7A) while a control peptide devoid of the RKC motif, peptide #8, was without effect; the cells also responded to the 15F11 MAb indicating the specificity of αvβ5 integrin in mediating cell growth (*data not shown*). Secondly, both sCD23 and LP promote growth of ALL cells (αvβ5+ see supplementary Figure 3) above background levels (Figure 7B) and co-stimulation with SDF-1 (Figure 7C) or PDGF (Figure 7D) with lower concentrations of sCD23 resulted in significant enhancement in cell growth. The data demonstrate that normal bone marrow-derived B cell precursors and patient-derived ALL cells can respond to sCD23 and related ligands. The growth responses were not as robust as those noted in the cell line models and although we observed enhancement in cell growth with the addition of SDF-1 and PDGF, the [^{3}H]-TdR incorporation induced by suboptimal concentration of sCD23 in these cases was minimal.

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 that 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-ICAM1 interaction (31, 32). This report demonstrates 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 peptides derived from sCD23 provokes ERK phosphorylation and growth responses in B cell precursors that are enhanced by SDF-1. PDGF also enhances signalling delivered via soluble ligation of αvβ5, suggesting that both tyrosine kinase and G protein-coupled receptor-linked pathways modulate adhesion-independent signalling by αvβ5.

Growth of SMS-SB cells stimulated with sCD23 was enhanced by addition of SDF-1 and similar responses were noted with sCD23-derived peptides (containing the RKC motif recognised by αvβ5) and the anti- αvβ5 MAb 15F-11, all of which promote growth of these cells. SDF-1 acts through CXCR7 as well as via CXCR4 and in order to address the specificity of CXCR4 in enhancing αvβ5-mediated signals, the binding of SDF-1 to CXCR4 was blocked using the CXCR4 antagonist AMD3100 (49, 50). AMD3100 completely abolished the ability of SDF-1 to enhance αvβ5-mediated SMS-SB cell growth (Figure 1F); pre-treatment of SMS-SB cells with the anti-CXCR4 MAb (12G5) also inhibited the action of SDF-1 (*data not shown*). There is growth of SMS-SB cells with AMD3100 treatment alone (Figure 1F). AMD3100 has been extensively used as CXCR4 antagonist however it has also been suggested to be a weak partial agonist (51), which might partly explain the small apparent growth effect with AMD3100 alone. However, in the presence of SDF-1, AMD3100 clearly acts as a CXCR4 antagonist. Importantly, AMD3100 has no effect on LP-induced cell growth but blocks the enhancement in growth with LP and SDF-1. Finally, assessment of expression of CXCR7 expression illustrated that SMS-SB cells have modest level of CXCR7, Blin-1 cells have no CXCR7 but express CXCR4, while IB4 cells lack CXCR4 but express CXCR7; IB4 cells do not respond to SDF-1 stimulation (*data not shown*). Taken together the data are consistent with the interpretation that SDF-1 mediates its effect on αvβ5-stimulated cell growth via CXCR4.

Higher levels of CXCR4 expression do not influence the lack of response to αvβ5 ligation noted in the more mature cell lines, and 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. Thus, CXCR4 is continuously expressed by B cells and, as the cells mature and begin to express surface IgM, the chemotactic response to SDF-1 is lost despite sustained expression of CXCR4 (52-54). In agreement with these results our data support the notion that αvβ5-mediated cell growth is important at particular stages of B cell development and is modulated by SDF-1 in a stage-specific manner.

SMS-SB cells undergo apoptosis in low-density culture conditions (3). The inhibition of cell growth by U0126 shows that basal ERK phosphorylation is necessary for the survival of SMS-SB cells; in the presence of U0126 the cells rapidly undergo apoptosis. ERK phosphorylation observed following sCD23, RKC-containing peptide or 15F-11 treatment demonstrates that growth induced by ligation of αvβ5 with sCD23 is regulated via the ERK signalling pathway. The enhancement in cell growth noted with SDF-1 and sCD23 correlated well with a sustained increase in ERK phosphorylation. This was verified by similar enhancements observed when CD23-derived peptides and 15F-11 antibody were used as stimuli. However it cannot be excluded that other signalling pathways might be involved in regulating SMS-SB cell growth, and the observation that LY294002 inhibited the enhancement of αvβ5 -mediated cell growth by SDF-1 indicates that AKT has the potential to regulate adhesion-independent signalling by αvβ5. This would be consistent with studies that demonstrate that SDF-1 activates both ERK and AKT (38, 55, 56) and merits further investigation.

Many cytokines are known to modulate integrin function and B-cell precursor survival, but no similar enhancement of αvβ5-mediated cell growth was promoted by the cytokines IL-7, IL-3, IL-4 and IL-11 at the concentrations used. It has been shown that SDF-1, IL-3 and IL-7 induce proliferation of ALL cells co-cultured with stromal cells; IL-7 alone did not induce ERK activation but there was a synergistic ERK activation following IL-7 and SDF-1 co-stimulation (57). However, in the co-culture studies, the

ALL cells were propagated on stromal cells and effects on ERK and other signalling pathways would have been affected by multiple adhesion contacts between the ALL cells and stromal cells. Therefore, the data from the ALL-stromal cell co-culture model illustrate convincingly that ERK phosphorylation can occur in an adhesion-dependent manner (57).

In our own studies of normal bone marrow B cells and ALL-derived cells, cultures that lacked serum or stromal cell support failed to support any growth of the cells. Using bone marrow B cell precursors propagated on irradiated stromal cells we were able to show that αvβ5 ligation by sCD23 induces growth response in these precursors, similar to that seen in cell lines. The importance of this was further assessed in ALL cells to investigate if SDF-1 could enhance this αvβ5-mediated growth response. Cultures with stromal cells supported modest growth of ALL cells exposed to αvβ5-stimulatnts alone and some degree of enhancement of sCD23-driven growth of ALL cells by SDF-1 was achieved. The data indicate that normal marrowcytes respond to sCD23 and that in ALL cells some additive effect of αvβ5-directed ligands and other receptors can be elicited. The finding that sCD23 can induce ERK phosphorylation in B cell precursor cell lines is consistent with data from studies of monocytes that showed strong ERK responses to integrin ligation and sCD23 stimulation (58, 59), and it remains to be determined whether sCD23 and other αvβ5 ligands can induce ERK phosphorylation in normal B cell precursors and ALL cells.

Finally, PDGF enhanced the cell growth induced via αvβ5 in a manner similar to that observed with SDF-1. Interestingly, a previous study shows that early pre-B cells, but not mature B cells, express the PDGFRβ and PDGF was shown to promote proliferation of these cells but not immature B cells (60). A number of studies, particularly in adherent cell systems, have demonstrated a cross-talk between growth factors and integrin signalling, and synergy between growth factors and cell adhesion has been observed in the activation of the MAPK (61, 62) and PI3K/AKT pathways (63). Moreover, PDGF has previously been shown to interact physically with the αvβ3 integrin to stimulate proliferation of oligodendrocytes by activation of the integrin in an adhesion-dependent

manner (64). Therefore, it is plausible that PDGF and αvβ5 also interact to promote adhesion-independent proliferation in B cell precursors. Our data indicate that αvβ5 synergises with two distinct signalling pathways, via a tyrosine kinase receptor (PDGFR-β) and a 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 in order to understand further the mechanisms involved in αvβ5 -mediated cell growth.

The model emerging from these data suggests a role for αvβ5 integrin in B cell precursor survival and growth that can be modulated by other biological effectors such as SDF-1 and PDGF. This may have important implications for growth of normal and neoplastic B cells. B cell development in different compartments of the hematopoietic microenvironment is accompanied by stage-specific changes that are critical for proliferation and differentiation 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.

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Titles & Legends to Figures

Figure 1: SDF-1 enhances αvβ5 -mediated growth of SMS-SB cells.

(A) Low cell density (LCD) cultures of SMS-SB cells were propagated with the indicated concentrations of recombinant sCD23, and [^{3}H]-TdR incorporation assessed after 72hr. **(B)** Growth 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) is shown. **Panels C-E** illustrate the growth response of SMS-SB cells to stimulation with 5μg/ml 15F-11 MAb **(C),** 5μg/ml peptide #9 **(D),** and 1μg/ml long peptide (LP) **(E),** 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 **(C)** or irrelevant peptide #34 **(D)** shown as a white bar. **Figure 1F** represents growth with a 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 SDF-1 addition. 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. Bars represent the standard deviation of triplicate determinations. On the figures, *$p<0.05$ or **$p<0.01$ indicate the statistical significance of the increase in growth of cells treated with different concentrations of SDF-1 and αvβ5 stimulants (sCD23, 15F-11MAb, peptide #9 or LP) in comparison to SDF-1 treatment alone, #$p<0.05$ and ##$p<0.01$ indicate the significance in enhancement of proliferation with 250ng/ml SDF-1 and αvβ5 stimulants when compared with αvβ5 stimulants alone. Data are representative of three independent experiments**.** **(G)** 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 P1F-6 and 15F-11. Fluorescence intensity histograms for the relevant αvβ5 MAb are represented by the grey shaded area and isotype control antibody staining is represented by the black line.

Figure 2: Levels of CXCR4 expression do not influence cell growth

Panels **A** and **B** show staining of CXCR4 (12G5) and αvβ5 (P1F6) respectively, in five cell lines. Antibody staining is shown in the grey shaded area and the isotype control

staining by the black line. **(C)** In the different cell lines shown, [^{3}H]-TdR incorporation driven by αvβ5 ligation (either with 2ng/ml sCD23 or with 1μg/ml LP) and SDF-1 is shown by black bars, and the growth response induced with SDF-1 alone is shown as hatched bars. Bars represent the standard deviation of triplicate determinations; *$p<0.05$ or **$p<0.01$ indicates a statistically significant different increase in growth of cells treated with different concentrations of SDF-1 and αvβ5 stimulants (sCD23 or LP) in comparison to SDF-1 treatment alone, and #$p<0.05$ and ##$p<0.01$ indicates a significant enhancement of cell growth with 250ng/ml SDF-1 and αvβ5 stimulants when compared with αvβ5 stimulants alone. Data are representative of three independent experiments.

Figure 3: Regulation of αvβ5-mediated cell growth by other cytokines.

Figures 3A, B, C and **D** represent growth 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, growth induced by the peptides and the cytokine is shown in black bars, by cytokine alone is shown as hatched bars and by cytokine with negative control peptide # 41 or #8 is shown as white bars. The concentrations of each cytokine are equivalent molar concentrations. Data are representative of three independent experiments.

Figure 4: SDF-1 enhances ERK phosphorylation in cells treated 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). **Panel A** shows 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. **(B)** The increase in phosphorylation was quantitated by densitometric analysis of the bands. **Panel C** represents western blots for phosphorylation of ERK and for total actin in SMS-SB cells treated with 15F-11 MAb alone or in combination with SDF-1. **(D)** Western blots for phosphorylated and total ERK in SMS-SB cells treated with peptide #9 (5μg/ml), negative control peptide #8 (5μg/ml).

Panel E represents western blots for phosphorylation of ERK and for total actin in SMS-SB cells treated with LP (1μg/ml) alone or in combination with SDF-1.

Figure 5: Effect of inhibition of ERK and PI-3K signalling on αvβ5-mediated growth 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; cell growth was assessed by [^{3}H]-TdR incorporation after 72 h of culture. **Panel A** shows cell proliferation with LP and/or SDF-1 in untreated cells and cells treated with U0126. **Panel B** shows cell growth stimulated by LP and/or SDF-1 in untreated cells or cells treated with LY294002. Unstimulated cells are represented as grey bars, LP treated as black bars, the LP and SDF-1 combination as white bars and SDF-1 treatment alone as hatched bars. Bars represent the standard deviation of triplicate determinations, with *$p<0.05$ or **$p<0.01$ indicating a statistically significant increase in proliferation of cells treated with different concentrations of SDF-1 and αvβ5 stimulants (LP) in comparison to SDF-1 treatment alone; #$p<0.05$ and ##$p<0.01$ denote the significance of enhancement in cell growth with 250ng/ml SDF-1 and αvβ5 stimulants when compared with αvβ5 stimulants alone. Data are representative of three independent experiments. **Panel C** illustrates western blot analysis of phosphorylated ERK and total actin in SMS-SB cells pre-treated with 10μM U0126, 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 10μM U0126, 50μM LY294002, DMSO (vehicle) or no treatment for 12 h. Quadrant gates in the insets are based on unstained, annexinV or PI stains alone.

Figure 6: PDGF enhances αvβ5 -driven growth and ERK phosphorylation in SMS-SB cells.

LCD SMS-SB cultures were stimulated with the indicated concentrations of PDGFβ and 1μg/ml LP (**Panel A**) or 160ng/ml sCD23 (**Panel B**) for 72 h prior to determination of [^{3}H]-TdR incorporation. Growth induced by PDGF and LP or sCD23 in combination with PDGF is shown as black bars and that driven by PDGF alone is shown as hatched

bars. Bars represent the standard deviation of triplicate determinations, with *p<0.05 or **p<0.01 indicating the statistical significance of the increase in growth of cells treated with different concentrations of SDF-1 and αvβ5 stimulants (sCD23 or LP) in comparison to SDF-1 treatment alone, #p<0.05 and ##p<0.01 indicate the significance of the enhancement of cell growth with 250ng/ml SDF-1 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 P1F-6 and 15F-11. Staining with the relevant αvβ5 MAbs is represented by the grey shaded area and isotype control antibody staining is represented by the black line. **Panel D** shows western blots for phosphorylated ERK and ERK2 in SMS-SB cells treated with PDGF (250ng/ml) alone or PDGF (250ng/ml) with sCD23 (160ng/ml).

Figure 7. Response of Normal B cell precursors and ALL Cells to αvβ5 Ligands

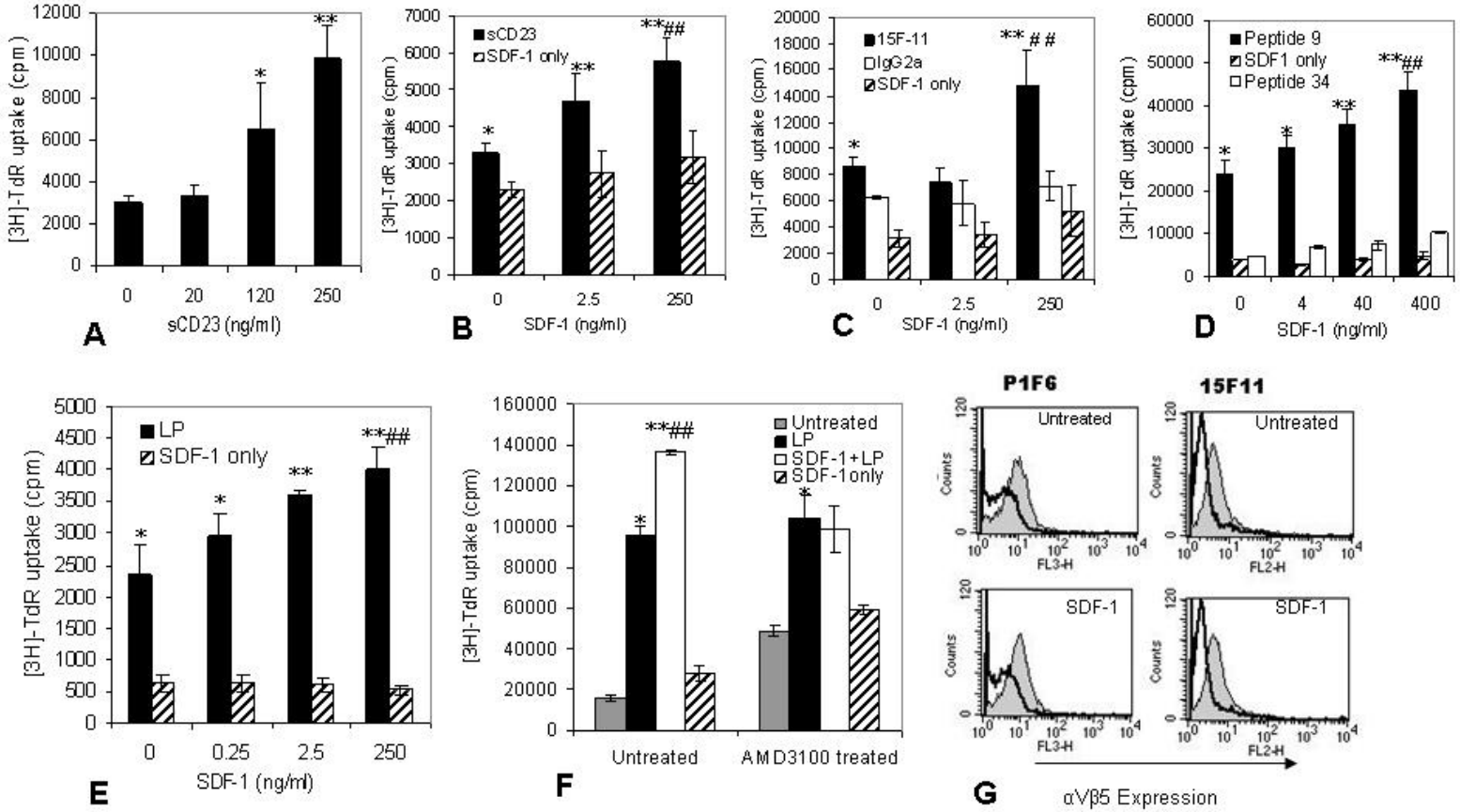
Aliquots of κ^- FACS-sorted human bone marrow B cells (**Panel A**) or cells derived from samples from ALL patients (**Panel B**) were stimulated with 5μg/ml peptide #8, LP or with 250ng/ml sCD23 for 48 hr prior to pulse with tritiated thymidine. Aliquots of ALL cells were also stimulated with 150ng/ml sCD23 in the presence or absence of 250ng/ml SDF-1 (**Panel C**) or PDGF (**Panel D**), prior to assessment of cell growth. Bars represent the standard deviation of triplicate determinations, with *p<0.05.

Supplementary Figure 1

Panel A:- SMS-SB cells were incubated overnight in the presence or absence of SDF-1 then treated with CD23-derived, RKC-containing biotinylated peptide #9 or #11, or with peptide #41 that lacks RKC, and binding visualized using PE-streptavidin as described previously (2). The upper pair of panels shows staining of untreated SMS-SB cells, while the lower two panels show the equivalent staining of SDF-1-treated cells; the solid black line shows staining with peptide #41 while the filled grey area illustrates staining with the RKC peptides #9 or #11.

Panel B:- SMS-SB cells were stimulated with LP or sCD23 or peptide #8, which lacks the RKC motif, as described in the legend to Figure 1 and stained with biotinylated-12G5 anti-CXCR4 antibody; binding was visualized with PE-streptavidin. For each indicated treatment, the histograms show background staining with IgG2a immunoglobulin as a black line and staining with 12G5 in the filled grey area.

Figure 1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sCD23
SDF-1 only
SDF-1 (ng/ml)
B
15F-11
IgG2a
SDF-1 only
SDF-1 (ng/ml)
C
Peptide 9
SDF1 only
Peptide 34
SDF-1 (ng/ml)
D
LP
SDF-1 only
SDF-1 (ng/ml)
E
Untreated
LP
SDF-1+LP
SDF-1only
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P1F6
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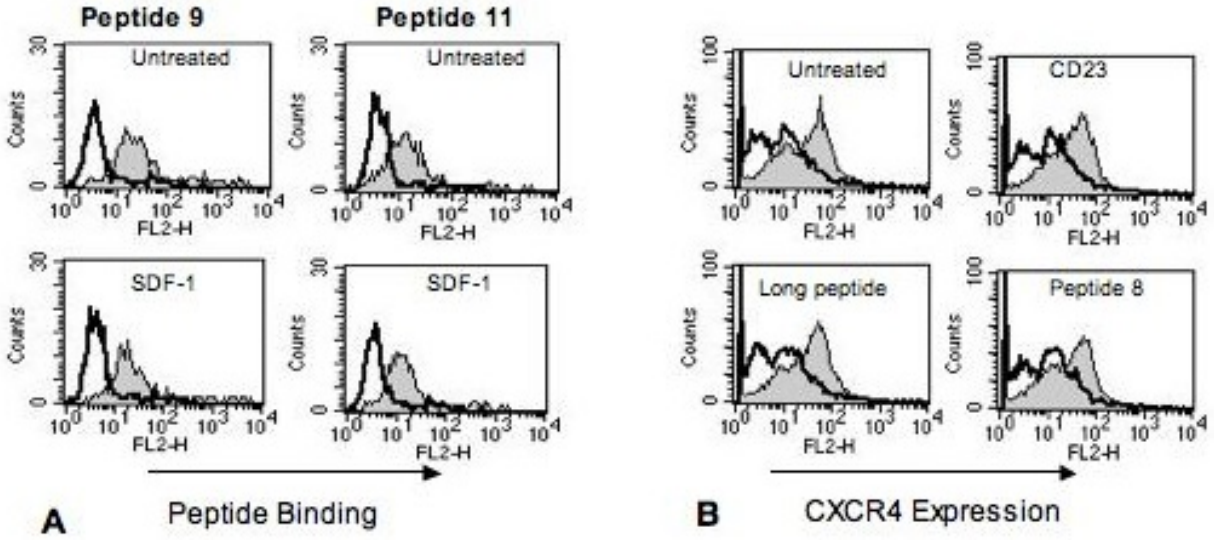
SDF-1
Counts
FL3-H
FL2-H
αVβ5 Expression
G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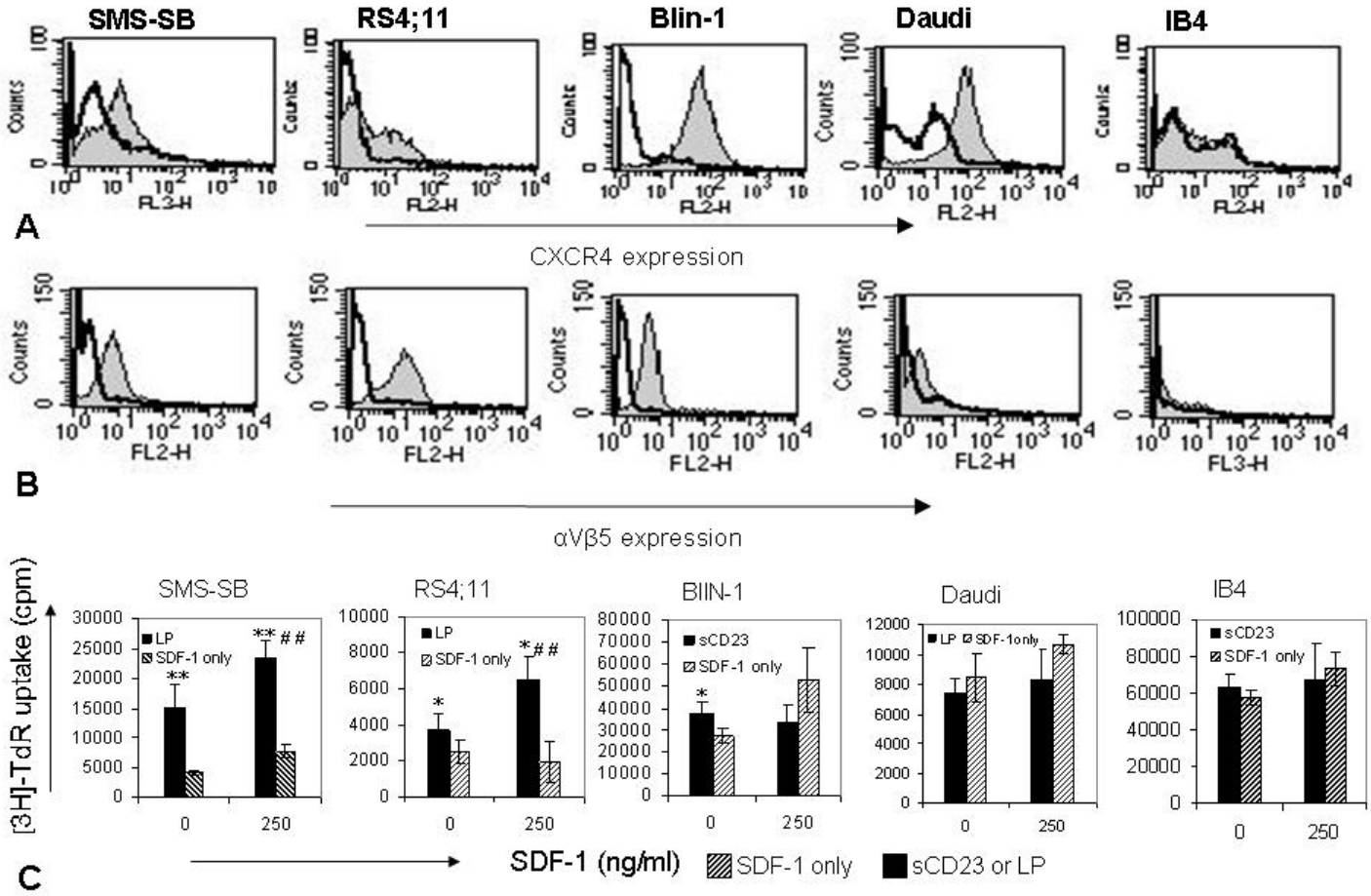
Counts
FL2-H
B
CXCR4 Expression

Figure 2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CXCR4 expression
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αVβ5 expression
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RS4;11
BIIN-1
Daudi
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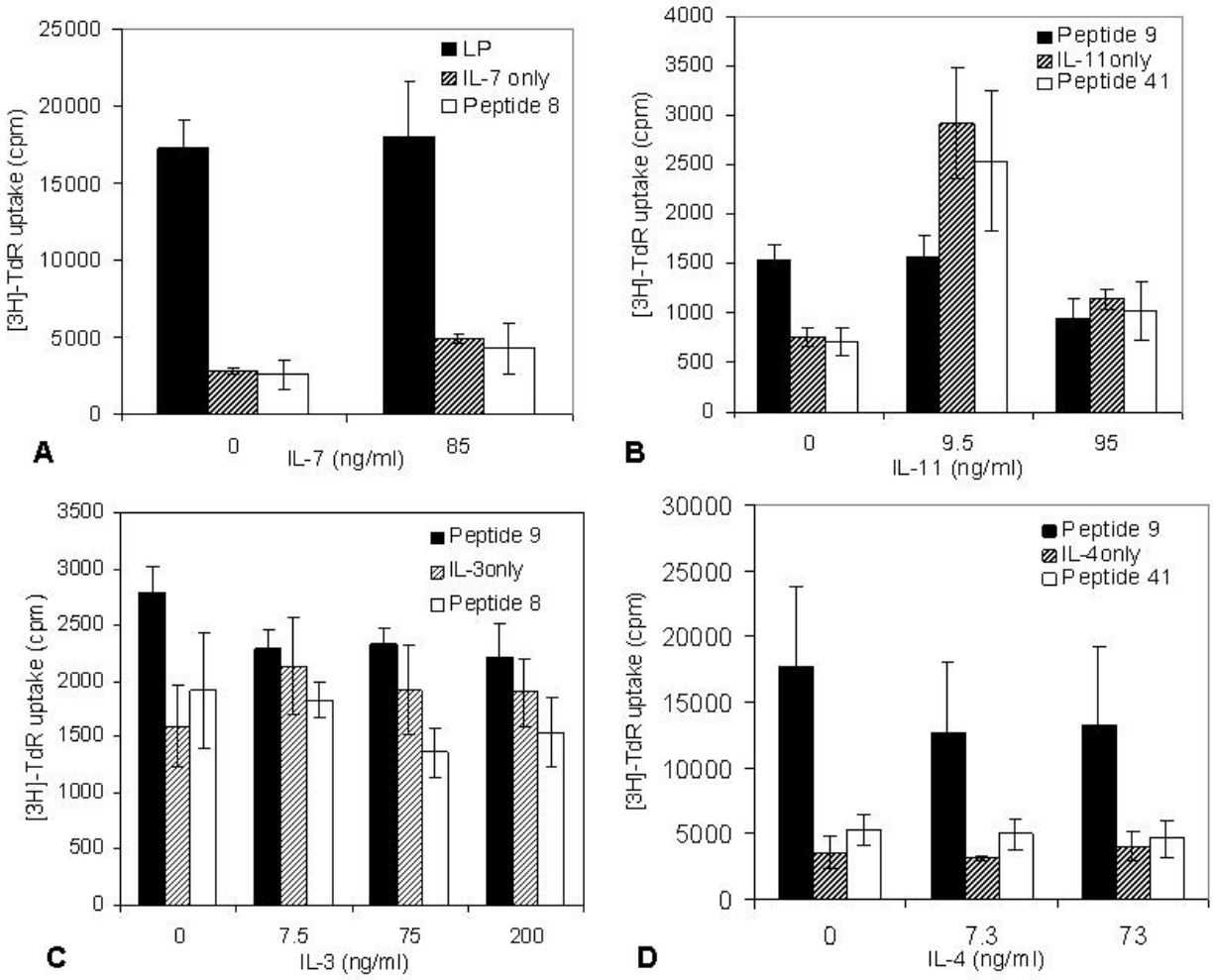
LP
SDF-1 only
sCD23
sCD23 or LP
SDF-1 (ng/ml)
C

Figure 3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15000
10000
5000
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IL-7 (ng/ml)
B
[3H]-TdR uptake (cpm)
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3500
3000
2500
2000
1500
1000
500
0
Peptide 9
IL-11only
Peptide 41
0
9.5
95
IL-11 (ng/ml)
C
[3H]-TdR uptake (cpm)
3500
3000
2500
2000
1500
1000
500
0
Peptide 9
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Peptide 8
0
7.5
75
200
IL-3 (ng/ml)
D
[3H]-TdR uptake (cpm)
30000
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Peptide 41
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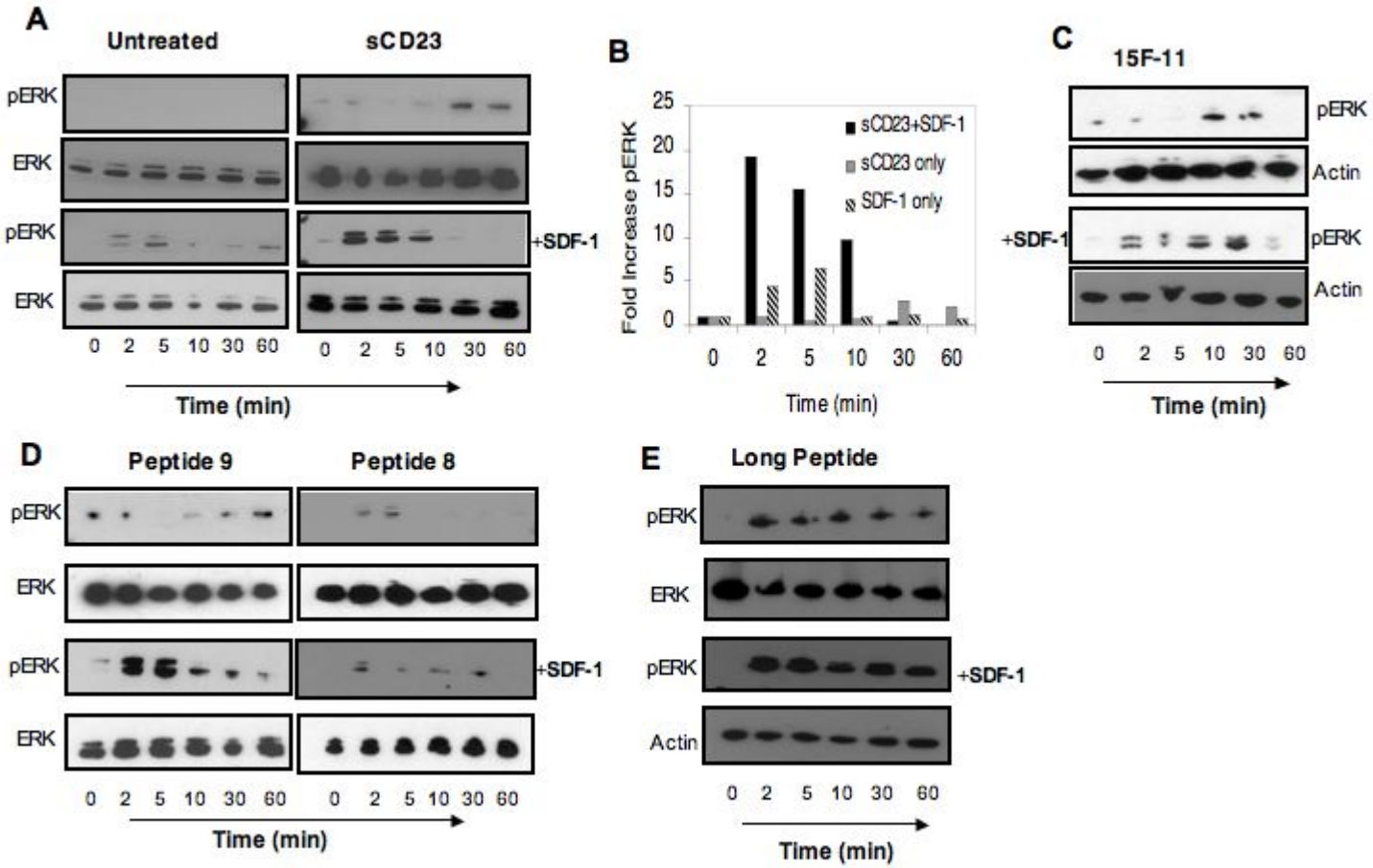
73
IL-4 (ng/ml)

Figure 4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0 2 5 10 30 60
0 2 5 10 30 60
Time (min)
B
Fold Increase pERK
25
20
15
10
5
0
sCD23+SDF-1
sCD23 only
SDF-1 only
0 2 5 10 30 60
Time (min)
C
15F-11
pERK
Actin
+SDF-1
pERK
Actin
0 2 5 10 30 60
Time (min)
D
Peptide 9
Peptide 8
pERK
ERK
pERK
ERK
+SDF-1
0 2 5 10 30 60 0 2 5 10 30 60
Time (min)
E
Long Peptide
pERK
ERK
pERK
Actin
+SDF-1
0 2 5 10 30 60
Time (min)

Figure 5

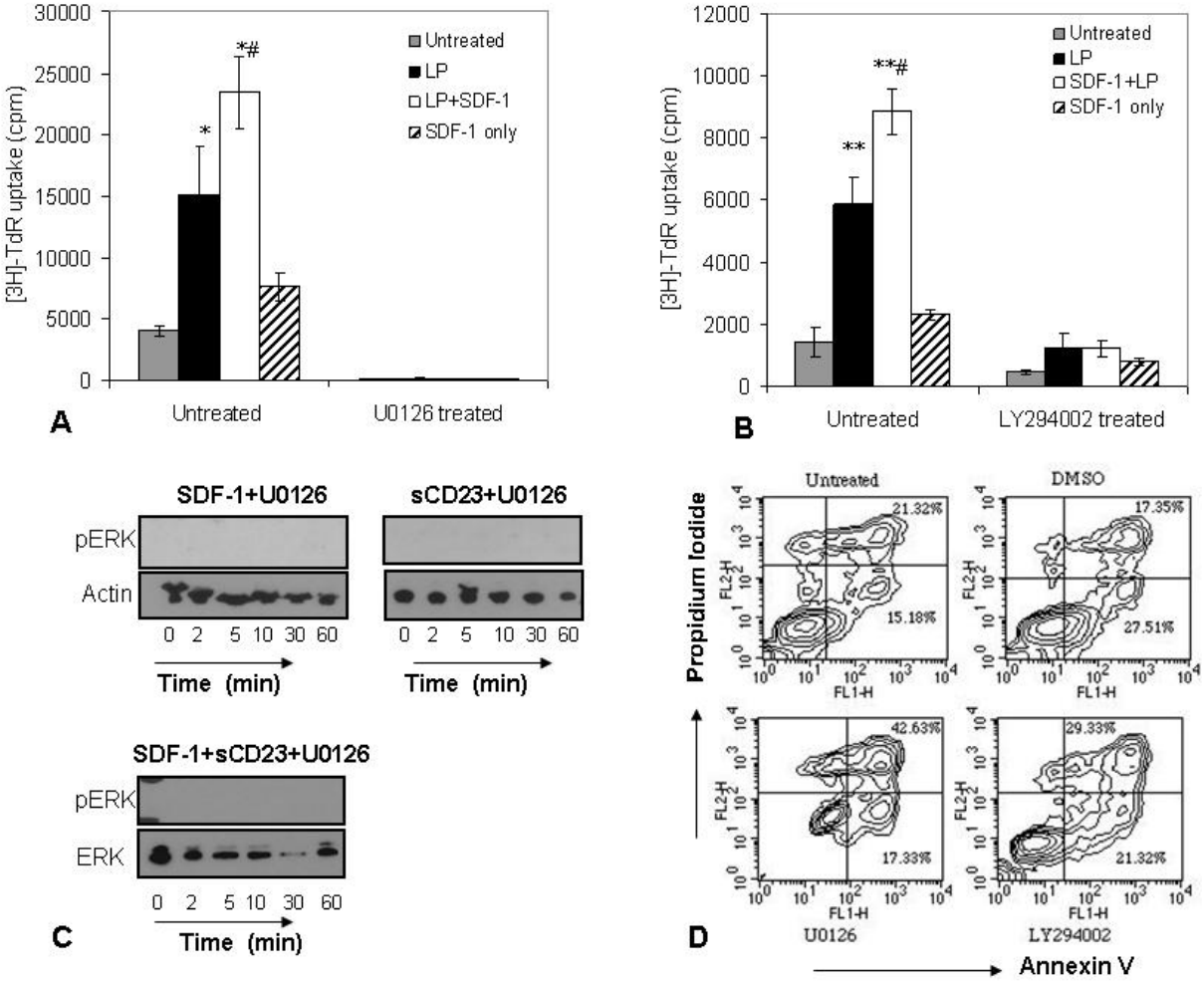

[3H]-TdR uptake (cpm)
30000
25000
20000
15000
10000
5000
0
*
*#
Untreated
LP
LP+SDF-1
SDF-1 only
Untreated
U0126 treated
A
12000
10000
8000
6000
4000
2000
0
**
**#
Untreated
LP
SDF-1+LP
SDF-1 only
Untreated
LY294002 treated
B
SDF-1+U0126
sCD23+U0126
pERK
Actin
0 2 5 10 30 60
Time (min)
SDF-1+sCD23+U0126
pERK
ERK
0 2 5 10 30 60
Time (min)
C
Untreated
DMSO
Propidium Iodide
FL2-H
FL1-H
21.32%
15.18%
17.35%
27.51%
42.63%
17.33%
29.33%
21.32%
U0126
LY294002
Annexin V
D

Figure 6

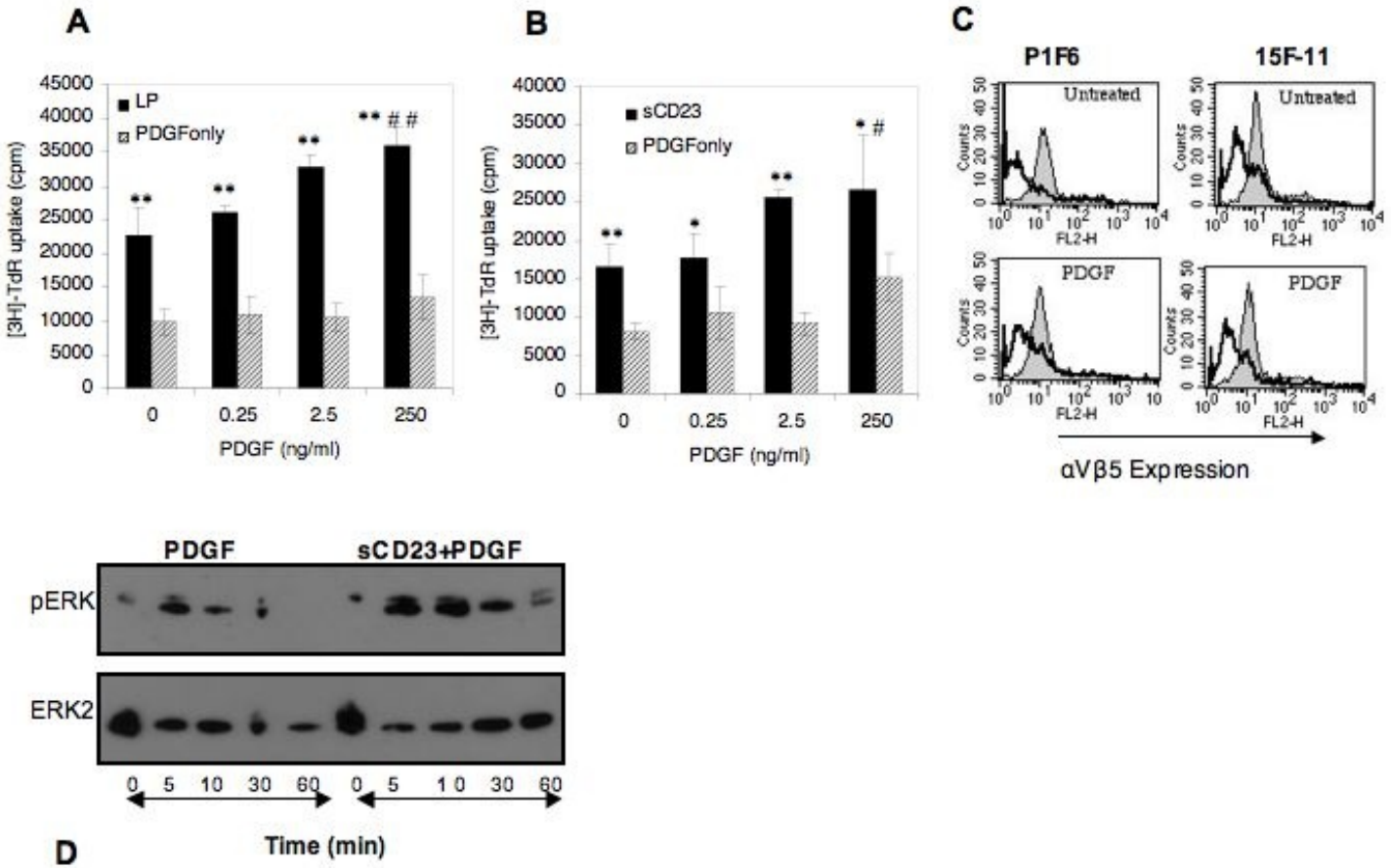
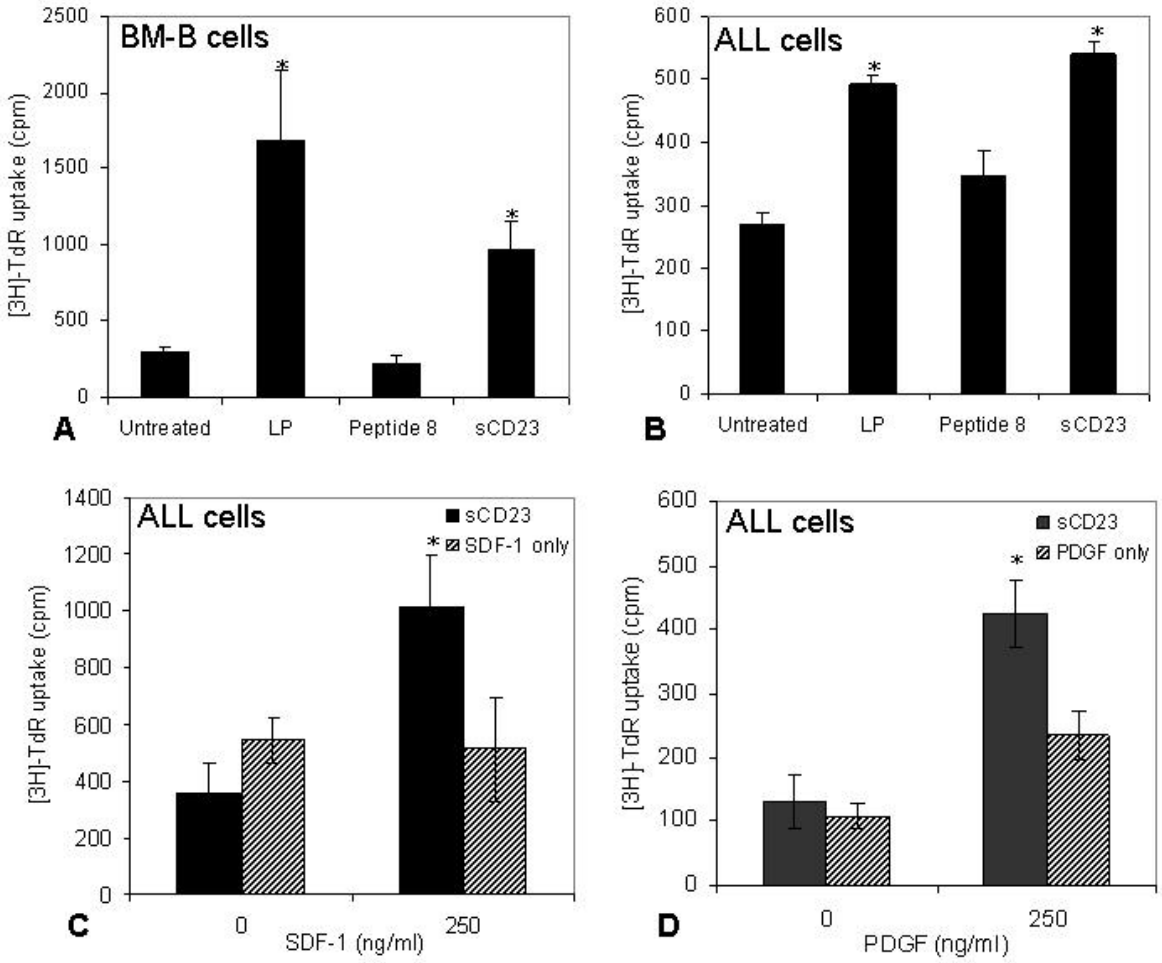

A
B
C
LP
PDGFonly
sCD23
[3H]-TdR uptake (cpm)
PDGF (ng/ml)
0
0.25
2.5
250
P1F6
15F-11
Untreated
PDGF
Counts
FL2-H
αVβ5 Expression
PDGF
sCD23+PDGF
pERK
ERK2
0 5 10 30 60 0 5 10 30 60
Time (min)
D

Figure 7


BM-B cells
[3H]-TdR uptake (cpm)
2500
2000
1500
1000
500
0
Untreated
LP
Peptide 8
sCD23
A
ALL cells
600
500
400
300
200
100
0
B
ALL cells
sCD23
SDF-1 only
1400
1200
1000
800
600
400
200
0
SDF-1 (ng/ml)
C
ALL cells
sCD23
PDGF only
PDGF (ng/ml)
D