The cytoplasmic domains of TNF- α converting enzyme (TACE/ADAM17) and L-selectin are regulated differently by p38 MAPK and PKC to promote ectodomain shedding

David J Killock^{*†} and Aleksandar Ivetic^{*†§}

^{*} Membrane/cytoskeleton research group, Cardiovascular Division, BHF/James Black Centre, King's College London, 125 Coldharbour Lane, London SE5 9NU, UK

[†] Department of Cardiovascular Sciences, National Heart and Lung Institute, Imperial College, Hammersmith Campus, DuCane Road, London W12 0NN, UK

[§] Corresponding author:

e-mail: alex.ivetic@kcl.ac.uk

Telephone: 0044 (0)20 7848 5196

Fax: 0044 (0)20 7848 5193

Running title: Divergent mechanisms of TACE-dependent L-selectin shedding

Keywords : Leukocyte, L-selectin, Ezrin/Radixin/Moesin, phosphatases, ectodomain shedding

L-selectin mediates the initial tethering and subsequent rolling of leukocytes along luminal walls of inflamed venules. TNF- α converting enzyme (TACE) is responsible for cleaving the membrane-proximal extracellular domain of L-selectin (also known as shedding), which reduces the efficiency of leukocyte recruitment to sites of inflammation. Many reports have highlighted roles for PKC and p38 MAPK in promoting L-selectin shedding with little insight in to the mechanism involved. By using phorbol myristate acetate (PMA) and the phosphatase inhibitors cantharidin and calyculin A, we could selectively activate PKC or p38 MAPK, respectively, to promote TACE-dependent shedding of L-selectin. Interestingly, the intracellular mechanisms leading to the shedding event differed dramatically. For example, regulatory elements within the L-selectin cytoplasmic tail, such as Ezrin/Radixin/Moesin (ERM)-binding and serine residues, were important for PKC- but not p38 MAPK-dependent shedding. Also, increased and sustained cell surface levels of TACE, and phosphorylation of its cytoplasmic tail (a hallmark of TACE activation), occurred in lymphocytes and monocytes following p38 MAPK activation. Finally, we showed that TNF- α -induced shedding of L-selectin in monocytes was strikingly similar to cantharidin-induced shedding and suggest that this newly characterised mechanism could be physiologically relevant in inflammatory cells.

Introduction

L-selectin is a cell adhesion molecule that mediates the initial capture and subsequent rolling of leukocytes along endothelial cell-expressed ligands that line the luminal wall of post-capillary venules [1, 2]. Leukocyte trafficking is strongly dependent on the expression levels of cell adhesion molecules on both leukocytes and endothelial cells [3]. Surface levels of L-selectin can be modulated at the transcriptional level [4] or post-translationally via ectodomain shedding [5], for example in response to TNF- α or lipopolysaccharide (LPS) challenge [6, 7]. The protease that mediates shedding of L-selectin is called TNF- α Converting Enzyme (TACE) [8], and studies using leukocytes and fibroblasts devoid of TACE activity (by virtue of a mutation engineered within the zinc-binding domain - termed TACE ^{Δ Zn/ Δ Zn}) reveal a role for this sheddase in both activation-dependent and basal shedding [9, 10]. The biological significance of L-selectin shedding is not well understood, but recent reports reveal a role in regulating the rolling velocity of neutrophils [5] as well as redirecting the traffic of antigen-activated effector memory T-cells away from lymph nodes [11]. Furthermore, significant levels of

soluble L-selectin is present in the plasma of healthy mice and humans (between 1.5 - 2 μg per ml) [12, 13], which increases significantly under certain disease states [14, 15]. Therefore, the presence of higher levels of soluble L-selectin in the circulation could potentially hinder leukocyte recruitment by reducing ligand availability for cell-associated L-selectin. Finally, shedding of L-selectin can be very rapid (within minutes), providing a potential mechanism to downregulate signalling through L-selectin during adhesion.

The use of artificial inducers of L-selectin shedding, such as phorbol myristate acetate (PMA), has contributed significantly to our understanding of the molecular basis underlying shedding [16]. PMA directly activates PKC isozymes, which have been shown to phosphorylate serine residues in the L-selectin tail [17, 18]. Other inducers of L-selectin shedding, which include hypertonic stress [19], intracellular acidification [20] and mechanical shedding of L-selectin (i.e. the loss of L-selectin during neutrophil rolling) [21] have all been shown to be p38 MAPK-dependent. However, the intracellular mechanisms that lead to shedding are poorly understood. In light of this, understanding how PKCs and p38 MAPK contribute to L-selectin shedding became the main focus of this report. Using cantharidin and PMA to selectively activate p38 MAPK and PKC, respectively, we show that TACE-dependent shedding of L-selectin occurs via different signalling pathways. This suggests that shedding can occur via two distinct mechanisms. Our findings are of particular importance to understanding how L-selectin is shed in response to diverse outside-in signals, for example during neutrophil rolling [21] or during T-cell receptor activation [18].

Experimental

Chemicals

All chemicals were purchased from Sigma unless otherwise stated. PMA was dissolved in ethanol, cantharidin and calyculin A were dissolved in DMSO. Stock concentrations of cantharidin, calyculin A and PMA were kept at 250 mM, 20 μM and 32.5 μM , respectively. Phosphatidic acid was obtained from Avanti Polar Lipids, Inc. Ro-31-8220 was obtained from Calbiochem, and SB202190 and PD98059 from LC Laboratories. Ro-31-9790 was a kind gift from A. Ager (Cardiff University). Inhibitors were dissolved in DMSO with the exception of Ro-31-8220, which was dissolved in sterile water.

Cells

The murine 300.19 pre-B cell line, stably expressing WT and R357A forms of human L-selectin have been described previously [22]. Stable 300.19 pre-B cell lines expressing sheddase resistant ($\Delta\text{M-N}$) and cytoplasmic serine mutated forms of L-selectin were generated and cultured as previously described for R357A cells [22]. These cells were grown in RPMI-1640 medium containing L-glutamine (Invitrogen) supplemented with 10% foetal calf serum, 1% v/v of 100 units/mL penicillin and streptomycin, 50 μM β -mercaptoethanol and 2 $\mu\text{g}/\text{mL}$ puromycin. Cells were maintained at 37°C, 5% CO_2 under humidifying conditions and seeded overnight at a density of 0.5×10^6 cells per ml before each experiment.

Peripheral blood leukocytes were isolated from human peripheral blood collected from healthy volunteer donors. 25 mL of neat or modified Hank's Buffered Saline Solution (mHBSS) (Invitrogen) diluted blood was layered on 15 mL of Histopaque-1077 in sterile 50 mL falcon tubes and sedimented by centrifugation for 30 minutes at room temperature (with no break). Mononucleated cells were aspirated from a Buffy coat layer and washed three times in modified HBSS. The erythrocyte/neutrophil containing 'pellet' was resuspended in mHBSS (1:1) and then diluted with sterile filtered 2% Dextran-500 (Amersham Biosciences) in mHBSS (1:1). The erythrocytes were allowed to sediment for 20 minutes at room temperature before the upper, neutrophil containing layer was collected, diluted in mHBSS and harvested by centrifugation. The cell pellet was then resuspended in 3mL of sterile water for 30 seconds to lyse remaining red blood cells. After lysis the

suspension was diluted to 50mL with mHBSS and centrifuged at 1150rpm for 5 minutes with a low break. Neutrophil and mononuclear cell pellets were resuspended in 10% FCS in Hank's Buffered Saline Solution (HBSS) (Invitrogen) supplemented with 25 mM HEPES, counted and used immediately

MEFs derived from wildtype or TACE^{ΔZn/ΔZn} murine embryos (kind gift from Roy Black – Amgen, Seattle) were maintained in RPMI-1640 medium containing L-glutamine supplemented with 10% foetal calf serum, 1% v/v of 100 units/mL penicillin and streptomycin. Further information on these cells can be found in [23].

Antibodies and plasmids

The murine anti-human L-selectin antibody, DREG56, which recognises the lectin domain, was purified from the HB-300 hybridoma. A mouse IgG₁ isotype control antibody was obtained from Santa Cruz Biotechnology, Inc. Rabbit anti-mouse immunoglobulin F(ab')₂ conjugated to R-Phycoerythrin (RPE) (DAKO) was used as a secondary for DREG56 or isotype control antibody. Phycoerythrin-conjugated DREG56 (DREG56-PE) and isotype control antibodies were purchased from Santa Cruz. Mouse IgG_{2a}-FITC isotype control and mouse anti-human CD11a-FITC antibodies were both obtained from Serotec. Antibody recognising the cytosolic portion of TACE (Clone C15 sc-6410) was used for immuno-precipitation and western blotting (Santa Cruz). Anti-moesin clone C-15 sc-6416 (Santa Cruz), anti-p38, anti-phospho-p38 (which recognises phospho-Thr 180/Tyr 182), anti-MAPK substrate (anti-phospho-threonine-proline) and anti-phosphorylated ERM (Cell Signalling Technology) antibodies were used for western blotting. Anti-human TACE ectodomain antibody was obtained from R&D systems. Goat anti-rabbit and rabbit anti-goat horseradish peroxidase conjugated secondary antibodies were purchased from DAKO. For western blotting of immuno-precipitated proteins anti-rabbit TrueblotTM secondary antibodies (eBioscience), which preferentially recognise the non-reduced primary antibody rather than the reduced immuno-precipitating antibody, were used where possible.

Expression vectors for WT and R357A human L-selectin were described previously [22]. ΔM-N and serine mutants of L-selectin were generated using standard mutagenesis techniques as instructed by Stratagene's Quick Change® XL II kit. Briefly, the cDNA of WT human L-selectin was amplified by PCR from a plasmid containing the cDNA of WT human L-selectin (Origene, Rockville MD) in to pMT2 vector. Subsequently, complementary primers were designed to exclude codons for methionine 325 up to asparagine 332. Resultant plasmids were sequenced to verify successful deletion/substitution of amino acids within the L-selectin tail..

L-selectin and LFA-1 shedding assays

Approximately 5x10⁵ to 1x10⁶ cells were treated in 200 μL of medium (10% FCS in HBSS with 25 mM HEPES or growth medium for leukocytes/U937s and 300.19/MEF cells, respectively) supplemented with either PMA, cantharidin, calyculin A, phosphatidic acid, human TNF-α (R&D systems), LPS (Invitrogen) or equivalent amounts of the appropriate carrier at 37°C and 5% CO₂ for 30 minutes. Any inhibitor pre-treatment was also carried out at 37°C and 5% CO₂ for 30 minutes but in 100 μL of medium before another 100 μL of 2x concentrated solutions of the desired stimulus (PMA, cantharidin, etc.) was added for another 30 minutes. The stimulation solutions also contained the appropriate inhibitor to maintain the concentration upon dilution of the cell suspension. Inhibitors used were: 100μM Ro-31-9790 (metalloprotease inhibitor); pan-PKC inhibitor Ro-31-8220 (5μM); p38 inhibitor, SB202190 (25μM); MEK inhibitor, PD98059 (50μM). Cell surface L-selectin or LFA-1 expression was then determined by flow cytometry.

Flow cytometric analysis

Cells were labelled with antibody on ice for 30 minutes. All washes were performed using 10% FCS in HBSS with 25mM HEPES for leukocytes/U937 cells or 1% BSA in RPMI-1640 for 300.19/MEF cell lines. Cells were first washed and then incubated with 150 μL 10% FCS in HBSS for

leukocytes/U937s in order to block any Fc receptors for 30 minutes on ice. Cells were subsequently washed and labelled with either DREG56-PE or IgG₁-PE isotype control both at 1:80 in 50 μ L (approximately 250 ng/million cells). For dual labelling experiments anti-CD11a-FITC or a FITC-labelled isotype control was also used at 1:80. Alternatively, cells were resuspended in 50 μ L of anti-TACE ectodomain (1 μ g/well) or an equivalent concentration of mouse IgG₁ control antibody, followed by incubation in 50 μ L of anti-mouse RPE-labelled secondary antibody at 1:20. MEF and 300.19 cell lines were incubated with 50 μ L of DREG56 or mouse IgG₁ isotype control antibody at 1:80 followed by incubation in 50 μ L of anti-mouse RPE-labelled secondary at 1:20. Cells were washed three times before analysis of protein expression by flow cytometry. Neutrophil, peripheral blood lymphocyte and monocyte populations were identified based on their characteristic forward and side scatter profiles. For cell lines, only live cells were analysed and were determined based on their distinctive scatter pattern (confirmed by annexin V staining). A minimum of 10,000 cells were counted for each experiment. Data from flow cytometry experiments are represented as percentage of L-selectin remaining relative to carrier-only treated wildtype cells after correction for background fluorescence with isotype control antibodies. For kinase inhibitor studies data is displayed as percentage of L-selectin shed, determined by subtracting the percentage of L-selectin remaining after stimulation and kinase inhibitor pre-treatment from the percentage of L-selectin remaining after kinase inhibitor pre-treatment without cell stimulation. This calculation normalised for changes in cell fluorescence derived from individual inhibitors. Finally, all values were normalised against IgG isotype controls.

Enzyme-linked Immunosorbent Assay (ELISA) for soluble L-selectin

500 μ L of 300.19 cells expressing WT human L-selectin resuspended in 1% BSA in RPMI at a density of 5×10^5 cells/mL were left untreated or treated with 1 nM, 10 nM, 100 nM or 1 μ M PMA, 100 μ M, 250 μ M, 500 μ M or 1 mM cantharidin or 20 nM, 40 nM or 80 nM Calyculin A for 30 minutes at 37°C and 5% CO₂. Cell-free supernatants were then obtained by centrifugation at 14,000 rpm for 5 minutes at 4°C. Equal volumes (100 μ L) of cell-free supernatant from each sample were assayed for the presence of soluble L-selectin (sL-selectin) using a commercially available ELISA kit (R & D systems). All samples were performed in triplicate. Soluble L-selectin levels were determined by comparison to a standard curve created using known quantities of soluble L-selectin standards. All standards were run in duplicate and the mean value used for each experiment.

Immunoprecipitation

For the immunoprecipitation of TACE approximately $0.5-1 \times 10^8$ U937 cells were harvested by centrifugation at 1200 rpm after stimulation in 10 mL of growth media for 30 minutes at 37°C and 5% CO₂ (with or without prior incubation of SB202190 for 30 minutes). The pellet was subsequently lysed in 1 mL of ice-cold lysis buffer (1% NP40, 20 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM NaF, protease inhibitor cocktail (Roche), 0.1 mM Na₃VO₄, 1 mM PMSF, 4 mM EDTA) supplemented with 25 nM Calyculin A on ice for 15 minutes (note that calyculin A was supplemented in to the lysis buffer to prevent dephosphorylation of endogenous ERMs after stimulation). Lysates were clarified by centrifugation at 14,000 rpm and 4°C for 10 minutes. A small fraction of the lysate was retained for loading control whilst the remaining lysate was pre-cleared with 25 μ L of pre-equilibrated protein G-agarose beads (Santa Cruz) at 4°C under continuous agitation for 1 hour. The beads were pelleted by centrifugation at 14,000 rpm for 5 minutes at 4°C and the lysate transferred to a fresh tube containing 5 μ g of anti-TACE cytoplasmic domain antibody, or an unrelated isotype matched control (goat polyclonal anti-moesin), before the addition of 25 μ L of pre-equilibrated protein G-agarose beads followed by an incubation for a further 2 hours (under continuous agitation at +4°C). The immunoprecipitate was washed once in lysis buffer supplemented with calyculin A (1 mL) and then a further three times with lysis buffer. Proteins bound to the beads were solubilised by boiling in protein

sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.2% bromophenol blue, 1.8% β -mercaptoethanol).

Immunoblotting

This procedure was conducted as previously described [24].

In brief, cells were stimulated in 1 mL of growth media at a density 5×10^6 per mL for 30 minutes at 37°C and 5% CO₂. Cells were subsequently lysed and the equivalent of approximately 1×10^6 cells was resolved in each lane. All primary antibodies were used at 1:1,000 as per manufacturers' instructions and all secondary antibodies were used at 1:5,000.

Transfection of U937s and MEFs

Microporation was performed using a Digital Bio in-tip electroporation apparatus. Cells were grown in antibiotics free medium for at least 24 hours before microporation to increase viability and transfection efficiency. For microporation of MEFs, cells were seeded the previous day at 70% confluency and harvested using trypsin-EDTA, centrifugation at 1,000 rpm, and washed once in PBS. Transfection was performed as instructed by the manufacturers. 1×10^6 cells in 10 μ L were transfected with 2 μ g of WT or Δ M-N L-selectin plasmid. Each 10 μ L electroporation was placed into a separate well of a 6-well plate containing 2 mL of antibiotics free medium and allowed to recover overnight. All transfected cells were pooled in to a single population prior to each shedding assay to ensure that a single equivalent population of cells was used for each treatment. Trypsinisation of MEFs had no effect on L-selectin expression (data not shown).

Results

Inhibition of serine/threonine phosphatases induces the rapid loss of cell surface-associated L-selectin

The role of serine/threonine phosphatases in regulating the shedding of L-selectin has never been addressed before. We therefore sought to determine whether inhibition of phosphatases could lead to L-selectin shedding. Cantharidin is a natural compound derived from the blister beetle and has been used in a study to elicit the *in vivo* migration of leukocytes from human blood in to skin blisters [25]. Cantharidin is known to inhibit the PP1/PP2A family of phosphatases [26]. Interestingly, a recent study has shown that ectodomain shedding of the integrin $\alpha_L\beta_2$ (LFA-1) and L-selectin occurs in blister-resident neutrophils and monocytes [25]. However, the study never determined whether the effect was due to direct exposure to cantharidin [25, 27]. To this end, freshly isolated primary leukocytes were challenged with increasing doses of cantharidin for 30 minutes. The dose of cantharidin required to induce skin blisters was calculated to be approximately 4 mM [25]. Therefore, doses below 2mM were used to determine the direct effect of cantharidin on L-selectin expression. It should be noted that cantharidin was poorly soluble in DMSO, which resulted in high working concentrations. The doses of cantharidin used within a 30 min time scale was non-toxic as replenishing cells in normal growth media after stimulation did not result in cell death (see supplementary figure 1).

Flow cytometric analysis revealed a dose-dependent reduction of surface L-selectin in response to increasing concentrations of cantharidin (Figure 1A-C). In contrast, cell surface levels of LFA-1 remained relatively unchanged in response to cantharidin stimulation (Figure 1A-C). These results suggest that direct exposure of cantharidin to primary leukocytes results in the potent loss of L-selectin but not LFA-1 expression. Cantharidin treatment also led to a dose-dependent loss in L-selectin expression in murine 300.19 pre-B cells stably expressing human L-selectin (supplementary figure 1). Finally, the use of an alternative PP1/PP2A inhibitor, calyculin A, produced similar results (supplementary figure 1), suggesting that phosphatase inhibitors can dramatically affect L-selectin expression levels. Interestingly, the concentration range of calyculin A used to induce the loss in L-

selectin expression was close to concentrations known to inhibit phosphatase activity (i.e. between 10-40 nM, see supplementary figure 1I).

Further evidence for the phosphatase inhibitory effects of cantharidin was demonstrated by analysing C-terminal phosphorylation of ERM. Treatment of lymphocytes with calyculin A has been shown to induce rapid C-terminal phosphorylation of ERM proteins [28], which is a hallmark of ERM activation [29]. Replacing calyculin A with cantharidin (100-500 μ M) also led to a marked increase in C-terminal phosphorylation of ERM in 300.19 pre-B lymphocytes (Figure 1D), which was concordant with the loss of L-selectin expression (compare results in Fig. 1D with results in supplementary figure 1). Based on this approach it was apparent that phosphatase inhibition could be achieved using cantharidin concentrations ranging between 100-500 μ M. This concentration range was therefore used in subsequent studies to effectively inhibit phosphatase activity. Collectively, these results confirm that inhibition of serine/threonine phosphatases promotes the rapid loss of cell surface-expressed L-selectin, which implies that such phosphatases are constitutively active in resting leukocytes and likely act, in part, to negatively regulate shedding.

Cantharidin-induced loss of L-selectin expression is mediated by a metalloproteinase activity

Antigen-capture ELISA was next used to determine whether the loss of surface-expressed L-selectin in response to phosphatase inhibition was due to the release of soluble L-selectin. Stimulation of 300.19 pre-B cells stably expressing WT L-selectin with increasing doses of PMA, calyculin A or cantharidin led to a dose-dependent increase in the liberation of soluble L-selectin (Figure 2A). Interestingly, the levels of soluble L-selectin liberated in to the cell-free supernatant was independent of the stimulus used, suggesting that the extent of shedding was comparable.

Shedding of L-selectin is known to occur via TACE, which can be inhibited by the zinc-dependent metalloproteinase inhibitor, Ro-31-9790 [30]. To verify that cantharidin-induced loss of L-selectin expression occurs via the action of a metalloproteinase, 300.19 pre-B cells stably expressing WT L-selectin were preincubated with 100 μ M Ro-31-9790 for 30 minutes prior to stimulation with cantharidin. Pretreatment with Ro-31-9790 markedly reduced the extent to which L-selectin was downregulated in response to either PMA, calyculin A or cantharidin stimulation (supplementary Figure 2). Shedding of L-selectin occurs at a defined extracellular membrane-proximal domain that is located approximately ten amino acids from the plasma membrane [31]. To further confirm that the shedding of L-selectin occurs at the known cleavage site, 300.19 pre-B cell lines stably expressing either WT L-selectin or a sheddase-resistant form of L-selectin (called Δ M-N) were subjected to either cantharidin or PMA stimulation. This construct has been characterised in previous studies and was demonstrated to effectively inhibit PMA-induced shedding [32]. As expected, WT L-selectin was efficiently shed in response to challenge with either stimulus (Figure 2B&C). In contrast, neither cantharidin nor PMA could induce shedding in cell lines expressing Δ M-N L-selectin, suggesting that correct positioning of the L-selectin cleavage site is critical for cantharidin-induced shedding (Figure 2B&C). Moreover, our results exclude the possibility that cantharidin-induced loss of L-selectin expression could be due to internalisation or microparticle release. Collectively, these results strongly imply that cantharidin induces *bona fide* shedding of L-selectin, and suggests that the mechanism of cleavage may be similar to PMA-induced shedding of L-selectin.

Cantharidin-induced shedding of L-selectin is TACE-dependent

Attempts to knock down TACE using siRNA has proved to be challenging in suspension cell lines (for example in U937 promonocytic cells – unpublished, Killock and Ivetic 2009). In addition, Ro-31-9790 is known to inhibit other metalloproteinases other than TACE. We therefore used TACE ^{Δ Zn/ Δ Zn} MEFs as an alternative approach to determine the specificity of the metalloproteinase responsible for cantharidin-induced shedding of L-selectin. Indeed, transfection of L-selectin in MEFs has been used in previous studies to monitor differences between basal and activation-induced shedding of L-selectin

[9]. Mammalian expression vectors harbouring the open reading frames of either WT or Δ M-N human L-selectin were microporated in to WT or TACE^{Zn^{-/-}} MEFs, and subsequently challenged with either PMA or cantharidin. Shedding of WT L-selectin was dramatically suppressed when TACE ^{Δ Zn/ Δ Zn} MEFs were challenged with cantharidin, and similar outcomes were observed in response to PMA stimulation (supplementary Figure 3). As expected, no shedding was observed in either WT or TACE ^{Δ Zn/ Δ Zn} MEFs expressing Δ M-N L-selectin (supplementary Figure 3). Conversely, cantharidin and PMA stimulation led to the concomitant shedding of WT L-selectin only in WT MEFs, further highlighting a role for TACE in cantharidin-induced shedding of L-selectin.

Cantharidin-induced shedding occurs independently of L-selectin/ERM interaction

The data presented thus far show strong observational parallels between PMA-induced shedding and cantharidin-induced shedding, suggesting that both processes could be mechanistically similar. The cytoplasmic tail of L-selectin has been shown to be important in regulating PMA-induced shedding. For example, we have previously shown that abrogating L-selectin/ERM interaction results in reduced PMA-induced shedding of L-selectin [22]. To assign a putative role for L-selectin/ERM interaction in cantharidin-induced shedding, 300.19 pre-B cells stably expressing either WT L-selectin, or an ERM-binding mutant of L-selectin, called R357A, were challenged with increasing concentrations of either PMA or cantharidin. As anticipated, shedding of R357A L-selectin was markedly reduced following PMA stimulation (Figure 3A). Surprisingly, treatment with cantharidin resulted in shedding of R357A L-selectin that was indistinguishable from WT L-selectin (Figure 3B). This result suggests that L-selectin/ERM interaction is dispensable for cantharidin-induced shedding.

Mutation of serine residues in the L-selectin tail affects PMA- but not cantharidin-induced shedding

The cytoplasmic tail of human L-selectin has two serine residues at positions 364 and 367 (S364 and S367, see Figure 3C). Previous reports have shown that these residues become phosphorylated in response to either PMA or T-cell receptor stimulation [17] [18], suggesting that they may play a role in regulating shedding. We therefore explored whether S364 and S367 were required for regulating cantharidin- or PMA-induced shedding. Stable cell lines were generated in 300.19 pre-B cells to express either non-phosphorylatable alanine (A) mutants or phospho-mimicking aspartate (D) mutants of L-selectin (see supplementary figure 4). Each cell line was subjected to increasing amounts of PMA or cantharidin, and surface levels of L-selectin were monitored by flow cytometry. Significant changes in the expression levels of L-selectin were seen in all cell lines as a consequence of PMA stimulation (Figure 3D&E). Of particular interest was the finding that PMA-induced shedding of S367A L-selectin had reduced, and, in contrast, shedding of S367D L-selectin had increased (Figure 3E). Interestingly, we found that mutation of S364 to either alanine or aspartate resulted in reduced shedding of L-selectin (Figure 3D). These results suggest more complex roles for the serine residues in regulating PMA-induced shedding, and/or that substitution mutagenesis of serine to aspartate at position 364 does not necessarily mimic phosphorylation. Antigen capture ELISA revealed that the basal turnover of WT and mutant L-selectins displayed no significant differences from one another (see supplementary figure 5).

Curiously, the extent of L-selectin shedding induced by cantharidin in all the cell lines tested did not deviate from the shedding of WT L-selectin (Figure 3F). However, a similar trend in increased loss of S367D L-selectin in response to middling doses of cantharidin was observed, but this was not statistically significant. Stimulation with calyculin A also promoted normal shedding in all cell lines tested, suggesting that this mode of shedding was not dependent on regulatory elements within the L-selectin tail (see supplementary figure 5). In summary, these data suggest that serine residues in the L-selectin tail play a central role in regulating PMA-induced shedding, but are redundant for cantharidin- or calyculin A-induced shedding.

Cantharidin-induced shedding of L-selectin is p38 MAPK-dependent

Both PKC and MAPK isozymes have been implicated in mediating signals that lead to shedding of L-selectin [19, 21, 33]. For example, p38 MAPK regulates shedding of L-selectin in response to osmotic cell stress and mechanical shedding [19, 21], and PKC-dependent shedding of L-selectin has been shown to occur in response to stimulation of the T-cell receptor [18]. A range of kinase inhibitors were therefore used to target PKCs (Ro-31-8220), and MAP kinases; p38 MAPK (SB202190) and MEK1 (PD98059) – an upstream activator of ERK. Primary lymphocytes, neutrophils and monocytes were isolated from whole blood and preincubated with each kinase inhibitor prior to stimulation with either PMA or cantharidin. As expected, PMA-induced shedding was almost completely blocked by Ro-31-8220 in all primary leukocytes tested (Figure 4A-C). In contrast, SB202190 and PD98059 had little effect on inhibiting PMA-induced shedding. Moreover, the extent of L-selectin shedding increased beyond 100% in all leukocytes pre-treated with SB202190 (see second bar of each histogram Figure 4A-C). This implies that inhibition of p38 MAPK could lead to increased accumulation of L-selectin at the cell surface by preventing the basal turnover of L-selectin at the plasma membrane. Cantharidin-induced shedding remained unaffected by Ro-31-8220 pretreatment, suggesting that PKCs are not involved in mediating the shedding response (Figure 4D-F). However, cantharidin-induced shedding was strongly inhibited by SB202190, but not PD98059, implying that p38 MAPK, and not MEK/ERK, plays an important role in this pathway (Figure 4D-F).

To test this further, anti-phospho-p38 MAPK antibody (which recognises the phosphorylation of both Thr180/Tyr182) was used to probe Western blots of whole cell lysates of neutrophils and peripheral blood mononuclear cells (PBMCs) that had been stimulated with either PMA or cantharidin over time (Figure 5). Interestingly, PMA stimulation affected p38 MAPK phosphorylation differently in neutrophils and PBMCs (compare Figure 5 A&B). Phospho-p38 levels rose rapidly in response to PMA stimulation, whereas no increase was seen in neutrophils, suggesting cell type variation. This increase in phospho-p38 MAPK levels had no effect on shedding L-selectin as this could not be blocked with SB202190 (Figure 4B&C), suggesting a p38 MAPK-independent mechanism. In contrast, phosphorylation of p38 MAPK in response to cantharidin stimulation was delayed in both neutrophils and PBMCs (Figure 5C&D). As PBMCs contain a mixture of PBLs and monocytes, we tested responses of the monocytic U937 cell line to PMA and cantharidin stimulation. As with neutrophils, no increase in phospho-p38 MAPK was observed following PMA stimulation. However, a significant and delayed increase in phospho-p38 MAPK was observed following cantharidin, which was similar to the other cell types tested (Figure 5E). No gross changes in total TACE levels in U937 cells was observed as judged by Western blotting, suggesting that stimulation by either PMA or cantharidin has no effect on TACE at the protein level (Figure 5E).

Cantharidin induces sustained surface expression and cytoplasmic tail phosphorylation of TACE via p38 MAPK

Based on the tail-dependent and tail-independent mechanisms of L-selectin shedding observed in response to respective PMA and cantharidin stimulation, we next sought to determine if TACE itself was regulated differently in response to cantharidin and PMA. Mobilisation of TACE to the plasma membrane is deemed a hallmark of TACE maturation [34, 35]. To this end, flow cytometry was used to monitor the surface levels of L-selectin and TACE in PBLs and monocytes over a 60 minute time course of PMA and cantharidin stimulation (Figure 6A-D). Irrespective of cell type, shedding of L-selectin occurred more rapidly in response to PMA than cantharidin treatment (i.e. 5 minutes versus 15 minutes, respectively – see Figure 6A-D). Interestingly, L-selectin shedding and surface expression of TACE appeared to be inversely correlated, suggesting a possible causal link between the two events (Figure 6A&B), although this was less apparent in monocytes (Figure 6C&D). Furthermore, PMA stimulation led to transient increases in TACE expression (Figure 6A&C) whereas cantharidin stimulation resulted in continued and sustained surface expression of TACE (Figure 6B&D). Increased and sustained expression of TACE was also observed in U937 cells following cantharidin but not PMA stimulation (see supplementary Figure 7). We next determined whether blocking p38 MAPK

activity would have an effect on surface levels of TACE expression following cantharidin stimulation. Indeed, in all cases, TACE mobilisation to the plasma membrane was blocked in cells pretreated with SB202190, implying the importance of p38 MAPK in regulating the traffic of TACE to the plasma membrane.

A recent report has highlighted a role for MEK/ERK in targeting threonine residues within the cytoplasmic tail of TACE [34, 35]. Threonine phosphorylation of the TACE cytoplasmic tail has since been interpreted as a hallmark of its activity. In an attempt to determine if the TACE cytoplasmic tail was threonine phosphorylated in response to either PMA or cantharidin stimulation, TACE was immunoprecipitated from lysates of U937 cells that were pre-treated with either stimulus over various time points. No threonine phosphorylation of the TACE cytoplasmic tail was detected in immunoprecipitates derived from lysates of PMA-stimulated U937 cells (Figure 7A, lanes 1 and 2). On the other hand, TACE cytoplasmic tail phosphorylation was detected at low levels in cantharidin-treated U937 cells at 30 minutes and more robustly at 60 minutes, which is concordant with when TACE surface expression was greatest and when L-selectin surface expression was lowest (Figure 7A, lanes 5-9). Collectively, these observations further highlight mechanistic differences between cantharidin- and PMA-induced shedding. Interestingly, phosphorylation of the cytoplasmic tail of TACE was blocked if cells were pretreated with SB202190. This implies that p38 could be involved in directly phosphorylating the tail of TACE, although further experiments are required to verify this.

TNF- α -induced shedding of L-selectin is mechanistically similar to cantharidin-induced shedding

We next sought to determine if our findings were physiologically relevant. TNF- α has previously been shown to induce the shedding of L-selectin in neutrophils [6]. It is also known that p38 MAPK participates in signalling downstream of TNF receptor stimulation. Indeed, Western blotting of lysates derived from TNF- α -treated PBMCs and neutrophils resulted in increased phospho-p38 MAPK levels (Figure 8A). Interestingly, pre-treatment of monocytes and neutrophils with SB202190 led to a marked suppression of TNF- α -induced shedding of L-selectin (Figure 8B&C), suggesting that p38 MAPK was involved in mediating the shedding response. A modest, but statistically significant level of PKC activity was also involved in the shedding response in monocytes, suggesting cell-type variation and possible redundancies between the kinases (Figure 8C). However, the effect of blocking p38 MAPK activity was far more robust, suggesting a greater involvement of p38 MAPK in the shedding response. No increase in the expression of TACE was seen in neutrophils as a consequence of TNF- α stimulation. This was rather unexpected, but one explanation could be due to increased turnover of active TACE at the plasma membrane, which has been previously described in another cell type [34]. However, increased and sustained expression of TACE was observed in monocytes challenged with TNF- α (Figure 8D), which was similar to what had been observed in response to cantharidin (Figure 6D) but not PMA stimulation (Figure 6C). Moreover, surface expression of TACE was concordant with L-selectin shedding, which ensued at approximately 15 minutes (Figure 8D). Finally, pretreatment of monocytes with SB202190 led to a marked reduction in surface expression of TACE following stimulation with TNF- α (Figure 8E). Taken together, these results imply that TNF- α -induced shedding of L-selectin could be driven by mechanisms akin to cantharidin-induced shedding rather than PMA-induced shedding. Finally, we further confirmed that LPS-induced p38 MAPK activation in monocytes led to increased and sustained expression of TACE, and was concordant with L-selectin shedding (see supplementary figure 8).

Discussion

This report is the first to describe two divergent mechanisms for L-selectin shedding that are guided differently by PKC and p38 MAPK. Activation of p38 MAPK through the use of phosphatase inhibitors leads to threonine phosphorylation of the TACE cytoplasmic tail, and increased and sustained expression of TACE at the plasma membrane. Furthermore, ERM binding and serine

residues of the L-selectin tail are redundant in this mechanism. Paradoxically, selective activation of PKC by PMA requires ERM binding and serine residues in the L-selectin tail to mediate shedding. Phosphorylation of the TACE cytoplasmic tail and increased sustained expression of TACE at the plasma membrane were not observed in response to PMA stimulation.

The role of p38 MAPK in regulating L-selectin shedding has been previously explored in a number of reports [19, 21], but the intracellular mechanism regulating this has been poorly defined. It is possible that the mechanism postulated in this report could apply to other p38 MAPK-dependent mechanisms of L-selectin shedding, although this will require further investigation. What is clear from our current study is that threonine phosphorylation of the TACE cytoplasmic tail and mobilisation of TACE to the plasma membrane is p38 MAPK-dependent. A proposed model for p38 MAPK-dependent shedding of L-selectin is shown in supplementary Figure 9. It is noteworthy that the cytoplasmic tail of TACE has previously been shown to be phosphorylated by ERK at threonine (T) 735 [34, 35]. This has been shown to increase maturation and surface expression of TACE [34]. Interestingly, the amino acids surrounding T735 (PQTP) provide a highly conserved p38 MAPK phosphorylation motif. It is therefore likely that p38 MAPK and ERK target a similar site in TACE, which may be stimulus-dependent or cell type-dependent. It is believed that phosphorylation of T735 is the only motif within the TACE tail that can be recognised by the anti-MAPK substrate (anti-phospho-threonine-proline) antibody [35]. It is also interesting to note that the turnover of TACE at the plasma membrane differed over time between PMA and cantharidin stimulation, which may reflect how p38 MAPK and PKC regulate the membrane traffic of TACE (Figure 6). During the writing of this report, work from Wang and colleagues described a redundant role for the cytoplasmic tail of TACE in PMA-induced shedding of L-selectin [36]. This further corroborates with our data to suggest that the cytoplasmic tail of TACE is regulated by p38 MAPK, but not PKC activity – highlighting divergent mechanisms in TACE-dependent shedding of L-selectin.

It is tempting to speculate that the difference in surface expression of TACE could be partly dictated by changes in the cortical actin-based cytoskeleton. For example, we and others [28] have observed an early (5 min) increase in ERM activation following cantharidin stimulation (see Figure 1D), which is in stark contrast to the early inactivation of ERM in response to PMA stimulation (Ivetic & Killock 2009, unpublished data). Net increases in ERM activity has been linked to increased cortical cellular rigidity [37], which may play a role in restricting vesicular traffic to and from the plasma membrane. As such, cantharidin stimulation may lead to the sustained expression of TACE at the plasma membrane, whereas PMA stimulation could lead to reduced plasma membrane rigidity to restrict the transient traffic of TACE to the plasma membrane within a short period of time. Determining whether the cortical actin cytoskeleton plays a role in the turnover of TACE at the leukocyte plasma membrane will be the focus of future studies.

Our current work has highlighted a central role for S364 and S367 in regulating PMA but not cantharidin-induced shedding. The outcome of shedding from the various L-selectin mutants was not easily predictable, particularly for the S364 mutants. For example, shedding of S364A L-selectin in response to PMA stimulation was much reduced compared to WT L-selectin. Strangely, shedding of S364D L-selectin in response to PMA stimulation was also much reduced. Our results could be interpreted in at least two different ways: (i) The serine-to-aspartate mutant does not act as a phospho-mimicking mutant, but behaves more like a non-phosphorylatable mutant. (ii) Phosphorylation of L-selectin at position S364 may require a subsequent dephosphorylation step to complete the shedding process effectively.

In contrast to the S364 mutants, S367A and S367D mutants of L-selectin were respectively resistant and sensitised to PMA-induced shedding. It is possible that phosphorylation of S367 could influence phosphorylation/dephosphorylation of S364 by kinases/phosphatases. Although a recent report has

identified a number of PKC isozymes to bind to and phosphorylate L-selectin at positions S364 and S367 [18], it would be necessary to determine if interaction with such kinases occurs *in vivo*. Microscopy techniques using fluorescence resonance energy transfer (or FRET) have recently been employed in determining L-selectin/binding partner interaction [38], which could potentially address this issue in intact cells. In addition, identification of a phosphatase that could potentially dephosphorylate the tail of L-selectin at S364 would be required to determine whether a dephosphorylation step is synonymous with resolving PMA-induced shedding.

In summary, we have identified two distinct mechanisms by which L-selectin can be cleaved by TACE. In each case, the cytoplasmic tails of TACE and L-selectin are involved in promoting either p38 MAPK- or PKC-dependent shedding, respectively. How these kinases might be involved in regulating L-selectin-dependent trafficking within the innate and acquired immune system will be an interesting focus for further study.

Acknowledgements

We wish to thank Prof. Roy Black (Amgen) for the kind gift of TACE^{ΔZn/ΔZn} MEFs. We thank Dr Ann Ager for providing Ro-31-9790.

Funding

This work was generously supported by the Wellcome Trust (076834/Z/05/Z).

References

- 1 Springer, T. A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. **76**, 301-314
- 2 Rosen, S. D. (2004) Ligands for L-selectin: homing, inflammation, and beyond. *Annu Rev Immunol*. **22**, 129-156
- 3 Ley, K., Laudanna, C., Cybulsky, M. I. and Nourshargh, S. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. **7**, 678-689
- 4 Christopherson, I., Piechoki, M., Liu, G., Ratner, S. and Galy, A. (2001) Regulation of L-selectin expression by a dominant negative Ikaros protein. *J Leukoc Biol*. **69**, 675-683
- 5 Smalley, D. M. and Ley, K. (2005) L-selectin: mechanisms and physiological significance of ectodomain cleavage. *J Cell Mol Med*. **9**, 255-266
- 6 Kaba, N. K. and Knauf, P. A. (2001) Hypotonicity induces L-selectin shedding in human neutrophils. *Am J Physiol Cell Physiol*. **281**, C1403-1407
- 7 Crockett-Torabi, E., Sulenbarger, B., Smith, C. W. and Fantone, J. C. (1995) Activation of human neutrophils through L-selectin and Mac-1 molecules. *J Immunol*. **154**, 2291-2302
- 8 Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N., Boyce, R. W., Nelson, N., Kozlosky, C. J., Wolfson, M. F., Rauch, C. T., Cerretti, D. P., Paxton, R. J., March, C. J. and Black, R. A. (1998) An essential role for ectodomain shedding in mammalian development. *Science*. **282**, 1281-1284
- 9 Walcheck, B., Alexander, S. R., St Hill, C. A. and Matala, E. (2003) ADAM-17-independent shedding of L-selectin. *J Leukoc Biol*. **74**, 389-394
- 10 Li, Y., Brazzell, J., Herrera, A. and Walcheck, B. (2006) ADAM17 deficiency by mature neutrophils has differential effects on L-selectin shedding. *Blood*. **108**, 2275-2279
- 11 Galkina, E., Tanousis, K., Preece, G., Tolaini, M., Kioussis, D., Florey, O., Haskard, D. O., Tedder, T. F. and Ager, A. (2003) L-Selectin Shedding Does Not Regulate Constitutive T Cell Trafficking but Controls the Migration Pathways of Antigen-activated T Lymphocytes. *J Exp Med*. **198**, 1323-1335
- 12 Schleiffenbaum, B., Spertini, O. and Tedder, T. F. (1992) Soluble L-selectin is present in human plasma at high levels and retains functional activity. *J Cell Biol*. **119**, 229-238
- 13 Tu, L., Poe, J. C., Kadono, T., Venturi, G. M., Bullard, D. C., Tedder, T. F. and Steeber, D. A. (2002) A functional role for circulating mouse L-selectin in regulating leukocyte/endothelial cell interactions in vivo. *J Immunol*. **169**, 2034-2043

- 14 Font, J., Pizcueta, P., Ramos-Casals, M., Cervera, R., Garcia-Carrasco, M., Navarro, M., Ingelmo, M. and Engel, P. (2000) Increased serum levels of soluble L-selectin (CD62L) in patients with active systemic lupus erythematosus (SLE). *Clin Exp Immunol.* **119**, 169-174
- 15 Kretowski, A., Gillespie, K. M., Bingley, P. J. and Kinalska, I. (2000) Soluble L-selectin levels in type I diabetes mellitus: a surrogate marker for disease activity? *Immunology.* **99**, 320-325
- 16 Dello Sbarba, P. and Rovida, E. (2002) Transmodulation of cell surface regulatory molecules via ectodomain shedding. *Biol Chem.* **383**, 69-83
- 17 Haribabu, B., Steeber, D. A., Ali, H., Richardson, R. M., Snyderman, R. and Tedder, T. F. (1997) Chemoattractant receptor-induced phosphorylation of L-selectin. *J Biol Chem.* **272**, 13961-13965
- 18 Kilian, K., Dervedde, J., Mueller, E. C., Bahr, I. and Tauber, R. (2004) The interaction of protein kinase C isozymes alpha, iota, and theta with the cytoplasmic domain of L-selectin is modulated by phosphorylation of the receptor. *J Biol Chem.* **279**, 34472-34480
- 19 Rizoli, S. B., Rotstein, O. D. and Kapus, A. (1999) Cell volume-dependent regulation of L-selectin shedding in neutrophils. A role for p38 mitogen-activated protein kinase. *J Biol Chem.* **274**, 22072-22080
- 20 Kaba, N. K., Schultz, J., Law, F. Y., Lefort, C. T., Martel-Gallegos, G., Kim, M., Waugh, R. E., Arreola, J. and Knauf, P. A. (2008) Inhibition of Na⁺/H⁺ exchanger enhances low pH-induced L-selectin shedding and beta2-integrin surface expression in human neutrophils. *Am J Physiol Cell Physiol.* **295**, C1454-1463
- 21 Lee, D., Schultz, J. B., Knauf, P. A. and King, M. R. (2007) Mechanical shedding of L-selectin from the neutrophil surface during rolling on sialyl Lewis x under flow. *J Biol Chem.* **282**, 4812-4820
- 22 Ivetic, A., Florey, O., Deka, J., Haskard, D. O., Ager, A. and Ridley, A. J. (2004) Mutagenesis of the ezrin-radixin-moesin binding domain of L-selectin tail affects shedding, microvillar positioning, and leukocyte tethering. *J Biol Chem.* **279**, 33263-33272
- 23 Reddy, P., Slack, J. L., Davis, R., Cerretti, D. P., Kozlosky, C. J., Blanton, R. A., Shows, D., Peschon, J. J. and Black, R. A. (2000) Functional analysis of the domain structure of tumor necrosis factor-alpha converting enzyme. *J Biol Chem.* **275**, 14608-14614
- 24 Ivetic, A., Deka, J., Ridley, A. and Ager, A. (2002) The cytoplasmic tail of L-selectin interacts with members of the Ezrin-Radixin-Moesin (ERM) family of proteins: cell activation-dependent binding of Moesin but not Ezrin. *J Biol Chem.* **277**, 2321-2329
- 25 Evans, B. J., McDowall, A., Taylor, P. C., Hogg, N., Haskard, D. O. and Landis, R. C. (2006) Shedding of lymphocyte function-associated antigen-1 (LFA-1) in a human inflammatory response. *Blood.* **107**, 3593-3599
- 26 Knapp, J., Boknik, P., Luss, I., Huke, S., Linck, B., Luss, H., Muller, F. U., Muller, T., Nacke, P., Noll, T., Piper, H. M., Schmitz, W., Vahlensieck, U. and Neumann, J. (1999) The protein phosphatase inhibitor cantharidin alters vascular endothelial cell permeability. *J Pharmacol Exp Ther.* **289**, 1480-1486
- 27 Hemler, M. (2006) Shedding of heterodimeric leukocyte integrin *Blood.* **107**, 3417-3418
- 28 Brown, M. J., Nijhara, R., Hallam, J. A., Gignac, M., Yamada, K. M., Erlandsen, S. L., Delon, J., Kruhlak, M. and Shaw, S. (2003) Chemokine stimulation

- of human peripheral blood T lymphocytes induces rapid dephosphorylation of ERM proteins, which facilitates loss of microvilli and polarization. *Blood*. **102**, 3890-3899
- 29 Ivetic, A. and Ridley, A. J. (2004) Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. *Immunology*. **112**, 165-176
- 30 Preece, G., Murphy, G. and Ager, A. (1996) Metalloproteinase-mediated regulation of L-selectin levels on leucocytes. *J Biol Chem*. **271**, 11634-11640
- 31 Kahn, J., Ingraham, R. H., Shirley, F., Migaki, G. I. and Kishimoto, T. K. (1994) Membrane proximal cleavage of L-selectin: identification of the cleavage site and a 6-kD transmembrane peptide fragment of L-selectin. *J Cell Biol*. **125**, 461-470
- 32 Chen, A., Engel, P. and Tedder, T. F. (1995) Structural requirements regulate endoproteolytic release of the L-selectin (CD62L) adhesion receptor from the cell surface of leukocytes. *J Exp Med*. **182**, 519-530
- 33 Alexander, S. R., Kishimoto, T. K. and Walcheck, B. (2000) Effects of selective protein kinase C inhibitors on the proteolytic down-regulation of L-selectin from chemoattractant-activated neutrophils. *J Leukoc Biol*. **67**, 415-422
- 34 Soond, S. M., Everson, B., Riches, D. W. and Murphy, G. (2005) ERK-mediated phosphorylation of Thr735 in TNFalpha-converting enzyme and its potential role in TACE protein trafficking. *J Cell Sci*. **118**, 2371-2380
- 35 Diaz-Rodriguez, E., Montero, J. C., Esparis-Ogando, A., Yuste, L. and Pandiella, A. (2002) Extracellular signal-regulated kinase phosphorylates tumor necrosis factor alpha-converting enzyme at threonine 735: a potential role in regulated shedding. *Mol Biol Cell*. **13**, 2031-2044
- 36 Wang, Y., Herrera, A. H., Li, Y., Belani, K. K. and Walcheck, B. (2009) Regulation of mature ADAM17 by redox agents for L-selectin shedding. *J Immunol*. **182**, 2449-2457
- 37 Kunda, P., Pelling, A. E., Liu, T. and Baum, B. (2008) Moesin controls cortical rigidity, cell rounding, and spindle morphogenesis during mitosis. *Curr Biol*. **18**, 91-101
- 38 Killock, D. J., Parsons, M., Zarrouk, M., Ameer-Beg, S. M., Ridley, A. J., Haskard, D. O., Zvelebil, M. and Ivetic, A. (2009) In Vitro and in Vivo Characterization of Molecular Interactions between Calmodulin, Ezrin/Radixin/Moesin, and L-selectin. *J Biol Chem*. **284**, 8833-8845

Figure Legends

Figure 1

Phosphatase inhibitors cantharidin and calyculin A induce rapid down-regulation of L-selectin expression

(A-C) Flow cytometry was used to monitor the surface levels of LFA-1 (solid black line) and L-selectin (dashed line) in primary leukocytes following stimulation for 30 minutes at 37°C with increasing cantharidin concentrations. Response profiles are shown for neutrophils (A), peripheral blood lymphocytes (B) and monocytes (C). (D) Western blotting of cell extracts derived from pre-B cells treated with either calyculin A or cantharidin for 30 min at 37°C. Blots were probed using antibody against C-terminal phospho-threonine ERM. Upper panel: ERM phosphorylation in response to calyculin A stimulation. Lower panel: ERM

phosphorylation in response to cantharidin stimulation. Western blots were developed in seconds by exposure to X-ray film. Over-exposure (1 min) of PVDF membrane to X-ray film reveals that ERMs are phosphorylated in response to 100 μ M cantharidin. All experiments were performed at least three times in triplicate.

Figure 2

Inhibition of phosphatases results in the release of soluble L-selectin

(A) Cell free supernatants from 0.5×10^6 300.19 pre-B cells stably expressing WT human L-selectin were analysed following 30 minutes of challenge with a concentration range of either PMA, calyculin A or cantharidin at 37 °C. ELISA techniques were employed (as outlined in *Materials and Methods*) to determine the amount of soluble L-selectin released in to the cell-free supernatant. (B and C) 300.19 cells stably expressing a sheddase resistant form of L-selectin, called Δ M-N, were challenged with increasing concentrations of PMA or cantharidin for 30 minutes at 37°C. Shedding profiles are shown for Δ M-N (solid black line) and WT (dashed line) L-selectin and are expressed as the percentage of L-selectin remaining after treatment. Percentage of L-selectin expression was calculated by comparing L-selectin expression levels in untreated cells treated with carrier alone. All experiments were performed at least three times in triplicate.

Figure 3

Arginine 357 and serines 364 and 367 are required for PMA-induced shedding, but not for cantharidin-induced shedding

Cell lines expressing either WT or R357A L-selectin were challenged with increasing doses of PMA (A) or cantharidin (B). Flow cytometric analysis was used to evaluate the amount of surface L-selectin remaining after stimulation for 30 minutes at 37°C. (C) Single amino acid sequence of the L-selectin tail, indicating the relative positions of S364 and S367. (D) Stable 300.19 pre-B cell lines were generated to express single alanine substitution mutations in either serine 364 or serine 367. Stable cell lines expressing non-phosphorylatable, or phospho-mimicking mutants of L-selectin were challenged with increasing doses of either PMA (D and E) or cantharidin (F). Levels of L-selectin were measured using flow cytometry, and remaining L-selectin levels were expressed as a percentage of untreated controls (i.e. carrier alone). Statistical significance for serine mutant forms of L-selectin in comparison to WT at the same concentration of stimulus was determined by unpaired student's *t* test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. All experiments were performed at least three times in triplicate.

Figure 4

Inhibition of serine/threonine phosphatases in resting leukocytes results in p38 MAPK-dependent shedding of L-selectin

Neutrophils, PBLs and monocytes were isolated from fresh whole blood and pre-incubated with an inhibitor targeting: p38 MAPK (SB202190), PKC isozymes (Ro-31-8220) or MEK/ERK (PD98059) in order to assign the involvement of a specific family of kinases involved in regulating PMA- (A-C) and cantharidin- (D-F) induced shedding. This study used 100 nM PMA and 500 μ M cantharidin. Statistical significance was calculated between carrier and inhibitor treated cells by unpaired student's *t* test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. All experiments were performed at least three times, and in triplicate.

Figure 5

P38 MAPK is activated differently in leukocytes stimulated with PMA, but not cantharidin

(A-D) Neutrophils and PBMCs were isolated from whole blood and stimulated with either PMA (A&B) or cantharidin (Canth – C&D) for the indicated time points. Cells were lysed, resolved on polyacrylamide gels and transferred to PVDF membrane for Western blotting. Immunoblots were probed with either a phospho-specific phospho-p38 MAPK antibody (p-p38), or an anti-p38 antibody to monitor total p38 levels from whole cell lysates. (E) The relative activity of p38 MAPK was monitored by Western blot following stimulation of U937 cells with 100 nM PMA or 500 μ M cantharidin. Total TACE levels were monitored by Western blotting to determine if any gross changes at the protein level were seen following stimulation. The relative levels of β -actin served as loading control. All experiments were performed at least three times.

Figure 6

Time course of TACE and p38 MAPK activation in response to cantharidin stimulation

PBLs (A and B) and monocytes (C and D) were isolated from fresh blood and stimulated with either PMA (A and C) or cantharidin (B and D). Surface levels of TACE (light grey line) and L-selectin (black line depicted as “LAM”) were monitored by flow cytometry over time (0, 5, 10, 15, 30 and 60 minutes). Surface levels of TACE derived from untreated cells (i.e. carrier alone) are shown in dark grey dashed line. Values were recorded as percentage of expression levels relative to untreated controls. All experiments were performed at least three times in triplicate. (E) U937 cells, primary neutrophils, monocytes and PBLs were preincubated with or without SB202190 prior to treatment with 500 μ M cantharidin for 30 minutes at 37 °C. Surface levels of TACE were monitored using flow cytometry as in previous experiments. All statistical evaluations were performed using an unpaired student's *t* test: # $p \leq 0.05$, **/### $p \leq 0.01$, ***/#### $p \leq 0.001$. “*” indicates statistical significance comparing carrier versus cantharidin treatment (in the absence of SB202190). “#”: statistical significance compares treatments with and without pre-incubation of SB202190.

Figure 7

p38 MAPK regulates phosphorylation of the TACE cytoplasmic tail

(A) U937 cells were either stimulated with 100 nM PMA for 5 and 15 minutes (lanes 1 and 2, respectively). Lanes 5-9 correspond to: 0, 5, 10, 30 and 60 min incubation with 500 μ M cantharidin. Cells left treated with carrier alone for 30 minutes at 37°C is shown in lane 4. TACE was immunoprecipitated from lysates and resolved on polyacrylamide gels for subsequent immunoblotting with anti-TACE antibody (upper blot) or anti-p-Thr antibody (lower panel). An isotype control antibody was used in lane 3, which failed to immunoprecipitate endogenous TACE. (B) U937 cells were incubated with SB202190 or carrier alone before stimulation with either PMA or cantharidin. After stimulation anti-TACE antibodies were used to immunoprecipitate TACE from U937 cell lysates. Immunoprecipitates were resolved on polyacrylamide gels and subsequently transferred to PVDF membrane. The same antibody that was used to immunoprecipitate TACE was used to probe for TACE (upper blot). Anti-phospho-threonine antibody was used to determine if the cytoplasmic tail of TACE was threonine phosphorylated in response to cantharidin treatment (lower blot).

Figure 8

TNF- α -induced shedding of L-selectin mimics responses akin to cantharidin but not PMA stimulation

(A) Neutrophils and monocytes were purified from whole blood and challenged with carrier alone “u” (for “untreated”), cantharidin “c”, or TNF- α “t”. Upper panel shows phospho-p38 MAPK levels and lower panel shows total p38 MAPK levels for loading control. Monocytes

(B) and neutrophils (C) were pre-treated with SB202190, Ro-31-8220 or PD98059 prior to stimulation with or without TNF- α . Flow cytometry was used to monitor surface levels of L-selectin before and after stimulation. Data is expressed as percentage of L-selectin shed. (D) Surface levels of TACE and L-selectin were monitored in monocytes in response to TNF- α stimulation. (E) Surface expression of TACE is prevented by pre-incubation with SB202190. All experiments were performed at least three times. Experiments in B-E were additionally performed in triplicate. $p = * \leq 0.05$, $** \leq 0.01$, $*** \leq 0.001$.

Figure 1

Figure 2

Figure 3

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091611

Figure 3 cont....

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 8 cont...