Two sites on P-selectin (the lectin and epidermal growth factor-like domains) are involved in the adhesion of monocytes to thrombin-activated endothelial cells

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P-selectin, also known as GMP-140, PADGEM or CD62, is expressed on the surface of thrombin-activated platelets and endothelial cells (EC). It is a member of the selectin family of adhesion molecules that regulate leucocyte interactions with the blood vessel wall. In this study we have found that peptides derived from both the lectin (residues 19–34 and 51–61) and epidermal growth factor (EGF)-like (residues 127–139) domains inhibit the adhesion of peripheral blood mononuclear cells (PBMC), elutriated monocytes and a monocytic cell line (U937) to thrombin-activated EC. This inhibition occurred in a concentration-dependent manner and the peptide most active at the

lowest concentrations was the one derived from the EGF-like motif (127–139). The scrambled forms of these peptides, identical in amino acid composition to the authentic peptides but with altered sequences, were not inhibitory. Thrombin-activated platelets supported adhesion of U937 cells and this adhesion was dramatically inhibited by the two peptides derived from the lectin-like domain (residues 19–34 and 51–61). All three peptides, when conjugated to BSA and coated on plastic plates, mediated U937 cell adhesion. This study shows, for the first time, that two sites on P-selectin, the lectin and EGF-like domains, are involved in the adhesion of monocytes to thrombin-activated EC.

INTRODUCTION

The adhesion of leucocytes to the vascular endothelium is a critical event taking place before their extravasation and migration to inflammatory sites. This adhesion is regulated by several classes of adhesion molecules of which the most recently described are the selectins. These are a family of molecules composed of three structurally related membrane proteins. L-selectin (LECAM-1, LAM-1), an adhesion protein on myeloid cells and subsets of lymphocytes, participates in mobilization of neutrophils to sites of acute inflammation and homing of lymphocytes to peripheral lymph nodes [1–4]. E-selectin (ELAM-1) is a cytokine-inducible endothelial cell (EC) adhesion molecule for neutrophils [5], monocytes [6] and memory T-cells [7,8]. P-selectin, an integral membrane protein found in the α -granules of platelets [9,10] and the Weibel–Palade bodies of ECs, is translocated and expressed on the plasma membrane upon stimulation by agonists such as thrombin or histamine [11,12]. Adhesion proteins are lectins that bind to fucosylated oligosaccharide ligands on the surface of certain leucocytes [13–17]; however, the protein(s) that carry these ligands are unknown [18]. Each selectin contains an N-terminal lectin-like domain of approx. 121 residues followed by an epidermal growth factor (EGF)-like motif, a series of consensus repeats related to those in complement-regulatory proteins, a transmembrane domain, and a cytoplasmic tail [1,2,19,20].

To date, most work relating to the role of P-selectin mediating leucocyte adhesion to ECs has focused on the sites mediating the interaction with neutrophils, whereas little is known about the sites mediating the interaction with monocytes. It has been shown that the lectin-like domain on P-selectin is involved in binding neutrophils [18]. Differences between the two cell types, such as the expression of very late activation antigen (VLA-4) by

neutrophils, but not monocytes, may require different epitopes on P-selectin for interaction [21]. In this study, we show that two sites, the lectin-like and EGF-like domains of P-selectin, are involved in mediating monocyte adhesion to thrombin-activated ECs.

MATERIALS AND METHODS

Materials

All chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise stated. Tissue culture medium 199 and supplements [fetal calf serum (FCS), horse serum, goat serum, penicillin/streptomycin, glutamine, sodium bicarbonate] were from Gibco Life Technologies Ltd. (Paisley, Scotland, U.K.).

Peptides

Hydrophilic peptides on the known P-selectin structure, obtained by sequencing the cDNA [16], were selected by Appligene (Strasbourg, France) using previously published techniques [22,23]. All peptides were synthesized on an Applied Biosystem 431A peptide synthesizer (Paris, France) by fluorenyl-methoxycarbonyl (Fmoc) chemistry, purified by h.p.l.c. and analysed on a Kontron Chromakon 500 amino acid analyser and by mass spectrometry. The numbering of the peptides corresponds to the published amino acid sequence of human P-selectin [5,16,17] and is as follows: lectin domains, 19–34 (CQNYTDLVAIQN-KNE), 51–61 (IGIRKNNKTWT), and 76–90 (ADNEPNNKR-NNEDC); EGF domain, 127–139 (CSKQGECLLETIGN); complement protein repeat domains, 221–232 (CPPLKIPERGNI), and 537–551 (PEQGSLLDCSDTRGEF); cytoplasmic tail, 755–766 (RKRFRQKDDGKC). Control peptides for residues 19–34, 51–61 and 127–139 were identical in amino acid composition to

Abbreviations used: EC, endothelial cell; EGF, epidermal growth factor; FCS, fetal-calf serum; HUVECs, human umbilical vein endothelial cells; PBMCs, peripheral blood mononuclear cells; ACD, acid/citrate/dextrose; HBSS, Hanks' balanced salt solution.

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the authentic peptides except their sequence was altered. Stock solutions (2 mM) were prepared in PBS (0.1 M, pH 7.2) and appropriate dilutions were made in RPMI medium/1% FCS (Flow Labs., Irvine, Scotland, U.K.) before each experiment.

Monoclonal antibody (mAb)

LYP20 is an anti-P-selectin mAb that is directed against a disulphide-dependent epitope, most probably the lectin-EGF region, and is known to inhibit platelet aggregation and platelet-monocyte interaction [24] and bind to rat platelets [25]. This antibody was used as purified immunoglobulin and diluted in RPMI medium as described.

Culture of human umbilical vein endothelial cells (HUVECs)

ECs were isolated and grown in culture as described by Jaffe et al. [26]. Briefly, cells were isolated from umbilical cords treated with collagenase type II (Worthington Chemical Company, Freehold, NJ, U.S.A.) and grown to confluence on fibronectin (Boehringer-Mannheim, Mannheim, Germany) coated flasks in culture medium 199 at 37 °C in 5% CO₂. The culture medium was supplemented with 10% (v/v) de complemented FCS (Gibco), 300 µg/ml endothelial cell growth factor (ECGF) (Boehringer-Mannheim), 5 mM NaHCO₃, 2 mM glutamine, 100 units of penicillin/streptomycin and 1 mM sodium pyruvate. HUVECs were seeded either on to 24-well (Falcon Primarea, Meylan, France) or 96-well (Nunc Immunolon, Roskilde, Denmark) plates and left to grow to confluence (2 × 10⁵ cells per well for a 24-well plate) before being used in adhesion assays.

Isolation of peripheral blood mononuclear cells or monocytes

Routine isolation of peripheral blood mononuclear cells (PBMCs) was performed under sterile conditions using endotoxin-free Ficoll-Hypaque gradient medium in a gradient-density-centrifugation technique [27]. Whole blood was drawn from donors in acid/citrate/dextrose (ACD) and carefully layered on a Ficoll-Hypaque 1077 gradient. PBMCs present at the Ficoll-Hypaque interface, following centrifugation at 700 g for 30 min at 25 °C, were isolated and washed twice in 50 ml of PBS. Isolated PBMC pellets, gently resuspended in 1 ml of PBS, always showed more than 90% viability, normally more than 95%. Monocytes were isolated by elutriation centrifugation as previously described [28]. The preparation was > 90% pure, as controlled by flow cytometry (FACScan; Becton Dickinson and Co., Mountain View, CA, U.S.A.) with fluorescein isothiocyanate-conjugated anti-CD14 antibody (LeuM3; Becton Dickinson and Co.) and by May-Grunwald-Giemsa staining. The monocyte population was > 90% HLA-DR and < 1% CD25⁺ and viability was greater than 95% as determined by Trypan Blue dye exclusion. U937 cells, a monocytic cell line (European collection of animal cell cultures, Porton Down, Salisbury, Wilts., U.K.), were cultured in RPMI tissue culture medium (Flow Laboratories) supplemented with 10% FCS, 2 mM glutamine, 100 units of penicillin/streptomycin, and 1 mM sodium pyruvate. Cells were suspended in RPMI/1% FCS for adhesion experiments. All cells were labelled by sodium chromate (Na⁵¹CrO₄) incorporation as described previously [29].

Monocyte adhesion to HUVECs

⁵¹Cr-labelled PBMCs, U937 cells or elutriated monocytes were assayed for their adhesion to unstimulated or thrombin-stimu-

lated ECs following the method, with minor modifications, previously described [30–32]. Briefly, fully confluent ECs were stimulated with thrombin (2 units/ml) for a period of 5–10 min and the reaction terminated by plate inversion or by the addition of a thrombin antagonist, D-phenylalanyl-L-prolyl-L-argininyl chloromethane (PPACK; Calbiochem, La Jolla, CA, U.S.A.) to a final concentration of 1 × 10⁻⁶ M. ECs were then immediately incubated with RPMI/1% FCS in the presence or absence of mAb LYP20, followed by the addition of PBMC, elutriated monocytes or U937 cells [(4–6) × 10⁵ cells/well] for 10 min. The peptides were incubated with the monocytes for 5–10 min during thrombin activation of ECs and then added to wells immediately afterwards. All experiments were carried out at room temperature or at 37 °C under static conditions. Wells were then emptied 10 min later by inversion and non-adherent cells were removed by washing twice with RPMI/1% FCS. Well contents were solubilized with 50 mM Tris/0.1% SDS (pH 8.4) and radioactivity in cell lysates measured. For all adhesion experiments, the binding of PBMCs, elutriated monocytes or U937 cells to activated controls in the absence of peptide or antibody was normalized to 100% and the reduction in binding caused by the inhibitor was calculated as a percentage of this.

Platelet/U937 adhesion studies

Whole blood was collected on ACD and platelets isolated and washed according to the method of Massini and Luscher [33]. Platelets were then suspended in Tris/NaCl/EDTA (TENA) buffer at 1 × 10⁹ cells/ml and those to be activated were incubated with thrombin (1 unit/ml) for 5 min and the reaction stopped with PPACK (1 × 10⁻⁶ M final concentration). Both activated and non-activated platelets were then fixed with 2% HCHO and incubated for 30 min at 37 °C. They were then washed three times, resuspended in TENA buffer containing 0.1 M NH₄Cl, again washed three times and finally resuspended at 1 × 10⁹ cells/ml of TENA containing 0.04% NaN₃ final concentration. Platelets were then either plated directly on 96-well plates or stored at 4 °C for up to 2 weeks before use. Platelet fixation to plates was carried out as follows: platelets were gently stirred and 50 µl pipetted with 5 ml of TENA. An aliquot (50 µl) was then added to each well followed by the addition of 2% HCHO (50 µl). Plates were then incubated for 1 h at room temperature, washed three times, blocked with 5% FCS for 2 h, washed twice and then used in adhesion assays. These were carried out as described above. ⁵¹Cr-labelled U937 cells were incubated on plates for 10 min, unbound cells removed by two washings in PBS and radioactivity measured directly in a radioactivity counter.

Monocyte adhesion to peptide-BSA conjugates

The P-selectin peptides derived from those found to inhibit monocyte cell adhesion to thrombin-activated ECs were conjugated to BSA via a cysteine residue attached at either the N- or C-terminus. The active peptides 19–34 (CQNYRTDLVAIQNKNE), 127–139 (CSKQBECLETIGN) and control 755–766 (RKRFRQKDDGKC) already contained an authentic cysteine residue. However, a cysteine residue was added during synthesis to the N-terminus of peptide 51–61 (CIGIRKNNKTWT). The peptides were conjugated via the free sulphhydryl in the cysteine to maleimide-activated BSA at 10 mg of BSA/2–2.5 mg of peptide [34]. Material was dialysed four times against 0.1 mM PBS, pH 7.2, to remove unconjugated material and the amount of conjugated material was estimated by means of a Bio-Rad protein assay. Conjugation was confirmed by Ellman's analysis [35] which measured the free cysteine available after coupling.

Material was pipetted on to 96-well e.l.i.s.a. plates (Falcon) (100 μ l/well in carbonate-bicarbonate buffer, pH 9) and left overnight at 4 °C. Unconjugated BSA was also coated on to plates at the same concentration and used as a negative control in adhesion assays. The wells were washed three times in Hanks' balanced salt solution (HBSS) containing 1.26 mM Ca^{2+} and 0.81 mM Mg^{2+} , and blocked with the same solution containing 5% FCS. Wells were washed again three times with HBSS and ^{51}Cr -labelled U937 cells were added at $(2-4) \times 10^5$ per well and incubated under static conditions for 20 min at room temperature. Wells were inverted and unbound cells removed with three washes in HBSS containing Ca^{2+} and Mg^{2+} . Bound cells were lysed with 50 mM Tris buffer/0.1% SDS and radioactivity measured in a γ counter.

Analysis of data

Each separate experiment was carried out in duplicate as described and means calculated. S.D.s were calculated from the mean of each experiment. The significance of the difference between mean values was determined using the Mann-Whitney *U* test.

RESULTS

Lectin and EGF-like domain peptides inhibit monocyte adhesion to thrombin-activated ECs

A PBMC population was used as the source of monocytes for the preliminary screening experiments. Previous results have shown that the majority of cells in PBMC populations binding to thrombin-activated ECs were monocytes, as judged by specific

Table 1 Inhibition of adhesion of PBMC to thrombin-activated ECs in the presence of P-selectin peptides

Peptides were added at a final concentration of 0.5 mM and adhesion assays carried out as described in the Materials and methods section. $7.1 \pm 3\%$ of cells bound activated control. Peptides inhibiting by 25% or less were considered to have no effect. Data represent the results of triplicate determinations from three or four independent experiments and are expressed as means $\pm$ S.D.

Peptide	Inhibition (%)
Lectin-like	
19-34	$41 \pm 18.18^*$
19-34S	14 ± 12
19-26	9 ± 6.2
27-34	11 ± 12
51-61	$31 \pm 15.4^*$
51-61S	20 ± 18
76-90	12.8 ± 13
EGF-like	
127-139	$37 \pm 9^*$
127-139S	9.2 ± 11
Protein repeats	
221-232	24 ± 18
531-542	0
Cytoplasmic tail	
755-766	0

* $P \leq 0.05$ as determined by Mann-Whitney *U* test.

Figure 1 Dose-response for peptide inhibition of U937 cell and elutriated monocyte adhesion to activated-ECs

(a) Adhesion of U937 cells to thrombin-activated ECs in the presence of the three active peptides 19-34 ($\square$), 51-61 ($\blacklozenge$) and 127-139 ($\blacksquare$). Peptides were incubated with U937 cells 10 min before being added to ECs. Control peptide 755-766 ($\diamond$) was not inhibitory. Each point represents the mean of duplicate determinations from four independent experiments where $10 \pm 7\%$ of added cells bound to the activated control. S.D. between experiments was less than 15%. (b) Adhesion of elutriated monocytes in the presence of the three peptides. Symbols as in (a). Each point represents the mean of duplicate determinations from three independent experiments where $20 \pm 2.3\%$ of added cells bound to the activated control. S.D. between experiments was less than 15%.

markers to monocytes, B and T lymphocytes [36]. Of the nine peptides tested, the two lectin domain peptides, 19-34 and 51-61, and the EGF-like peptide, 127-139, were seen to inhibit adhesion significantly (Table 1). Their scrambled forms, the other lectin-like domain peptide (76-90), the two complement-like protein repeat peptides (221-232 and 531-542) and the cytoplasmic tail sequence (755-766) did not inhibit adhesion. Following this initial screening the three active peptides were studied further in assays using U937 cells at concentrations ranging from 1 mM to 10 μ M (Figure 1a). It is seen that peptide 19-34 is the strongest inhibitor (64%) at the highest concentration, but its activity decreases sharply with decreasing concentrations. Peptide 51-61 shows much less inhibitory effect. Most interestingly, peptide 127-139 is the most active right across the concentration range,

Table 2 Inhibition of adhesion of U937 cells to thrombin-activated platelets in the presence of P-selectin peptides and their scrambled forms

Peptides were added at a final concentration of 0.5 mM and adhesion assays carried out as described in the Materials and methods section. For these assays $16.1 \pm 0.14\%$ of added U937 cells bound activated control compared with $3.68 \pm 1.44\%$ for resting platelets. Peptides inhibiting by 25% or less were considered to have no effect. Data represent results of quadruplicate determinations from three independent experiments and are expressed as means $\pm$ S.D.

Peptide	Inhibition (%)
19-34	$65.4 \pm 13^*$
19-34S	25 ± 10
51-61	$56 \pm 8.3^*$
51-61S	11 ± 9.5
127-139	16.4 ± 20
755-766	0.00

* $P \leq 0.05$ as determined by Mann-Whitney U test.

Table 3 Adhesion of U937 cells to wells coated with BSA and BSA-P-selectin peptide conjugates

Peptides were coupled to BSA as described in the Materials and methods section and coated on to 96-well plates. ^{51}Cr -labelled U937 cells were incubated in wells for 20 min, unbound cells were removed by washing and cell adhesion determined by measuring the radioactivity in cell lysates. Table 3 shows percentage of cells bound from an original cell number of $(2-4) \times 10^5$ cells/well. Data represent the mean $\pm$ S.D. of triplicate determinations from three independent experiments.

Peptide	U937 cell adhesion (%)
BSA	19.5 ± 7.7
19-34-BSA	$38.6 \pm 8.1^*$
51-61-BSA	$45.2 \pm 2.3^*$
127-139-BSA	$38.8 \pm 5.5^*$
755-766-BSA	27.6 ± 37

* $P \leq 0.05$ as determined by Mann-Whitney U test.

inhibiting by up to 30% at $10 \mu\text{M}$. The scrambled form of these peptides (19-34, 51-61 and 127-139) and the cytoplasmic tail (755-766) were not inhibitory at 0.5 mM (results not shown). Similar results were obtained in assays using elutriated monocytes (Figure 1b). We tested for synergistic effects by using each active peptide in the presence of each of the other active peptides at a final concentration together of 0.5 mM, but no potentiating effect was observed. An anti-(P-selectin) monoclonal antibody (LYP20), used as a positive control in assays, inhibited cell adhesion by over 50% (results not shown).

Lectin-like domain inhibits adhesion of monocytes to thrombin-activated platelets

In order to test whether or not the observed effect with peptides was specific to EC P-selectin we used human platelet P-selectin as a base for U937 cell adhesion. Isolated platelets were activated by thrombin, fixed and plated on to 96-well plates as described in the Materials and methods section. Lectin peptides (19-34 and

51-61) inhibited the level of adhesion of U937 cells to thrombin-activated platelets to that observed for resting platelets (Table 2). In contrast, the scrambled form of peptide 19-34 showed a greatly reduced and non-significant effect whereas the scrambled form of 51-61 had no effect. In these assays, the EGF-like domain peptide (127-139) did not inhibit adhesion. Control peptide, corresponding to the cytoplasmic tail peptide 755-766, was not inhibitory.

Immobilized peptides mediate U937 cell adhesion

To determine whether the P-selectin peptides could interact directly with cells we coupled the active peptides to albumin, as described in the Materials and methods section, and tested whether the immobilized conjugates could support adhesion of U937 cells. The three peptides supported significantly more U937 cell adhesion than both the BSA alone and the control peptide 755-766-BSA conjugate (Table 3). Peptides were not seen to support adhesion synergistically when more than one peptide was placed on each well. When peptide 51-61 was used in combination with either 19-34, 127-139 or the three together, the adhesion was same as that for 51-61 alone (results not shown). Although this peptide was the least active of the three in inhibiting in the dose-response studies, it was most active in supporting U937 cell adhesion.

DISCUSSION

In this study, we show for the first time that two sites on P-selectin, the lectin and the EGF-like domains, are involved in mediating the adhesion of monocytes to thrombin-activated ECs. Several lines of evidence support this statement: (1) out of a series of peptides, corresponding to the most hydrophilic regions of the P-selectin molecule, only those from the lectin (19-34, 51-61) and EGF-like (127-139) peptide domains have an inhibitory effect on the adhesion of PBMC to EC. (2) Dose-response studies with U937 cells and elutriated monocytes demonstrated that these peptides, derived from the lectin and EGF-like domains, were biologically functional. The EGF-like peptide was most active, even at the lower end of the concentration range. (3) Immobilized peptide-BSA conjugates directly supported U937 cell adhesion. (4) The scrambled forms of these peptides did not inhibit adhesion, and control peptide from the cytoplasmic tail had no effect.

Recent studies have found that the lectin domain is involved in binding to myeloid cells. Geng et al. [34] have shown that peptides derived from the lectin-like domain inhibit neutrophil and a neutrophil cell line (HL60) adhesion to adsorbed P-selectin. These peptides, when conjugated to BSA, also supported cell adhesion. The authors, Geng et al., also showed that the lectin-like domains are Ca^{2+} -binding sites which modulate their cell-binding ability. Hollenbaugh et al. [37] have shown that residues important for binding are located in a small groove on the surface of the molecule composed of residues from the β -2, -3 and -5 strands of P-selectin lectin domain. Moreover, a number of residues within this groove, which are conserved among all selectins, were found to be critical for binding. Substitution of these residues abolished P-selectin binding to myeloid cells by either altering the Ca^{2+} -binding site, essential for lectin-mediated adhesion, and therefore negating its adhesive properties, or by modulating the architecture of the binding site such that it was inaccessible to its ligands. In our study we have investigated the adhesion of monocytes and a monocytic cell line (U937) to P-selectin expressed by activated ECs. Neither peptide completely inhibited cell adhesion, the maximum inhibition observed was

with peptide 19–34 which resulted in 64 % inhibition of U937 cell adhesion and 63 % of monocyte adhesion (Figures 1a and 1b). In the study of Geng et al. [34], peptides 23–30 and 54–63 completely inhibited neutrophil and HL60 adhesion to immobilized P-selectin. Such differences can probably be explained by the fact that we used P-selectin expressed by ECs rather than adsorbed, purified P-selectin. Adhesive receptors, other than P-selectin, are known to be expressed by ECs and would be involved in mediating cell–cell interaction [18]. Partial difference could also be explained by the peptide residues used in our study not corresponding exactly to those used by Geng et al. [34]. Moreover, differences between neutrophils and monocytes, in terms of expressed adhesion receptors, may also account for our observations [21].

A very significant finding in our study was to identify a peptide (127–139), derived from the EGF-like domain, that inhibited monocyte or U937 cell adhesion to activated ECs. The 127–139 peptide was most effective, compared with peptides derived from the lectin-like domain (19–34 and 51–61), even at lower concentrations (down to 10 μ M). Several studies have previously implicated the EGF-like domain in mediating selectin recognition of target cells. The EGF domain, located next to the N-terminal lectin-like domain, may be required for selectin-mediated recognition of target cells. E-selectin deficient in the lectin or EGF-like domains does not support adhesion. Moreover, the lectin-like domain of L- and E-selectin requires an adjacent EGF-like domain for expression [38,39]. Kansas et al. [40] have reported that lymphocyte adhesion mediated by L-selectin may operate cooperatively between functionally distinct sites, suggesting a role for the EGF-like domain of LAM-1 in lymphocyte adhesion to high endothelial venules. Some EGF-like domains in other proteins have been demonstrated to contain high-affinity Ca^{2+} -binding sites and occupancy of these sites by Ca^{2+} can alter the conformation of the domain as well as the function of the protein. Alternatively, binding of Ca^{2+} might support direct interactions of the EGF-like domain with a protein component of the ligand target cells [41–44]. Our study, by showing that peptide 127–139 inhibits monocyte cell adhesion to ECs and directly supports cell adhesion, is the first to show that the EGF-like domain of P-selectin has a direct role in mediating monocyte adhesion to ECs. When studying U937 cell adhesion to activated platelets, as opposed to activated ECs, peptide 127–139 had no effect. The effect of the lectin domain peptides (19–34 and 51–61) was much more dramatic compared with that observed with U937-EC. It is conceivable that the relative role played by each portion of the P-selectin in mediating adhesion may differ between platelets and ECs. Concentrations of P-selectin in platelets are much higher (13-fold, 0.166 % of total platelet protein) compared with that present in ECs (0.016 % of EC protein) [45]. It is conceivable that the reduced concentration of P-selectin on ECs might allow a greater exposition of the molecule by ligands present on leucocytes. In contrast, platelets with a high density of P-selectin might allow interaction with the lectin-like domain and not the EGF-like domain. It must be noted also that both cell types possess other different receptors.

In conclusion, we report that specific lectin and EGF-like domain peptides block leucocyte adhesion to thrombin-activated ECs and directly support cell adhesion. Our data show that residues contained in sequences 19–34, 51–61 and 127–139 play essential roles in mediating adhesion between ECs and leucocyte cell-surface ligands. Results in this study may prove useful for the development of new therapeutic inhibitors to modulate leucocyte interactions in inflammatory disorders.

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