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$\alpha_9\beta_1$ integrin engagement inhibits neutrophil spontaneous apoptosis: Involvement of Bcl-2 family members

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ABSTRACT

Integrin signaling is comprised of well-characterized pathways generally involved in cell survival. $\alpha_9\beta_1$ integrin has recently become a target of study and has been shown to present pro-survival effects on neutrophils. However, there are no detailed studies on how $\alpha_9\beta_1$ integrin-coupled signaling pathways interact and how they converge to finally modulate spontaneous apoptosis in neutrophils. In this regard we sought to investigate the main signaling events triggered by $\alpha_9\beta_1$ integrin engagement and how these signaling pathways modulate the apoptotic program of human neutrophils. Using VLO5, a snake venom disintegrin shown to bind to $\alpha_9\beta_1$ integrin in neutrophils, we demonstrate that $\alpha_9\beta_1$ integrin engagement leads to the activation of integrin signaling pathways and potently reduces neutrophil spontaneous apoptosis. These effects are dependent on the activation of PI3K and MAPK pathways, since both LY294002 (PI3K inhibitor) or PD95059 (MEK inhibitor) reverted the effects of VLO5/$\alpha_9\beta_1$ interaction. Moreover we show that VLO5/$\alpha_9\beta_1$ engagement induces NF-κB nuclear translocation and increases the ratio between anti- and pro-apoptotic proteins by inducing the degradation of pro-apoptotic protein Bad and increasing the expression of anti-apoptotic protein Bcl-x_L. VLO5 also inhibited the early steps of neutrophil spontaneous apoptosis by preventing Bax translocation to the outer mitochondrial membrane and consequent cytochrome c release. In conclusion, as the mechanistic details of $\alpha_9\beta_1$ integrin signaling pathways in human neutrophils becomes clearer, it should become possible to develop new therapeutic agents for human diseases where neutrophils play a prominent role.

1. Introduction

During recruitment from circulation and throughout their migration towards sites of inflammation, neutrophils (PMNs) receive molecular cues from their surrounding environment that concomitantly expand their short life span and activate a series of molecular effector mechanisms. Together these cues promote the arrival of viable, fully competent cells that are fundamental for host protection by their capacity for ingesting and killing invading microorganisms [1,2]. Integrins mediate many of the PMN–endothelial cell and PMN–extracellular matrix (ECM) interactions that occur during PMN recruitment and migration. Therefore integrins represent an important source of signals received by PMNs throughout their exit from circulation and arrival into tissues [2,3]. PMN integrins can either be constitutively expressed ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_v\beta_3$ and $\alpha_9\beta_1$) or upregulated upon activation ($\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$). Moreover some constitutively expressed integrins, such as $\alpha_9\beta_1$ integrin, can be further upregulated upon stimulation [4]. The $\alpha_9\beta_1$ integrin is widely distributed in the human body and is expressed in airway epithelium, endothelial cells, hepatocytes, muscle cells and PMNs [5,6]. Multiple endogenous ligands have been identified for this integrin, including the inducible endothelial counter-receptor, vascular cell adhesion molecule-1 (VCAM-1) [7], and the extracellular matrix proteins tenascin C [8] and osteopontin [9]. This integrin was also shown to interact with ADAM (a disintegrin and metalloprotease) family members such as ADAM12 and ADAM15 through their disintegrin-like domain [10,11]. On PMNs the integrin $\alpha_9\beta_1$ is highly expressed and critical for adhesion and transendothelial migration through its interaction with VCAM-1 [7].

Integrin-coupled signaling events are generally initiated by activation of focal adhesion kinase (FAK). FAK is a non-receptor tyrosine kinase that upon activation can serve as a docking site for several signaling proteins such as c-Src, phosphatydylinositol-3-

Abbreviations: ADAM, a disintegrin and metalloprotease; ECM, extracellular matrix; Erk, extracellular signal-regulated kinase; FAK, focal adhesion kinase; JNK, c-Jun kinase; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol-3-kinase; PMN, polymorphonuclear leukocyte; VCAM-1, vascular cell adhesion molecule-1

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kinase (PI3K), Rho GTPase family members, Grb2, and p130^{CAS} [12-14]. Integrin-mediated cell adhesion and FAK activation can also activate the Ras/Raf/MAPK pathway in leukocytes including extracellular signal-regulated kinase (Erk) 1/2, c-Jun kinase (JNK), and p38 [15,16]. Activation of both PI3K and MAPK pathways in PMNs is largely associated with delayed spontaneous apoptosis. In fact, activation of integrin-coupled signaling pathways triggers intracellular events that are shared with classical anti-apoptotic signals. For example, granulocyte macrophage colony-stimulating factor (GM-CSF) delays PMN spontaneous apoptosis through the activation of PI3K and Erk [17], a signaling pathway also triggered by integrin engagement [18-20]. Moreover integrin engagement leads to activation and nuclear translocation of nuclear factor-κB (NF-κB) [21-23] an event triggered by cytokines and associated with inhibition of PMN apoptosis [24,25]. Further support for these findings comes from *in vitro* and *in vivo* studies showing that PMN-endothelial cell and PMN-ECM interactions mediated by integrins interfere with PMN life span, delaying their spontaneous apoptosis [26,27].

A recent study demonstrated that in PMNs mitochondria serves exclusively as a mediator of apoptosis [28]. The mitochondrial apoptotic pathway is initiated by translocation of pro-apoptotic proteins such as Bax from the cytosol to the mitochondrial membrane. Insertion of pro-apoptotic proteins on mitochondrial membrane leads to the release of cytochrome *c* (cyt c) from the mitochondria to the cytosol. Free cyt c associates with APAF-1 and triggers the assembly and activation of apoptosome, an event that culminates with the activation of caspase-9 and -3 [29,30]. Activation of the mitochondrial apoptotic pathway in PMNs can be modulated by upregulation of anti-apoptotic proteins such as Bcl-x_L. These anti-apoptotic proteins interact with pro-apoptotic proteins and prevent their insertion on mitochondrial membranes [30]. Synthesis of anti-apoptotic proteins in PMNs occurs in response to cytokines and other anti-apoptotic stimuli mainly through the activation of NF-κB [24,25].

These evidences suggest that integrin engagement can be a trigger to the activation of signaling pathways known to modulate PMN spontaneous apoptosis. Recently, Ross and colleagues have shown that $\alpha_9\beta_1$/VCAM-1 interaction inhibits PMNs apoptosis [23]. However the mechanistic details underling this process was left understudied and, to our knowledge, there is no detailed study on how $\alpha_9\beta_1$ integrin-coupled signaling pathways interact and how they converge to finally modulate PMN apoptosis. In this regard we sought to investigate the main signaling events triggered by $\alpha_9\beta_1$ integrin engagement and how these signaling pathways modulate the apoptotic program of human PMNs. To do so we used a recently described disintegrin, VLO5, isolated from *Vipera lebetina obtusa* venom [31]. Disintegrins are a family of low molecular weight, cysteine-rich peptides usually isolated from viper venoms. These peptides are able to recognize and bind with high affinity to specific integrins [32]. They are usually single-chain polypeptides (monomeric disintegrins) that express the Arg-Gly-Asp (RGD) motif within an amino acid hairpin maintained by disulfide bridges [33]. VLO5 is a member of the recently discovered group of dimeric disintegrins. VLO5 was shown to bind with high affinity to $\alpha_4\beta_1$, $\alpha_9\beta_1$ and $\alpha_4\beta_7$ integrins, of which only $\alpha_9\beta_1$ is expressed in human PMNs. Our data demonstrate that $\alpha_9\beta_1$ integrin engagement delays human PMN spontaneous apoptosis through the PI3K and MAPK-Erk pathways. We also show that integrin engagement leads to NF-κB nuclear translocation, upregulation of anti-apoptotic protein Bcl-x_L and degradation of pro-apoptotic protein Bad, which modulate the mitochondrial apoptotic pathway in human PMNs.

2. Materials and methods

2.1. Reagents

Freeze-dried *Vipera lebetina obtusa* venom was purchased from Latoxan Serpentarium (Rosans, France). VLO5 was purified from *V. l. obtusa* venom as described [31]. Annexin V–FITC was purchased from the Laboratório de Biologia Celular e Molecular (Universidade de São Paulo, SP, Brazil). RPMI-1640, 3,3-diaminobenzidine (DAB), N-formyl-methionyl-leucyl-phenylalanine (fMLP), DNAse and protease inhibitors were from Sigma-Aldrich. Anti-$\alpha_9\beta_1$ integrin (clone Y9A2) blocking antibody was from Chemicom International. Mouse monoclonal anti-actin, mouse monoclonal anti-phosphotyrosine, rabbit polyclonal anti-FAK, rabbit polyclonal anti-p85 PI3K, rabbit polyclonal anti-Erk-2, rabbit polyclonal anti-human NF-κB p65, rabbit polyclonal anti-Bcl-xL, rabbit polyclonal rabbit polyclonal anti-Bax (Δ21), goat polyclonal anti-cytochrome *c*, anti-α_M integrin blocking antibody and biotinilated donkey anti-goat IgG were from Santa Cruz Biotechnology. Percoll, biotinilated donkey anti-rabbit or biotinilated sheep anti-mouse IgG were from Amersham Biosciences. LY294002 and PD98059 were from Calbiochem. HRP-conjugated streptavidin was from Zymed.

2.2. Neutrophil isolation

PMNs were isolated from ethylenediaminetetraacetic acid (EDTA) (0.5%)-treated peripheral venous blood from healthy human volunteers using a four-step discontinuous Percoll gradient as described [34]. Residual erythrocytes were removed by hypotonic lysis. Isolated PMNs were resuspended in RPMI supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. PMN viability and purity were always >99% and >97%, respectively.

2.3. Assessment of Neutrophil Apoptosis

2.3.1. Morphology

PMNs (1×10^6 cells/mL) were analyzed using cytospin preparations (6 min, 400 rpm) of fresh or 18 h (37 °C, 5% CO_2) cultured cells. PMNs were stained with Diff-Quik™ and counted under light microscopy (×1000) to determine the proportion of cells showing characteristic apoptotic morphology. At least 300 cells were counted per slide. All experiments were performed at least in triplicate. The results are expressed as mean ± S D .

2.3.2. Annexin V binding assay

Phosphatidylserine exposure on apoptotic cell surface was analyzed by flow cytometry. Fresh or 18 h cultured PMNs were incubated with or without VLO5 (1 μM). Cells were then washed with binding buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) and incubated for 20 min with 100 μL of Annexin V–FITC (2 μg/mL) working solution. After incubation cells were stained with propidium iodide (PI, 100 μg/mL) for necrosis exclusion. PI and Annexin V binding were assessed on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using the CellQuest (BD Biosciences) software. All experiments were performed at least in triplicate.

2.3.3. DNA fragmentation

PMNs (5×10^6 cells/mL) were incubated for 8 h at 37 °C in the presence or absence of 1 μM VLO5. After incubation PMNs were lysed in 500 μL of lysis buffer (0.2% Triton X-100, 1 mM EDTA, pH 7.4). Cell lysates were then centrifuged at 13,000 g, and the supernatants (containing fragmented DNA) were collected. Supernatants were treated with 50 μL of 5 M NaCl and 500 μL of isopropanol and left for 12 h at −70 °C. DNA pellets were washed with 70% ethanol, air dried, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). Fragmented DNA was separated on a 1% agarose gel electrophoresis containing 1 μg/mL ethidium bromide. The products of DNA fragmentation were visualized and documented under UV light.

2.4. Immunoprecipitation

PMNs (5×10^6 cells/mL) were lysed in immunoprecipitation lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1.5 mM

EDTA, Triton X-100 1%, glycerol 10%, 10 μg/μL aprotinin, 10 μg/μL leupeptin, 2 μg/μL pepstatin and 1 mM PMSF). Lysates (2 μg of protein/μL) were incubated overnight at 4 °C with polyclonal anti-FAK (1:200) antibody. Protein A/G agarose (20 μL/mg protein; Santa Cruz Biotechnology) was then added, and samples were incubated at 4 °C on a shaker for 2 h. After incubation samples were centrifuged and pellets were resuspended in sample buffer. Samples were resolved on a 10% SDS-PAGE. FAK phosphorylation was assessed by Western blot using anti-phosphotyrosine antibody (1:200) and secondary polyclonal anti-mouse IgG (1:1000). Association of PI3K to FAK was assessed by Western blot using anti-p85 PI3K antibody (1:1000) and secondary polyclonal anti-IgG (1:1000).

2.5. Cellular nuclear extracts

PMNs (5×10^6 cells/mL) were lysed in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF). After a 15 min incubation on ice, Nonidet P-40 was added to a final concentration of 0.5% (v/v). After centrifugation (1810×g; 5 min at 4 °C), pellet was suspended in ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 μg/mL pepstatin, 1 μg/mL leupeptin, and 20% (v/v) glycerol) and incubated for 30 min at 4 °C. Supernatants containing nuclear proteins were collected after centrifugation (12,000×g; 10 min at 4 °C). Samples were resolved on a 12% SDS-PAGE and analyzed by Western blot using anti-human NF-κB p65 antibody (1:1000), anti-histone H3 antibody (1:1000) or anti-Erk-2 antibody (1:1000), and biotin-conjugated anti-rabbit IgG antibody (1:1000) or biotin-conjugated anti-goat IgG antibody (1:1000).

2.6. Subcellular fractionation

Subcellular fractions were obtained as previously described [28]. Briefly, PMNs (5×10^6 cells/mL) were washed and resuspended in ice-cold cytosol extraction buffer (250 nM sucrose, 70 mM KCl, 250 μg/mL digitonin, 10 μg/μL aprotinin, 10 μg/μL leupeptin, 2 μg/μL pepstatin and 1 mM PMSF in PBS). After a 15 min incubation on ice, samples were centrifuged at 1000× g for 5 min and supernatant was collected (cytosolic fraction). Pellets were then resuspended in mitochondrial lysis buffer (10 mM MgCl2, 2 mM EGTA, 2 mM EDTA, 1% Nonident P-40 (v/v), 10% glycerol (v/v), 10 μg/μL aprotinin, 10 μg/μL leupeptin, 2 μg/μL pepstatin and 1 mM PMSF in 50 mM Tris, pH 7.5), incubated for 10 min on ice and centrifuged at 10,000× g for 10 min. Supernatant was taken as the mitochondrial fraction. Cytosolic and mitochondrial fraction were resolved on a 15% SDS-PAGE and analyzed by Western blot using anti-human cytochrome *c* antibody (1:1000), anti-Bax (Δ21) antibody (1:1000) and secondary biotin-conjugated anti-rabbit IgG antibody (1:1000).

2.7. Total cell extracts

PMNs (5×10^6 cells/mL) were incubated with VLO5 (1 μM) at 37 °C in 5% CO_2 atmosphere for the indicated times. To obtain the whole cell extracts, cells were resuspended in lysis buffer (50 mM HEPES, pH 6.4, 1 mM MgCl2, 10 mM EDTA, 1% Triton X-100, 1 μg/μL Dnase, 10 μg/μL aprotinin, 10 μg/μL leupeptin, 2 μg/μL pepstatin and 1 mM PMSF) and incubated for 30 min at 4 °C. The contents of Bad and Bcl-xL proteins were analyzed by Western blotting as described bellow.

2.8. Western blotting analysis

The total protein content in samples was determined by the Bradford's method [35]. Samples were denatured in sample buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 5% 2-ME, 10% glycerol, and 0.001% bromophenol blue) and boiled for 3 min. Samples (30 μg of total protein) were resolved by SDS-PAGE and then transferred to polyvinylidenedifluoride (PVDF) membranes (Amersham Biosciences). Membranes were blocked with T-TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) containing 1% BSA and probed with the appropriate primary antibodies (1:1000). After extensive washing in T-TBS, membranes were incubated with the appropriate biotin-conjugated secondary antibodies (1:1000) for 1 h and then incubated with HRP-conjugated streptavidin (1:1000) for a further 1 h. Immunoreactive bands were visualized by DAB staining. Image capture was carried out using a HP PrecisionScan LTX and bands were quantified by densitometry using ImageJ 1.34 s software (Wayne Rasband, NIH, USA).

2.9. Determination of mitochondrial membrane potential ($\Delta\psi_m$)

The change in mitochondrial membrane potential was measured using the potentiometric dye JC-1 as described previously [36]. Briefly, PMNs (10^6 cells/mL) were incubated with VLO5 (1 μM) for 18 h at 37 °C. Cells were then incubated with 10 μg/mL JC-1 (Molecular Probes) for 30 min at 37 °C. Suspensions were analyzed immediately on a FACSCalibur flow cytometer for red and green fluorescence (Becton Dickinson). Data analysis was carried out using CellQuest software.

2.10. Statistical analysis

Statistical significance was assessed by ANOVA, followed by Bonferroni's *t* test, and $p<0.05$ was taken as statistically significant.

3. Results

3.1. VLO5 delays neutrophil spontaneous apoptosis through an $\alpha_9\beta_1$ integrin-dependent pathway

Adhesion to biological surfaces through cell adhesion molecules is a powerful activator of PMN, being primarily modulated by integrins. The integrin signaling pathways mediate important functions in leukocytes, including apoptosis [2]. The ability of VLO5 to modulate PMN spontaneous apoptosis was evaluated. As shown in Fig. 1A, VLO5 was able to inhibit PMN spontaneous apoptosis in a concentration-dependent manner as accessed morphologically. This effect was dependent on VLO5 interaction with $\alpha_9\beta_1$ as it could be reverted by pre-treatment of the cells with an anti-$\alpha_9\beta_1$ monoclonal blocking antibody (clone Y9A2). To further confirm this data DNA fragmentation analysis and Annexin V binding assay were preformed. VLO5 was able to prevent DNA fragmentation observed after culturing cells for 8 h (Fig. 1B). Moreover VLO5 inhibited phosphatidylserine exposure as accessed by FACS analysis of Annexin V staining (Fig. 1C and D). Fig. 1C shows histograms from one representative experiment, and Fig. 1D shows means from four independent experiments. The effect of VLO5 on phosphatidylserine exposure could be blocked by treatment of cells with an anti-$\alpha_9\beta_1$ blocking antibody (Fig. 1C and D). Annexin V binding assay was performed in the presence of PI and the proportion of cells undergoing necrosis was always lower than 3% (data not shown). Together these data suggest that VLO5 rescues PMNs from spontaneous apoptosis through the interaction with $\alpha_9\beta_1$ integrins.

3.2. Activation of integrin-coupled signaling pathways by VLO5 in human neutrophils

Integrin activation triggers autophosphorylation and activation of FAK that converts FAK into a docking site for PI3K activation. Activated PI3K is able to regulate several cellular processes such as migration, adhesion and apoptosis [13]. We then sought to investigate whether VLO5 could trigger the activation of this integrin-coupled signaling

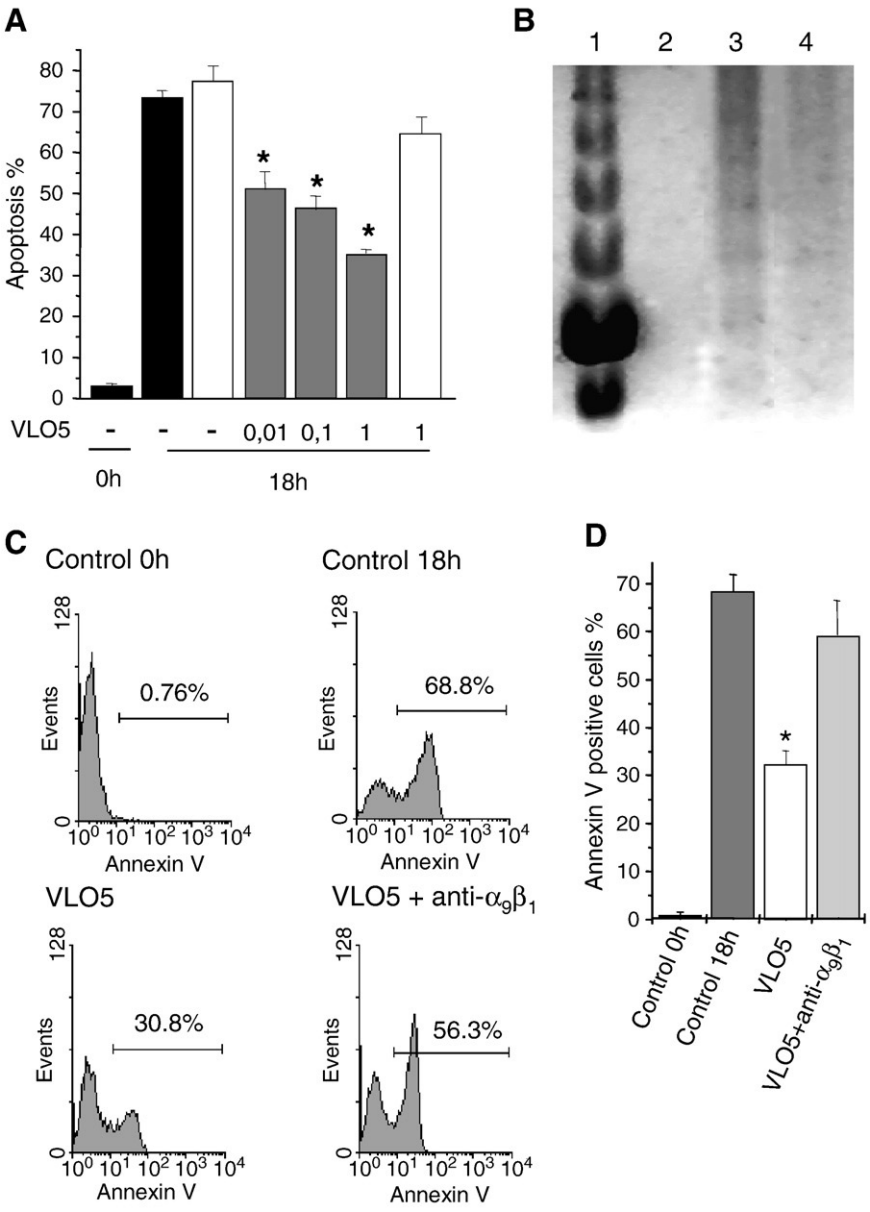

Fig. 1. VLO5 inhibit PMN spontaneous apoptosis. (A) Cells were pretreated for 5 min at 37 °C with medium alone (black and gray bars) or with the anti-$\alpha_9\beta_1$ blocking antibody (10 μg/mL, open bars) followed by incubation for 0 and 18 h at 37 °C in the absence (−) or presence of VLO5 (0 .01–1 μM). The number of apoptotic cells was assessed by cytology on cytocentrifuge preparations as described in Section 2. Data represent the means ± SD of at least 300 cells from four independent experiments. (B) Cells (5×10^6 cells) were incubated with RPMI medium (lane 3) or VLO5 (1 μM, lane 4) for 8 h, and then internucleossomal DNA fragmentation was determined as described in Section 2. Lane 2 represents nonapoptotic fresh PMNs, and lane 1 represents a molecular weight marker (kb). (C and D) Cells were pretreated for 5 min at 37 °C with medium alone or with the anti-$\alpha_9\beta_1$ blocking antibody followed by incubation for 0 and 18 h at 37 °C in the absence (Control 0 h, black bar and 18 h, gray bar) or presence of VLO5 (1 μM, VLO5, open bar and VLO5 + anti-$\alpha_9\beta_1$, light gray bar). The percentage of Annexin V-positive cells in each sample is indicated. C shows histograms from one representative experiment, and D shows means ± SD from four independent experiments. *P<0.05 compared to cells incubated for 18 h with medium alone or VLO5 + anti-$\alpha_9\beta_1$.

pathway in PMNs. Using immunoprecipitation and Western blotting analysis we could observe that VLO5 was able to induce FAK phosphorylation (Fig. 2A) and FAK–PI3K association (Fig. 2B).

Activation of FAK–PI3K through integrin signaling triggers the activation of the Ras–Raf–MAPK pathway leading to Erk-2 phosphorylation and subsequent nuclear translocation [16]. VLO5 was able to induce the translocation of Erk-2 to the nucleus in a PI3K-dependent manner as this effect could be blocked by the treatment of cells with the PI3K inhibitor LY294002. We could also block the effect of VLO5 on Erk1/2 nuclear translocation by the treatment of cells with anti-$\alpha_9\beta_1$ integrin blocking antibody (Fig. 2C). These data suggest that VLO5, interacting with $\alpha_9\beta_1$ integrin triggers integrin-coupled signaling pathways in PMNs.

3.3. VLO5 inhibition of neutrophil spontaneous apoptosis is dependent on PI3K and MAPK pathways

Activation of FAK-PI3K and MAPK pathways is known to inhibit apoptosis in several experimental settings and different cell types [37]. We tested whether the effects of VLO5 on PMN spontaneous apoptosis could be attributed to the activation of integrin-coupled signaling pathways. Pre-incubation of cells with PI3K inhibitor LY294002 or the MEK 1/2 inhibitor PD98059 reverted the VLO5 effect on PMN spontaneous apoptosis (Fig. 3A). As expected, PD98059 was able to inhibit Erk activation, as measured by Erk nuclear translocation (Fig. 3B) and LY294002 efficiently inhibited the activation of the PI3K downstream target, Akt, as measured by Akt

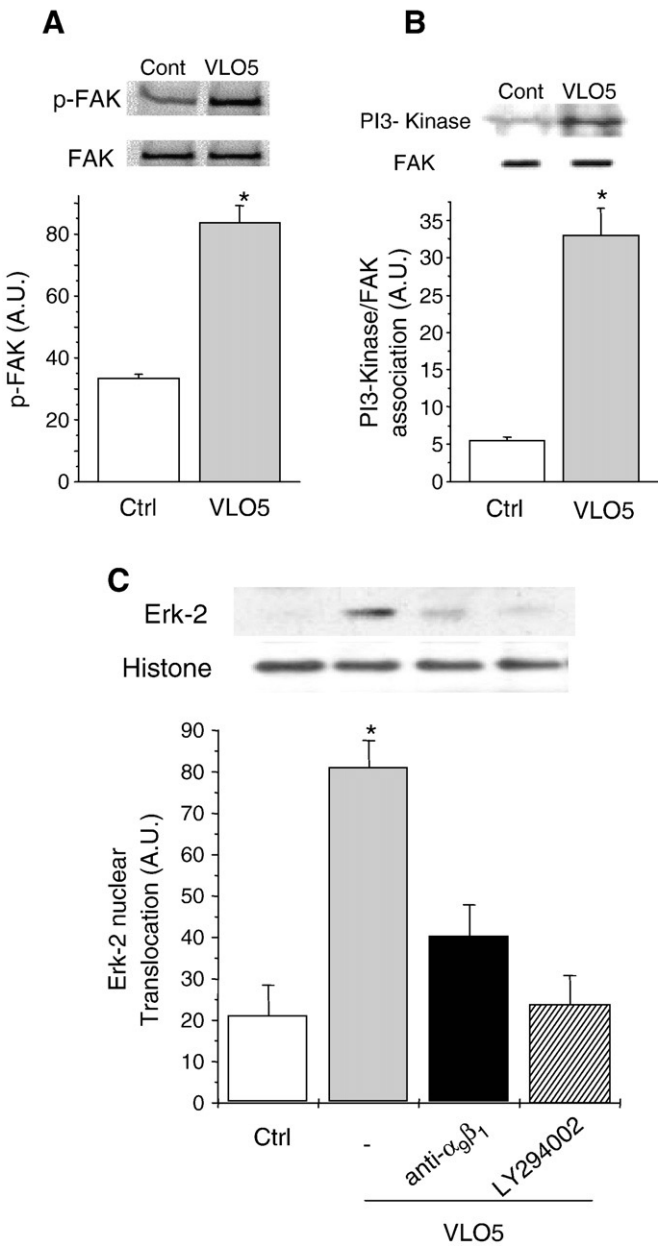

Fig. 2. VLO5 activates integrin-coupled signaling pathways in PMNs. (A and B) PMNs were treated with VLO5 (1 μM, gray bar) or medium (white bar) for 5 min at 37 °C, and immunoprecipitation was carried out as described in methods using an anti-FAK antibody. Samples were analyzed using an anti-phosphotyrosine antibody for p-FAK (A) and p85 PI3K (B), and total FAK was used as loading control for both samples. FAK phosphorylation and PI3K/FAK association were determined by immunoblotting and quantified by densitometry. (C) PMNs were pretreated for 5 min at 37 °C with medium alone or with LY294002 (3 μM), or with an anti-$\alpha_9\beta_1$ blocking antibody (10 μg/mL) followed by incubation for 1 h at 37 °C in the absence (white bar) or presence of VLO5 (1 μM, gray, black and dashed bars). Erk-2 nuclear content was determined by immunoblotting using an anti-Erk-2 antibody and quantified by densitometry. Data shows means ± SD of three similar experiments. *$P<0.05$ compared with control cells or compared with LY294002 and (C) anti-$\alpha_9\beta_1$ pretreated cells.

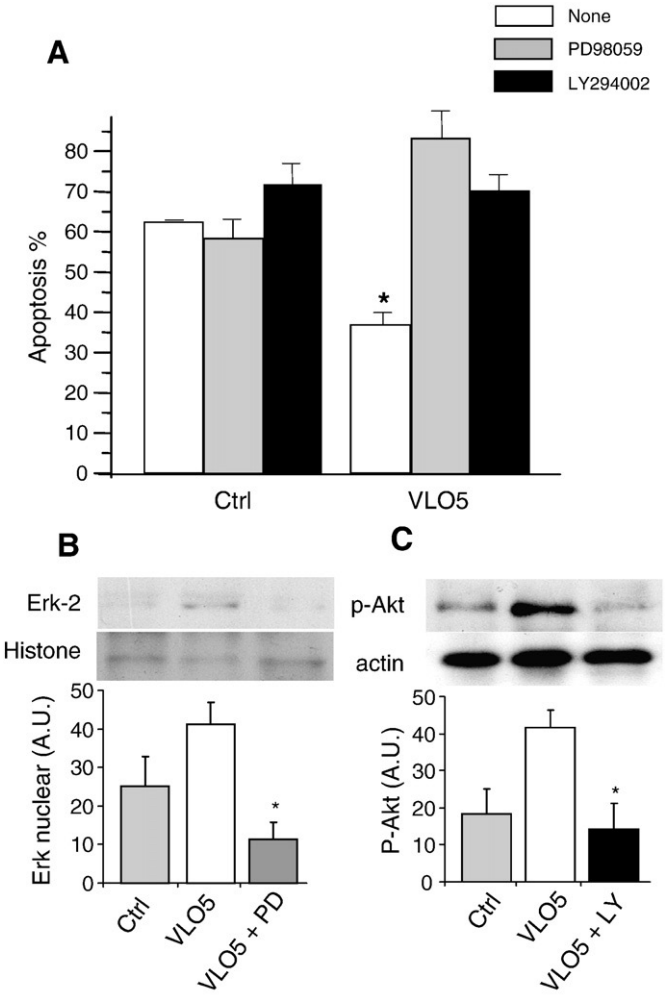

Fig. 3. Involvement of PI3K and MAPK pathways on the anti-apoptotic effects of VLO5. (A) Cells were pretreated for 5 min at 37 °C with medium alone (open bars), PD98059 (10 μM, light gray bars) or LY294002 (3 μM, dark gray bars) followed by incubation for 18 h at 37 °C in the absence (Ctrl) or in the presence of VLO5 (VLO5, 1 μM). The number of apoptotic cells was assessed by cytology on cytocentrifuge preparations as described in materials and methods. Data represent means ± SD of at least 300 cells from three independent experiments. *$P<0.05$ compared with control cells or compared with PD98059 and LY294002 pretreated cells. (B) Erk nuclear translocation in unstimulated PMNs (Ctrl), PMNs treated with VLO5 (VLO5) or VLO5 plus PD98059 (VLO5+PD). (C) Akt phosphorylation in unstimulated PMNs (Ctrl), PMNs treated with VLO5 (VLO5) or VLO5 plus LY294002 (VLO5+LY). Blots in B and C are from one representative out of three independent experiments. Graphs in B and C are the means ± SD of three independent experiments. * Represents significant differences relative to VLO5-stimulated PMNs.

phosphorylation (Fig. 3C). Furthermore PD98059 and LY294002 had no effect on the apoptosis of unstimulated PMNs (Fig. 3A).

3.4. VLO5 interferes with the mitochondrial apoptotic pathway

PMN spontaneous apoptosis involves the mitochondrial pathway which is triggered by the translocation of pro-apoptotic proteins such as Bax to the mitochondrial outer membrane. This leads to the dissipation of mitochondrial membrane potential ($\Delta\psi m$) and cytochrome *c* release which in turn triggers the formation of the apoptosome and the activation of caspase-9 and -3 [30]. To measure the $\Delta\psi m$ we used the potentiometric dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide). JC-1 exists as a monomer in the cytosol (green) and also accumulates as J aggregates in the mitochondria which stains red. On the other hand, in apoptotic cells mitochondrial membrane potential collapses and JC-1 does not accumulate as red aggregates in the mitochondria. Consequently apoptotic cells stain primarily green and can then be easily differentiated from healthy cells showing green and red staining [38]. As can be seen in Fig. 4, PMNs incubated for 18 h presented low red to green ratio, indicating $\Delta\psi m$ dissipation. VLO5 inhibited the dissipation of mitochondrial membrane potential increasing the ratio of red to green fluorescence intensity in flow cytometry analysis (Fig. 4, right panel). This effect was shown to be dependent on VLO5 and $\alpha_9\beta_1$ integrin interaction as it could be blocked by the anti-$\alpha_9\beta_1$ blocking antibody (Fig. 4). Dot-plot analysis

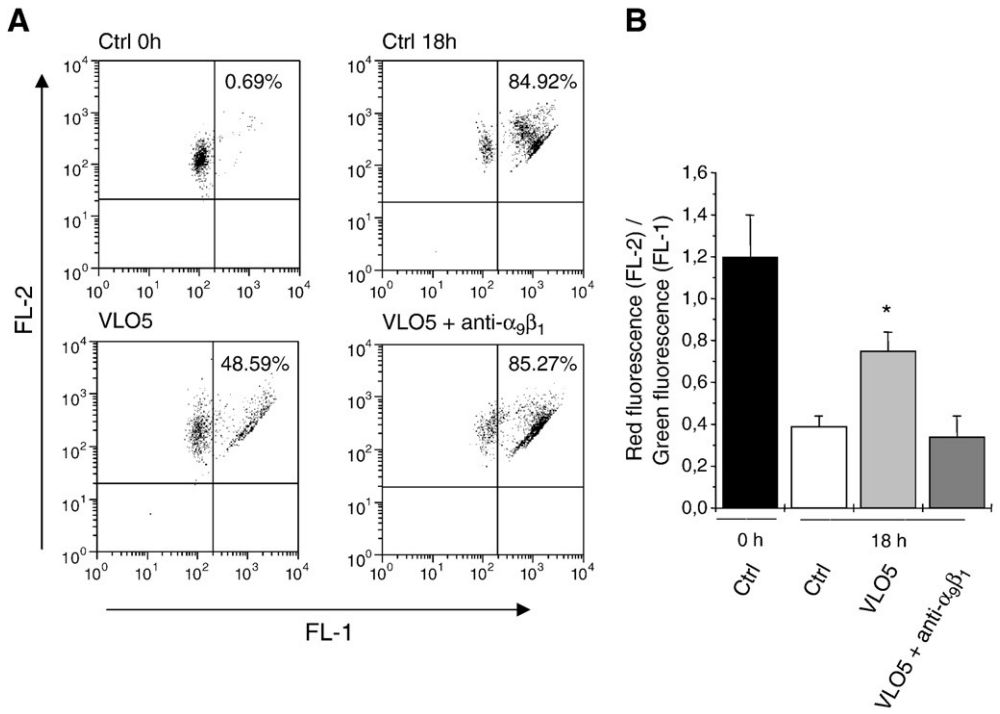

Fig. 4. Effect of VLO5 on mitochondrial membrane potential (Δψm). PMNs were pretreated for 5 min at 37 °C with medium alone (light gray and open bars) or with anti-$\alpha_9\beta_1$ blocking antibody (10 μg/mL, dark gray bar) followed by incubation for 18 h at 37 °C in absence (Cont 18 h) or in the presence of VLO5 (1 μM, VLO5 and VLO5 + $\alpha_9\beta_1$). Fresh PMNs (Cont 0 h, black bar) were used as control of mitochondrial membrane integrity. Cells were stained with 5 μg/mL of JC-1 dye for 30 min at 37 °C and analyzed by flow cytometry. The Δψm perturbation was determined by taking the ratio of mean fluorescence intensities of red to green florescence. Dot plots (left panel) are from one representative experiment and show the percent of double positive cells in fresh PMNs (ctrl 0 h), PMNs incubated for 18 h (ctrl 18hs), PMNs treated with VLO5 for 18 h (VLO5), and PMNs treated for 18 h with VLO5 plus anti-$\alpha_9\beta_1$ blocking antibody (VLO5 + anti-$\alpha_9\beta_1$). *P<0.05 compared to cells incubated for 18 h with medium alone or VLO5 + anti-$\alpha_9\beta_1$.

showed similar results. In this analysis, viable cells are represented by single green positivity cells while Δψm dissipation (or apoptosis) is represented by a shift to double-positive. As can be seen in Fig. 4 (left panel), PMNs incubated for 18 h present an increase in the percent of double-positive cells (viable cells) relative to freshly isolated PMNs, indicating increased Δψm dissipation. This could be reverted by incubation with VLO5. Consistently anti-$\alpha_9\beta_1$ blocking antibody inhibited the effects of VLO5.

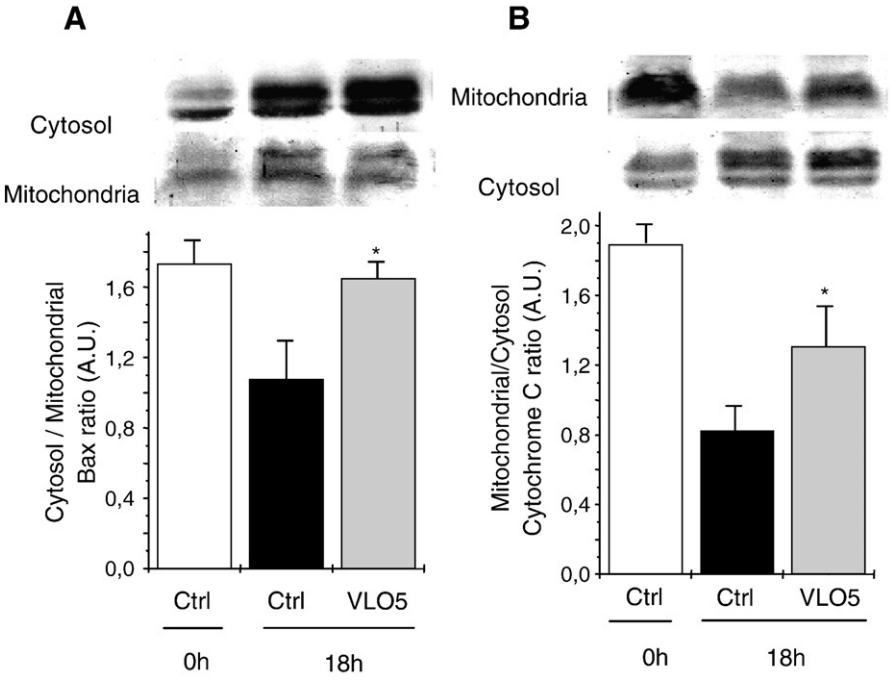

Fig. 5. Effects of VLO5 on Bax mitochondrial content and cytochrome *c* release to the cytosol. (A and B). PMNs were treated with VLO5 (1 μM, gray bar) or medium (white and black bars) for 0 and 18 h at 37 °C. PMN lysates were prepared by subcellular fractionation and cytosolic, and mitochondrial fractions were obtained as described in methods. The cytosolic and mitochondrial fractions were immunoblotted. (A) The samples were analyzed using anti-Bax antibody, and a cytosol/mitochondrial Bax ratio was obtained. (B) Samples were analyzed using anti-cytochrome *c* antibody, and mitochondrial/cytosol cytochrome *c* ratio was obtained. *P<0.05 compared to cells incubated for 18 h.

Dissipation of $\Delta\psi m$ can result from the translocation of pro-apoptotic proteins, such as Bax, to the mitochondrial membrane. This is an event that initiates early in apoptosis and increases throughout the process, being well detected in later time points [69]. Using subcellular fractionation we could further demonstrate that VLO5 was able to inhibit the translocation of pro-apoptotic protein Bax to the mitochondria (Fig. 5A) and cytochrome *c* release to the cytosol (Fig. 5B).

3.5. VLO5 induces NF-κB translocation to the nucleus

Activation of PI3K can lead to the activation of the Akt pathway which in turn can induce NF-κB nuclear translocation. NF-κB nuclear translocation triggers the expression of several target genes including the anti-apoptotic genes Bcl-2 and Bcl-x_L [21-25]. We showed that the anti-apoptotic effect of VLO5 was dependent on PI3K activation (Fig. 3). We then sought to investigate whether VLO5 could induce NF-κB nuclear translocation. As shown in Fig. 6, VLO5 increased NF-κB nuclear content relative to control. This effect was dependent on VLO5 interaction with $\alpha_9\beta_1$ as it could be reverted by pre-treatment of the cells with an anti-$\alpha_9\beta_1$ blocking antibody.

3.6. VLO5 promotes Bcl-x_L expression and Bad degradation

It is well demonstrated that activation of PI3K/Akt pathway results in a rescue from apoptosis by modulation of the balance between anti- and pro-apoptotic proteins in PMNs. PI3K/Akt pathway activation in PMNs results in Bad phosphorylation and proteasome-dependent degradation. This is a relatively rapid process that can be detected within 30 minutes. Akt activation also results in increased expression of the anti-apoptotic protein Bcl-x_L. This process depends on protein synthesis and is detected in later time points (2 and 4 h) [58]. We tested whether VLO5 could modulate the balance between anti- and pro-apoptotic proteins in PMNs. As shown in Fig. 7, VLO5 treatment diminished cellular content of the pro-apoptotic protein Bad (Fig. 7A)

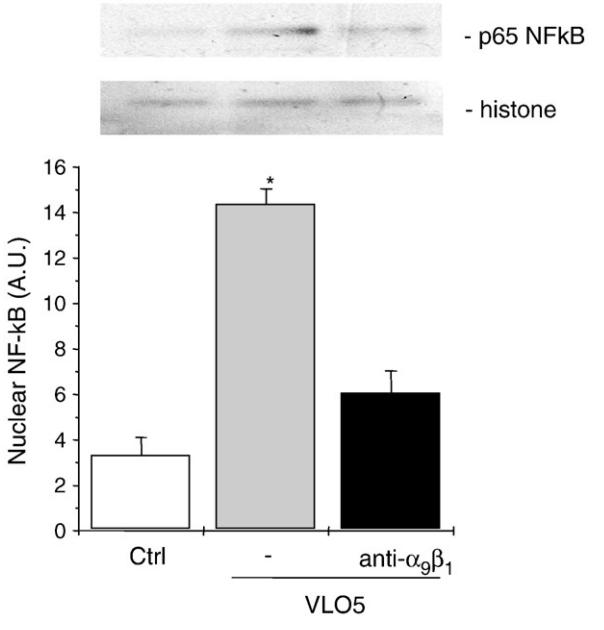

Fig. 6. Effect of VLO5 on NF-κB nuclear translocation. . PMNs were pretreated for 5 min at 37 °C with medium alone (open and gray bars) or with anti-$\alpha_9\beta_1$ blocking antibody (black bar) followed by incubation with VLO5 (1 μM, gray and black bars) or medium (open bar) for 90 min at 37 °C. Nuclear fractions were obtained as described in methods. Samples were separated on SDS-PAGE and immunoblotted for the detection of NK-κB p65 subunit. Histone H_3 content was used as loading control. Immunoblot figures are one representative out of three independent experiments. Graphs show means ± SD of bands intensity (arbitrary units) and * indicates significant difference compared to control or compared with anti-$\alpha_9\beta_1$ pretreated cells.

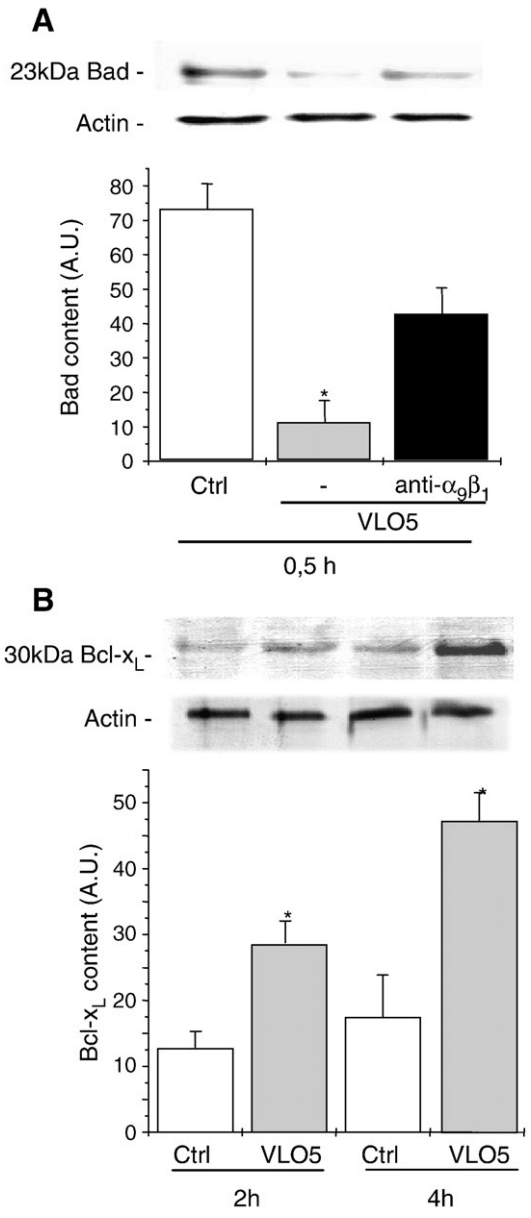

Fig. 7. Effect of VLO5 on total cellular content of Bad and Bcl-x_L. PMNs were pretreated for 5 min at 37 °C with medium alone (open and gray bars) or with anti-$\alpha_9\beta_1$ blocking antibody (black bar) followed by incubation with VLO5 (1 μM, gray and black bars) or medium (white bar) for the indicated times at 37 °C. Samples were subjected to SDS-PAGE and total Bad (A) or Bcl-x_L (B) content were evaluated by western blotting. Actin content was used as loading control. Immunoblot figures are one representative out of three independent experiments. Graphs show means ± SD of bands intensity (arbitrary units) and * indicates significant difference compared to control or compared with anti-$\alpha_9\beta_1$ pretreated cells.

and increased the content of the anti-apoptotic protein Bcl-x_L (Fig. 7B). The effect of VLO5 on Bad content was dependent on the engagement of $\alpha_9\beta_1$ integrin since pre-treatment with anti-$\alpha_9\beta_1$ blocking antibody could prevent the decrease in total Bad content. Taken together these data suggest that VLO5 modulates the mitochondrial apoptotic pathway in PMNs by increasing the anti- to pro-apoptotic protein ratio. This would inhibit pro-apoptotic protein translocation to the mitochondrial membrane, prevent the dissipation of mitochondrial membrane potential and cytochrome *c* release to the cytosol.

4. Discussion

PMN apoptosis is a critical cellular process that modulates PMN number and function playing an essential role in PMN homeostasis

and resolution of inflammation [39,40]. PMN lifespan can be extended by several stimuli including cytokines, growth factors, bacterial products [1]. Moreover PMN viability can be modulated by other molecular cues derived from the surrounding environment which include adhesion molecules expressed by endothelial cells and ECM proteins [1,2,41]. These factors are usually encountered *en route* and *in situ* during inflammatory conditions, and contribute to the maintenance of viable and fully functional PMNs at the site of inflammation.

PMNs express a large set of integrins including those constitutively expressed and those expressed upon activation [4]. The $\alpha_M\beta_2$ integrin is the most abundant integrin expressed by PMNs and therefore is the target of many studies addressing the potential modulatory role of integrin engagement on PMN function and viability [41]. The relative contribution of other integrins remains underinvestigated even though the possibility that their contribution as modulators of PMN functions can also be relevant.

In this sense $\alpha_9\beta_1$ integrin appears as a potentially important modulator of PMN function and viability. Expression of $\alpha_9\beta_1$ can be upregulated upon activation and it is a natural ligand for endothelial cell-expressed adhesion molecules and ECM-derived proteins. This implies that $\alpha_9\beta_1$ integrin engagement is likely to occur during all phases of PMN activity and therefore may contribute to the arrival and maintenance of long-lived tissue PMNs. Despite this the role of $\alpha_9\beta_1$ integrin as a modulator of PMN life span remains poorly studied. Ross and cols. have recently demonstrated that VCAM-1 inhibits PMN spontaneous apoptosis through the interaction with $\alpha_9\beta_1$ [23]. The molecular mechanisms involved in this process however were only superficially addressed and remained largely unknown. In the present study we evaluated the effects of $\alpha_9\beta_1$ integrin engagement on PMN apoptosis and the signaling pathways involved in this process. To do so we used a snake venom-derived disintegrin, VLO5, that binds to $\alpha_9\beta_1$, $\alpha_4\beta_1$, and $\alpha_4\beta_7$ integrins. We demonstrated that $\alpha_9\beta_1$/VLO5 interaction potently inhibited PMN spontaneous apoptosis through the activation of integrin-coupled signaling pathways.

In our preliminary experiments, we could not detect significant expression of $\alpha_4\beta_1$ integrin by resting PMNs (Supplementary Fig. 1), which is consistent with previous reports [23]. Other groups reported a low expression of $\alpha_4\beta_1$ integrin by resting PMNs that could be upregulated upon stimulation [70, 71]. However we could not detect significant expression of $\alpha_4\beta_1$ integrin by VLO5-stimulated PMNs (Suppl Fig. 1). On the other hand PMNs substantially expressed the integrin $\alpha_9\beta_1$ (Supplementary Fig. 1). Based on these results, we assumed that PMNs only express $\alpha_9\beta_1$ integrin among the three potential VLO5 ligands, and that the effects of VLO5 on PMNs were a consequence of $\alpha_9\beta_1$ engagement. This assumption found further support by the block of VLO5 effects when we used an anti-$\alpha_9\beta_1$ blocking antibody.

Initially described as potent antagonists of integrins deprived of intrinsic agonistic activity [42,43], disintegrins have recently been shown to interact with and activate integrin signaling pathways in some cell types [34,44,45]. These studies expanded the applications of disintegrins as tools for studying integrin biology demonstrating that these proteins can be used as integrin antagonists or integrin activators depending on the cell type investigated. Here we demonstrate that VLO5 can activate integrin-coupled signaling pathways on PMNs and that this effect could be blocked by co-incubation with blocking anti-$\alpha_9\beta_1$ integrin antibody. This suggests that VLO5 can be used as an $\alpha_9\beta_1$ integrin activator in PMN.

Recently our group demonstrated that the EC3 disintegrin interacts with and activates $\alpha_9\beta_1$ integrin in PMN but, in contrast to the observed inhibitory effect of VLO5, EC3 accelerated PMN apoptosis [46]. EC3 and VLO5 are heterodimeric disintegrins that present the Val-Gly-Asp (VGD) motif on its A-subunit and the Met-Leu-Asp (MLD) tripeptide in the B-subunit. The ligand selectivity and biological effects of EC3 and VLO5 has been attributed to the MLD sequence [47,48] while their differential effects have been attributed to amino acids surrounding the active MLD motif. Sequence analysis pointed to a single amino acid replacement N-terminally to the MLD motif: EC3 presenting an alanine and VLO5 a threonine (CKR*A*MLDGNDYC-EC3 and CKR*T*MLDGLNDYC-VLO5) [48]. Using synthetic peptides the authors demonstrated that VLO5 is a more potent inhibitor for $\alpha_9\beta_1$/VCAM-1 interaction than EC3, confirming data obtained for the native disintegrins [48]. The replacement of an apolar (alanine) by a polar (threonine) amino acid could result in the differential affinities observed that could explain their different biological effects despite the same specificities. Altogether these data suggest that when using disintegrins as tools for studying integrin biology it must be taken into account the cell type used and the nature of the interaction between the disintegrin and its cognate integrin.

VLO5 effects on PMN apoptosis were similar to those found using the natural $\alpha_9\beta_1$ integrin ligand VCAM-1 [23 and personal observation]. This suggests that VLO5 treatment mimics the events triggered *in vivo* by $\alpha_9\beta_1$ integrin engagement in PMNs. We focused on the effects of $\alpha_9\beta_1$ integrin engagement on PMN survival and demonstrate that VLO5 inhibited PMN spontaneous apoptosis through the activation of integrin-coupled signaling events.

FAK phosphorylation is pivotal to integrin-mediated signaling since this cytoplasmatic tyrosine kinase acts as a scaffold for several effector molecules such as Src, PI3K and the Ras/Raf/MAPK cascades [37,49]. FAK activation has been associated with survival signals due to the activation of the PI3K and the Ras/Raf/MAPK pathways [37,50-52]. Upon activation PI3K activates protein kinase B (Akt), which is intimately involved in apoptosis resistance, regulating the activity of several Bcl-2 family members [37,53-56].

We demonstrate that VLO5 induces FAK phosphorylation and PI3K-FAK association in PMNs. Moreover the delay in PMN spontaneous apoptosis triggered by VLO5 was dependent on PI3K activation. This adds new information to previous work by Ross and colleagues demonstrating that the inhibitory effect of VCAM-1 on PMN apoptosis was dependent on PI3K activation [23]. Furthermore we demonstrate that VLO5 induces Erk nuclear translocation and this effect is dependent on PI3K activation. The triggering of several intracellular signaling pathways linked to FAK and PI3K activation includes Ras/Raf-dependent Erk activation. Erk, a MAPK family member, is activated by tyrosine phosphorylation and translocates to the nucleus, affecting gene expression by directly phosphorylating transcription factors [57]. The MAPK pathway can control several PMN functions including cell survival [58-60].

PMN spontaneous apoptosis generally involves the intrinsic mitochondrial pathway. In fact the few mitochondria presented by PMNs appear to be sole modulators of PMN life span, having no apparent role in other PMN functions [28]. This intrinsic apoptotic pathway involves the translocation of pro-apoptotic proteins to the mitochondrial outer membrane and dissipation of mitochondrial membrane potential. These events lead to the leakage of cytochrome *c* to the cytosol and activation of the apoptosome, culminating in caspase-3 activation and apoptosis [61]. After 18 h of incubation a loss of mitochondrial transmembrane potential can be observed suggestive of a disruption of the outer membrane integrity and release of mitochondrial content into the cytosol. Activation of $\alpha_9\beta_1$ integrin signaling by VLO5 inhibits this process, preserving the transmembrane potential and consequently inhibits apoptosis. This effect seems to involve the modulation of cellular localization of the pro-apoptotic protein Bax, as VLO5 maintains Bax in the cytosol and prevents its translocation to the mitochondrial membrane. This consequently inhibits pore formation and cytochrome *c* release into the cytosol.

Different events can be involved in the cytosolic sequestration of pro-apoptotic proteins and consequent inhibition of apoptosis. Activation of the PI3K/Akt and the Ras/Raf/MAPK pathways can lead to Bad phosphorylation at specific serine residues. Phosphorylated Bad binds to 14-3-3ζ proteins in the cytosol that sequesters and tags Bad for subsequent degradation [62]. Alternatively, pro-apoptotic

proteins can be retained in the cytosol by binding to anti-apoptotic proteins as Bcl-2 and Bcl-xL [63]. We observed Bad degradation triggered by $\alpha_9\beta_1$ integrin activation in PMNs. This could be a consequence of PI3K/MAPK-dependent Bad phosphorylation and 14-3-3ζ-dependent degradation. On the other hand, we also found an increased expression of the anti-apoptotic protein Bcl-xL. In fact activation of the PI3K/Akt pathway was shown to increase Bcl-x_L transcription through NF-κB activation [64,65]. In this regard we show that VLO5 induces NF-κB nuclear translocation accompanied by increased Bcl-xL expression. Similarly Ross and cols. previously reported an $\alpha_9\beta_1$/VCAM-1-dependent NF-κB activation in PMNs [23]. This could increase the intracellular anti-/pro-apoptotic protein ratio and account for the inhibition of PMN apoptosis. Based in the above findings we may speculate that both 14-3-3ζ- and NF-κB-dependent processes can be operational in our system and both may respond to the anti-apoptotic effects of $\alpha_9\beta_1$ integrin engagement.

The molecular mechanisms triggered by $\alpha_9\beta_1$ integrin engagement that culminates in delayed PMN apoptosis resemble those described for other modulators of PMN life-span such as cytokines and growth factors. For example, granulocyte-macrophage-colony stimulating factor (GM-CSF)-inhibition of PMN apoptosis involves the activation of PI3K and MAPK pathways [66,67]. This suggests that cytokines, growth factors, integrins and other modulators of PMN survival can sum and potentiate their effects to maintain long-lived, functional PMNs in tissues. In fact it was recently demonstrated that granulocyte-colony stimulating factor (G-CSF) signaling can be enhanced by $\alpha_9\beta_1$ integrin signaling in PMNs [68]. However, how these factors cooperate in order to reach this goal in pathophysiological settings needs more profound studies.

5. Conclusion

By using the snake venom-derived disintegrin, VLO5, we could systematically study the role of $\alpha_9\beta_1$ integrin signaling in PMN survival. We provide strong evidences and detailed molecular mechanisms for the modulation of PMN spontaneous apoptosis by $\alpha_9\beta_1$ integrin engagement. This study has at least two important implications: (i) the detailed understanding of the mechanisms involved in the control of PMN life-span may provide potential targets for future therapies for diseases where PMNs play a determinant role; and (ii) snake venom disintegrins are suitable tools for the study of integrin biology and can be a potential template for the design of integrin modulating agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2010.03.012.

References

[1] H.U. Simon, Neutrophil apoptosis pathways and their modifications in inflammation, Immunol. Rev. 193 (2003) 101–110.
[2] A. Zarbock, K. Ley, Mechanisms and consequences of neutrophil interaction with the endothelium, Am. J. Pathol. 172 (2008) 1–7.
[3] K. Ley, C. Laudanna, M.I. Cybulsky, S. Nourshargh, Getting to the site of inflammation: the leukocyte adhesion cascade updated, Nat. Rev., Immunol. 7 (2007) 678–689.
[4] L. Lindbom, J. Werr, Integrin-dependent neutrophil migration in extravascular tissue, Semin. Immunol. 14 (2002) 115–121.
[5] E.L. Palmer, C. Ruegg, R. Ferrando, R. Pytela, D. Sheppard, Sequence and tissue distribution of the integrin alpha 9 subunit, a novel partner of beta 1 that is widely distributed in epithelia and muscle, J. Cell Biol. 123 (1993) 1289–1297.
[6] N.E. Vlahakis, B.A. Young, A. Atakilit, A.E. Hawkridge, R.B. Issaka, N. Bordreau, D. Sheppard, Integrin alpha9beta1 directly binds to vascular endothelial growth factor (VEGF)-A and contributes to VEGF-A-induced angiogenesis, J. Biol. Chem. 282 (2007) 15187–15196.
[7] Y.J. Taooka, T. Chen, T. Yednock, D. Sheppard, The integrin alpha9beta1 mediates adhesion to activated endothelial cells and transendothelial neutrophil migration through interaction with vascular cell adhesion molecule-1, J. Cell Biol. 145 (1999) 413–420.
[8] Y. Yokosaki, N. Matsuura, S. Higashiyama, I. Murakami, M. Obara, M. Yamakido, N. Shigeto, J. Chen, D. Sheppard, Identification of the ligand binding site for the integrin alpha9 beta1 in the third fibronectin type III repeat of tenascin-C, J. Biol. Chem. 273 (1998) 11423–11428.
[9] L.L. Smith, H.-K. Cheung, L.E. Ling, J. Chen, D. Sheppard, R. Pytela, C.M. Giachelli, Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by alpha9beta1 integrin, J. Biol. Chem. 271 (1996) 28485–28491.
[10] K. Eto, W. Puzon-McLaughlin, D. Sheppard, A. Sehara-Fujisawa, X.P. Zhang, Y. Takada, RGD-independent binding of integrin alpha9beta1 to the ADAM-12 and -15 disintegrin domains mediates cell-cell interaction, J. Biol. Chem. 275 (2002) 34922–34930.
[11] P. Lafuste, C. Sonnet, B. Chazaud, P.A. Dreyfus, R.K. Gherardi, U.M. Wewer, F.J. Authier, ADAM12 and alpha9beta1 integrin are instrumental in human myogenic cell differentiation, Mol. Biol.Cell 16 (2005) 861–870.
[12] D.D. Schlaepfer, S.K. Hanks, T. Hunter, P. van der Geer, Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase, Nature 372 (1994) 786–791.
[13] T.L. Shen, J.L. Guan, Differential regulation of cell migration and cell cycle progression by FAK complexes with Src, PI3K, Grb7 and Grb2 in focal contacts, FEBS Lett. 499 (2001) 176–181.
[14] R.L. Juliano, Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members, Annu. Rev. Pharmacol. Toxicol. 42 (2002) 283–323.
[15] E.H. Danen, K.M. Yamada, Fibronectin, integrins, and growth control, J. Cell Physiol. 189 (2001) 1–13.
[16] D.D. Schlaepfer, C.R. Hauck, D.J. Sieg, Signaling through focal adhesion kinase, Progr. Biophys. Mol. Biol. 71 (1999) 435–478.
[17] J.B. Klein, M.J. Rane, J.A. Sherzer, P.Y. Coxon, R. Kettritz, J.M. Mathiesen, A. Buridi, K. R. McLeish, Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways, J. Immunol. 164 (2000) 4286–4291.
[18] F.G. Giancotti, E. Ruoslahti, Integrin signaling, Science 285 (1999) 1028–1032.
[19] M.A. Williams, J.S. Solonkin, Integrin-mediated signaling in human neutrophil functioning, J. Leukoc Biol. 65 (1999) 725–736.
[20] B.B. Whitlock, S. Gardai, V. Fadok, D. Bratton, P.M. Henson, Differential roles for alpha(M)beta(2) integrin clustering or activation in the control of apoptosis via regulation of akt and ERK survival mechanisms, J. Cell Biol. 151 (2000) 1305–1320.
[21] C.H. Kim, K.H. Lee, C.T. Lee, Y.W. Kim, S.K. Han, Y.S. Shim, C.G. Yoo, Aggregation of beta2 integrins activates human neutrophils through the IkappaB/NF-kappaB pathway, J. Leukoc. Biol. 75 (2004) 286–292.
[22] R. Kettritz, M. Choi, S. Rolle, M. Wellner, F.C. Luft, Integrins and cytokines activate nuclear transcription factor-kappaB in human neutrophils, J. Biol. Chem. 279 (2004) 2657–2665.
[23] E.A Ross, M.R. Douglas, S.H. Wong, E.J. Ross, S.J. Curnow, G.B. Nash, E. Rainger, D. Scheel-Toellner, J.M. Lord, M. Salmon, C.D. Buckley, Interaction between integrin alpha9beta1 and vascular cell adhesion molecule-1 (VCAM-1) inhibits neutrophil apoptosis, Blood 107 (2006) 1178–1183.
[24] K. Wang, D. Scheel-Toellner, S.H. Wong, R. Craddock, J. Caamano, A.N. Akbar, M. Salmon, J.M. Lord, Inhibition of neutrophil apoptosis by type 1 IFN depends on cross-talk between phosphoinositol 3-kinase, protein kinase C-delta, and NF-kappa B signaling pathways, J. Immunol. 171 (2003) 1035–1041.
[25] S. François, J.E. Benna, P.M.C. Dang, E. Pedruzzi, M.A. Gougerot-Pocidalo, C. Elbim, Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kappaB signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated Bad, J. Immunol. 174 (2005) 3633–3642.
[26] R.W. Watson, O.D. Rotstein, A.B. Nathens, J. Parodo, J.C. Marshall, Neutrophil apoptosis is modulated by endothelial transmigration and adhesion molecule engagement, J. Immunol. 158 (1997) 945–953.
[27] H.M. McGettrick, J.M. Lord, K. Wang, G.E. Rainger, C.D. Buckley, G.B. Nash, Chemokine- and adhesion-dependent survival of neutrophils after transmigration through cytokine-stimulated endothelium, J. Leukoc. Biol. 79 (2006) 779–788.
[28] N.A. Maianski, J. Geissler, S.M. Srinivasula, E.S. Inemri, D. Roos, T.W. Kuijpers, Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis, Cell Death Differ 11 (2004) 143–153.
[29] S.J. Riedl, G.S. Salvesen, The apoptosome: signalling platform of cell death, Nat. Rev. Mol. Cell Biol. 8 (2007) 405–413.
[30] Y.L. Ow, D.R. Green, Z. Hao, T.W. Mak, Cytochrome c: functions beyond respiration, Nat. Rev., Mol. Cell Biol. 9 (2008) 532–542.
[31] J.J. Calvete, M.P. Moreno-Murciano, R.D.G. Theakston, D.G. Kisiel, C. Marcinkiewicz, Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulphide bond engineering, Biochem. J. 372 (2003) 725–734.
[32] M.A. McLane, C. Marcinkiewicz, S. Vijay-Kumar, I. Wierzbicka-Patynowski, S. Niewiarowski, Viper venom disintegrins and related molecules, Proc. Soc. Exp. Mol. Med. 219 (1998) 109–119.
[33] M.A. McLane, E.E. Sanchez, A. Wong, C. Paquette-Straub, J.C. Perez, Disintegrins, Curr. Drug Targets Cardiovasc. Haematol. Disord. 4 (2004) 327–355.

[34] A.L.J. Coelho, M.S. De Freitas, A.L. Oliveira-Carvalho, V. Moura-Neto, R.B. Zingali, C. Barja-Fidalgo, Effects of jarastatin, a novel snake venom disintegrin, on neutrophil migration and actin cytoskeleton dynamics, Exp. Cell Res. 251 (1999) 379–387.
[35] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[36] A. Cossarizza, M. Baccarani-Contri, G. Kalashnikova, C. Franceschi, A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), Biochem. Biophys. Res. Commun. 197 (1993) 40–45.
[37] D.G. Stupack, D.A. Cheresh, Get a ligand, get a life: integrins, signaling and cell survival, J. Cell Sci. 115 (2002) 3729–3738.
[38] F. Gianluca, D.A. Moulding, D.G. Spiller, R.J. Moots, M.H.R. White, S.W. Edwards, The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis, J. Immunol. 170 (2003) 1964–1972.
[39] M.A. Stark, Y. Huo, T.L. Burcin, M.A. Morris, T.S. Olson, K. Ley, Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17, Immunity 22 (2005) 285–294.
[40] P. Weinmann, K. Scharffetter-Kochanek, S.B. Forlow, T. Peters, B. Walzog, A role for apoptosis in the control of neutrophil homeostasis in the circulation: insights from CD18-deficient mice, Blood 101 (2003) 739–746.
[41] T.N. Mayadas, X. Cullere, Neutrophil beta2 integrins: moderators of life or death decisions, Trends Immunol 26 (2005) 388–395.
[42] R.J. Gould, M.A. Polokoff, P.A. Friedman, T.F. Huang, J.C. Holt, J.J. Cook, S. Niewiarowski, Disintegrins: a family of integrin inhibitory proteins from viper venoms, Proc. Soc. Exp. Biol. Med. 195 (1990) 168–171.
[43] B. Mercer, F Markland, C. Minkin, Contortrostatin, a homodimeric snake venom disintegrin, is a potent inhibitor of osteoclast attachment, J. Bone Miner. Res. 13 (1998) 409–414.
[44] M.R. Ritter, Q. Zhou, F.S. Markland Jr, Contortrostatin, a snake venom disintegrin, induces alphavbeta3-mediated tyrosine phosphorylation of CAS and FAK in tumor cells, J. Cell Biochem. 79 (2000) 28–37.
[45] A.L. Coelho, M.S. De Freitas, A. Mariano-Oliveira, A.L. Oliveira-Carvalho, R.B. Zingali, C. Barja-Fidalgo, Interaction of disintegrins with human neutrophils induces cytoskeleton reorganization, focal adhesion kinase activation, and extracellular-regulated kinase-2 nuclear translocation, interfering with the chemotactic function, FASEB J 5 (2001) 1643–1645.
[46] A.L. Coelho, M.S. De Freitas, A. Mariano-Oliveira, D.C. Rapozo, L.F. Pinto, S. Niewiarowski, R.B. Zingali, C. Marcinkiewicz, C. Barja-Fidalgo, RGD- and MLD-disintegrins, jarastatin and EC3, activate integrin-mediated signaling modulating the human neutrophils chemotaxis, apoptosis and IL-8 gene expression, Exp. Cell Res. 292 (2004) 371–384.
[47] C. Marcinkiewicz, Y. Taooka, Y. Yokosaki, J.J. Calvete, M.M. Marcinkiewicz, R.R. Lobb, S. Niewiarowski, D. Sheppard, Inhibitory effects of MLDG-containing heterodimeric disintegrins reveal distinct structural requirements for interaction of the integrin alpha 9beta 1 with VCAM-1, tenascin-C, and osteopontin, J. Biol. Chem. 275 (2000) 31930–31937.
[48] S. Bazan-Socha, D.G. Kisiel, B. Young, R.D. Theakston, J.J. Calvete, D. Sheppard, C. Marcinkiewicz, Structural requirements of MLD-containing disintegrins for functional interaction with alpha 4 beta 1 and alpha 9 beta1 integrins, Biochemistry 43 (2004) 1639–1647.
[49] J.L. Guan, D. Shalloway, Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation, Nature 358 (1992) 690–692.
[50] S.M. Frisch, K. Vuori, E. Ruoslahti, P.Y. Chan-Hui, Control of adhesion-dependent cell survival by focal adhesion kinase, J. Cell Biol. 134 (1996) 793–799.
[51] A.P. Gilmore, A.D. Metcalfe, L.H. Romer, C.H. Streuli, Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization, J. Cell Biol. 149 (2000) 431–446.
[52] J.H. Zhao, H. Reiske, J.L. Guan, Regulation of the cell cycle by focal adhesion kinase, J. Cell Biol. 143 (1998) 1997–2008.
[53] E. Kurenova, L.H. Xu, X. Yang, A.S. Baldwin Jr., R.J. Craven, S.K. Hanks, Z.G. Liu, W.G. Cance, Focal adhesion kinase suppresses apoptosis by binding to the death domain of receptor-interacting protein, Mol. Cell. Biol. 24 (2004) 4361–4371.
[54] Y. Leverrier, J. Thomas, A.L. Mathieu, W. Low, B. Blanquier, J. Marvel, Role of PI3-kinase in Bcl-X induction and apoptosis inhibition mediated by IL-3 or IGF-1 in Baf-3 cells, Cell Death Differ 6 (1999) 290–296.
[55] B.H. Lee, E. Ruoslahti, Alpha5beta1 integrin stimulates Bcl-2 expression and cell survival through Akt, focal adhesion kinase, and Ca2+/calmodulin-dependent protein kinase IV, J. Cell. Biochem. 95 (2005) 1214–1223.
[56] M.L. Matter, E. Ruoslahti, A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription, J. Biol. Chem. 276 (2001) 27757–27763.
[57] S. Yoon, R. Seger, The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions, Growth Factors 24 (2006) 21–44.
[58] M.A. Arruda, A.G. Rossi, M.S. de Freitas, C. Barja-Fidalgo, A.V. Graça-Souza, Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-kappaB, J. Immunol. 173 (2004) 2023–2030.
[59] M. Nakamae-Akahori, T. Kato, S. Masuda, E. Sakamoto, H. Kutsuna, F. Hato, Y. Nishizawa, M. Hino, S. Kitagawa, Enhanced neutrophil motility by granulocyte colony-stimulating factor: the role of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase, Immunology 119 (2006) 393–403.
[60] D.A. Sawatzky, D.A. Willoughby, P.R. Colville-Nash, A.G. Rossi, The involvement of the apoptosis-modulating proteins ERK 1/2, Bcl-xL and Bax in the resolution of acute inflammation in vivo, Am. J. Pathol. 168 (2006) 33–41.
[61] A. Oberst, C. Bender, D.R. Green, Living with death: the evolution of the mitochondrial pathway of apoptosis in animals, Cell Death Differ 15 (2008) 1139–1146.
[62] Q.B. She, D.B. Solit, Q. Ye, K.E. O'Reilly, J. Lobo, N. Rosen, The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells, Cancer Cell 8 (2005) 287–297.
[63] D.M. Finucane, E. Bossy-Wetzel, N.J. Waterhouse, T.G. Cotter, D.R. Green, Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL, J. Biol. Chem. 274 (1999) 2225–2233.
[64] S.J. Jeong, C.A. Pise-Masison, M.F. Radonovich, H.U. Park, J.N. Brady, Activated AKT regulates NF-kappaB activation, p53 inhibition and cell survival in HTLV-1-transformed cells, Oncogene 24 (2005) 6719–6728.
[65] E. Hatano, D.A. Brenner, Akt protects mouse hepatocytes from TNF-alpha- and Fas-mediated apoptosis through NK-kappa B activation, Am. J. Physiol. Gastrointest. Liver Physiol. 281 (2001) G1357–G1368.
[66] K. Yasui, Y. Sekiguchi, M. Ichikawa, H. Nagumo, T. Yamazaki, A. Komiyama, H. Suzuki, Granulocyte macrophage-colony stimulating factor delays neutrophil apoptosis and primes its function through Ia-type phosphoinositide 3-kinase, J. Leukoc. Biol. 72 (2002) 1020–1026.
[67] M. Derouet, L. Thomas, A. Cross, R.J. Moots, S.W. Edwards, Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis by increasing the stability of Mcl-1, J. Biol. Chem. 279 (2004) 26915–26921.
[68] C. Chen, X. Huang, A. Atakilit, Q-S Zhu, S.J. Corey, D. Sheppard, The Integrin alpha9beta1 contributes to granulopoiesis by enhancing granulocyte colony-stimulating factor receptor signaling, Immunity 25 (2006) 895–906.
[69] N.A. Maianski, F.P. Mul, J.D. van Buul, D. Roos, T.W. Kuijpers, Granulocyte colony-stimulating factor inhibits the mitochondria-dependent activation of caspase-3 in neutrophils, Blood 99 (2002) 672–679.
[70] J. Werr, X. Xie, P. Hedqvist, E. Ruoslahti, L. Lindbom, Beta1 integrins are critically involved in neutrophil locomotion in extravascular tissue In vivo, J Exp. Med. 187 (1998) 2091–2096.
[71] B. Johnston, P. Kubes, The alpha4-integrin: an alternative pathway for neutrophil recruitment? Immunol Today 20 (1999) 545–550.

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