

L-Selectin expression on neutrophils from allergic patients

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Summary

Background L-selectin (CD62L) is an adhesion molecule involved in leucocyte attachment to endothelium at sites of inflammation, and it has been demonstrated that L-selectin is rapidly shed after neutrophil activation. Recently, it has been reported that there is increasing evidence of neutrophil participation in asthma and the allergic process.

Objective The present study was designed to determine whether an IgE-dependent mechanism can modulate L-selectin expression on the surface of neutrophils. Moreover, we analyse the potential implication of intracellular signal-transduction pathways and whether specific immunotherapy (IT), glucocorticoids and antihistamines might regulate this process.

Methods Peripheral blood neutrophils from three groups of donors (asthmatic group without IT treatment, IT-treated asthmatic group and healthy group) were used. Cells were challenged *in vitro* with the specific allergen that produced clinical symptoms in asthmatic patients and also with the allergen to which the patients were not sensitive. Neutrophils from healthy donors were also challenged with allergens. Expression of CD62L on the neutrophil surface was analysed by flow cytometry, and soluble CD62L (sCD62L) in culture supernatant by ELISA. In an attempt to discover which IgE receptor is involved, we also challenged the neutrophils with monoclonal antibody to FcεRI, FcεRII (CD23) and galectin-3 receptors.

Results When neutrophils from allergic patients were challenged with specific allergens that produce clinical allergy symptoms, L-selectin was down-regulated from the surface of those cells, accompanied by a concomitant up-regulation of soluble L-selectin in the supernatant. The challenge with antibodies against FcεRI, FcεRII (CD23) and galectin-3, induces down-modulation of L-selectin on the surface of the neutrophils in all three cases. Calphostin C, wortmannin and mannoalide attenuated CD62L down-regulation, suggesting the potential implication of protein kinase C, phosphatidylinositol 3-kinase and phospholipase A₂ in the process. IT and glucocorticoids modulated allergen-dependent CD62L down-regulation, whereas antihistamines (terfenadine, loratadine and cetirizine) or nedocromil sodium did not affect the shedding of L-selectin.

Conclusions We present evidence that the neutrophil surface expression of CD62L can be modulated by an allergen-dependent mechanism. The modulation of CD62L expression can be induced through the three receptors of IgE. This process can be affected by IT.

Keywords adhesion molecule, allergy, asthma, CD23, CD62L, FcεRI, FcεRII, galectin-3, neutrophils, signal transduction

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Introduction

The localization of neutrophils at vascular sites of inflammation involves several discrete processes, including cell capture, rolling, activation and arrest. Following arrest, many leucocytes migrate between endothelial junctions into the perivascular tissue [1]. This co-ordinated sequence of events is mediated by at least three families of adhesion receptors: selectins, integrins and the Ig gene superfamily [2]. L-selectin is normally expressed on the surface of most leukocytes, including lymphocytes, neutrophils, monocytes, eosinophils,

haematopoietic progenitor cells and immature thymocytes [3]. L-selectin mediates the binding of lymphocytes to high endothelial venules of peripheral lymph nodes [4, 5], and is also involved in leukocyte attachment to endothelium at sites of inflammation [6]. In addition, L-selectin is thought to amplify the inflammatory process by permitting adherent neutrophils to recruit additional neutrophils [7–9]. L-selectin shedding is one important aspect of the normal physiological regulation of L-selectin adhesive function. Cell surface expression of this adhesion molecule is characteristically down-modulated in response to cell activation [3, 5, 10, 11]. It has been proposed that endoproteolytic release of L-selectin might provide a rapid means for the regulation of leukocyte de-adhesion necessary to enable leukocytes to detach from

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bound endothelial cells before transmigration between endothelial cells and into sites of inflammation [10].

There is increasing evidence of neutrophil participation in asthma and the allergic process. For instance, plasma chemotactic activity of neutrophils increases and peripheral-blood neutrophils show markers of activation during active asthma, after exercise-induced asthma and during early and late asthmatic reactions induced by allergens [12, 13]. In subjects with pollen-induced asthma, seasonal antigenic exposure is associated with an increase in the number of neutrophils shown in bronchial biopsies [14]. In other atopic asthmatics, local endobronchial allergen challenge leads to increased neutrophil infiltration of the airway mucosa [15]. Previous studies on human neutrophils have demonstrated the presence of the three forms of IgE receptor: FcεRI [16–18], FcεRII/CD23 [19, 20] and galectin-3 [21–24]. We have previously shown that specific allergens were able to activate functional responses by neutrophils from allergic patients sensitized to allergens of the same type as those which produce clinical allergic symptoms [25, 26]. However, it is unknown whether the expression of L-selectin on the surface of neutrophils can be modulated by an IgE-dependent mechanism. The present work was undertaken to analyse this question. We present evidence that specific allergens were able to regulate the expression of L-selectin on neutrophils from allergic patients. The modulation of CD62L expression can be induced through the three receptors of IgE. Incubation of neutrophils with calphostin C a specific protein kinase C (PKC) inhibitor [27], with wortmannin a specific phosphatidylinositol (PI) 3-kinase inhibitor [28], or with manoalide a phospholipase A₂ (PLA₂)-specific inhibitor [29], attenuates IgE-dependent down-regulation of CD62L. These data imply that IgE mediates CD62L down-regulation probably through PKC-, PI 3-kinase- and PLA₂-dependent pathways.

Material and Methods

Reagents

The allergens were commercially available antigen extracts, including D₁ (*Dermatophagoides pteronyssinus*), G₃ (*Dactylis glomerata*), T₉ (*Olea europaea*) and W₆ (*Artemisia vulgaris*). They were purchased from Bial-Aristegui (Bilbao, Spain). N-formyl-methionyl-leucyl-phenylalanine (fMLP), manoalide, wortmannin, calphostin C, herbimycin A, erbstatin, okadaic acid, ET-18-OCH₃ and D609 were from Sigma-Aldrich Co. (Madrid, Spain). Ficoll-Hypaque, phosphate-buffered saline (PBS), RPMI 1640, fetal bovine serum and penicillin/streptomycin were purchased from Bio-Whittaker (Verviers, Belgium). Monoclonal antibody (mAb) anti-FcεRI (α chain) clone AER-37 (CRA 1) was obtained from eBioscience (San Diego, CA, USA); mAb anti-FcεRII (CD23) clone 9P25 was from IZASA-Immunotech (Barcelona, Spain), and mAb anti-galectin-3 clone ab2785 was from Abcam (Cambridge, UK). All cultured reagents had endotoxin levels of ≤0.01 ng/mL, as tested by the *Limulus* lysate assay (Coatest, Chromogenix, Mölndal, Sweden).

Patients and controls

The studied group included adult atopic patients with intermittent [30] bronchial asthma (35 men and 45 women;

mean age 36 years; range 18–56 years), and healthy adult non-atopic volunteer controls (four men and six women; mean age 33 years; range 20–54 years). All subjects were lifelong non-smokers. Asthma was diagnosed on the basis of criteria previously described in detail [31]. One group of asthmatic patients had positive skin prick test (SPT) (Bial-Aristegui) and specific IgE (HYTEC 288, Hycor Biomedical Inc. – IZASA, Barcelona, Spain). The sensitivity of the assay was the 0.24 ng/mL to at least one common allergen (house-dust mites and pollens). The subjects received no specific hyposensitization (non-immunotherapy (IT) asthmatic group). Another group of asthmatic patients had been IT-treated with D₁ extract (Bial-Aristegui) for the previous three years and continued to receive a maintenance dose with the highest potency of the extract (IT-asthmatic group). The subjects of both asthmatic groups received no treatment; none of them had experienced episodes of asthma for at least 3 months, and none had had respiratory tract infections in the 4 weeks before blood sampling. The healthy controls had no history of allergy or bronchial symptoms, and had negative SPT (Bial-Aristegui) and specific IgE (HYTEC 288) to a battery of inhalant allergens (house-dust mites, pollens, moulds and animal danders). The Hospital Ethics Committee approved the study, and each subject gave informed consent.

Preparation of polymorphonuclear leucocytes

Human neutrophils were obtained from fresh heparinized blood as indicated [25, 32] and purified by dextran centrifugation, followed by Ficoll-Hypaque gradient centrifugation and hypotonic lysis of residual erythrocytes. Cells were washed twice with PBS. For further purification, neutrophil preparations were incubated with mouse anti-human CD9 antibody (Immunotech-IZASA) and goat anti-mouse IgG micromagnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) [33]. The purity of neutrophils was on average >99% (<0.1% eosinophil contamination). Purified cells were used immediately.

Dissociation of neutrophil-bound immunoglobulins

Immunoglobulins were dissociated from the surface of neutrophils as described previously [33]. Kita et al. [18] measured the amount of IgE binding on the surface of basophils, neutrophils, eosinophils and monocytes from peripheral blood. Neutrophils have the second highest amount of IgE binding on their surface (basophil > neutrophil > eosinophil > monocyte). Basophils have on their surface from 10 to 15 times more IgE than do neutrophils. To elute the IgE from the surface of the basophils, 0.5 to 1 × 10⁶ cells are employed [34, 35]. We used 1 × 10⁸ neutrophils to elute the IgE from the cell surface – i.e. from 100 to 200 times more cells than used with the basophils, to be able to obtain appreciable amounts of specific IgE from the neutrophil surface. After isolation, cells (1 × 10⁸ cells) were resuspended in 1 mL of acetate buffer (50 mM sodium acetate pH 4, 85 mM NaCl, 5 mM KCl, 0.03% human serum albumin) and incubated on ice for 3 min. An equal volume of gelatin veronal buffer (GVB) (1.8 mM sodium barbital, 3.1 mM barbituric acid, 0.1% gelatin, 0.05 mM MgCl₂, 141 mM NaCl, 0.15 mM CaCl₂ pH 7.3–7.4) was then added to the treated

cells, and the mixture was centrifuged at 500 *g* for 10 min. Immunoglobulin-containing supernatant was neutralized with 1 N NaOH and used for specific IgE and IgG determinations (HYTEC 288).

Neutrophil culture and treatment with different agents

Neutrophils were resuspended at 1×10^6 in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37 °C in an atmosphere of 5% CO₂ and 95% O₂. For stimulation treatments, the cells were incubated with allergens or agonists at 37 °C for times indicated in the legends of the figures. The inhibitors manolide, wortmannin, calphostin C, herbimycin A, erbstatin, okadaic acid, ET-18-OCH₃ and D609 were solubilized in dimethyl sulphoxide (DMSO) and added to cell suspensions 45 min prior to stimulation, in 1 µL aliquots. The final DMSO concentrations were less than 1%. Cells were pre-incubated for 2 h in the light with calphostin C as previously indicated [27]. None of the agents affected the viability of the cells at the concentrations used, as confirmed by the trypan blue dye exclusion test.

Flow cytometry analysis

For all experiments, cells were rapidly cooled on ice, and 1 mL of PBS was added. Cells were then centrifuged at 500 *g* for 10 min, supernatants removed and cell pellets washed three times with cold wash buffer before staining with 20 µL of phycoerythrin (PE)-labelled anti-CD62L; control cells were stained with PE-labelled IgG2b-non-specific control antibody (Coulter Corp. – IZASA, Barcelona, Spain). Flow cytometry was performed on an Epics Elite flow cytometer (Coulter Corp. – IZASA) and calibrated by standard techniques. For each stained sample, 2×10^4 cells were analysed for fluorescence intensity. The fluorescence distribution for both control and test cells was analysed using a

Coulter Elite software workstation. Results are expressed as the frequency of fluorescence-positive cells (%) and mean fluorescence intensity (MFI) [25].

Analysis of shed L-selectin (sL-selectin) by ELISA

Neutrophils were incubated on a 96-well plate in the absence or presence of stimuli at the times and doses indicated in each case. sL-selectin levels in culture supernatants were determined in replicate wells using a sandwich ELISA (Bender Med Systems, Vienna, Austria). Samples were diluted 1:100 before analysis. The limit of sensitivity determined by the manufacturer was 0.3 ng/mL.

Statistics

The data are expressed as the mean $\pm$ SE. A one-way ANOVA test was used, and statistical significance was defined at $P < 0.05$.

Results

Dose- and time-dependent effect of allergen on cell surface expression of CD62L

Experiments were designed to analyse whether the allergen affected in a dose-dependent manner the surface expression of CD62L. Figure 1a illustrates that when neutrophils from allergic patients were incubated with different doses of allergen, they triggered L-selectin down-regulation, as evidenced by the decrease in both the number of L-selectin-positive cells and L-selectin surface density. Figure 1b illustrates a time-dependent response in L-selectin surface expression on neutrophils challenged with allergen. Neutrophils from allergic patients were stimulated with 10 µg/mL of allergen. The down-modulation started at 10 min of treatment, and reached a maximum after 30 min of treatment. These effects were observed both in % of positive cells and in MFI.

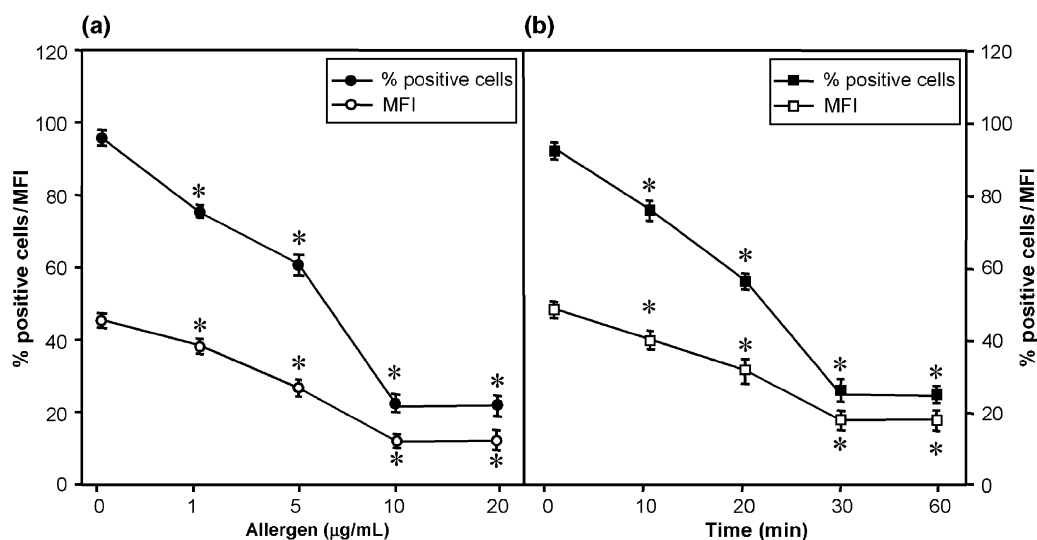


Fig. 1. Dose- and time-dependent effect of allergen on cell surface expression of L-selectin (CD62L) by neutrophils from allergic patients. When neutrophils from allergic patients ($n = 4$) were incubated with different doses of allergen, L-selectin down-regulation was triggered, as evidenced by the decrease in both number of L-selectin-positive cells and L-selectin surface density (a). Kinetic response in L-selectin surface expression on neutrophils challenged with specific allergen (b). * $P < 0.001$ vs. control.

Down-modulation of L-selectin on neutrophils after specific allergen challenge of cells from allergic patients

Subsequent studies were addressed to examining the specificity of L-selectin down-modulation of allergen-specific response on neutrophils. Consistent with our previous observations [25, 26, 33], the response of neutrophils to the allergen by was very specific. Neutrophils from healthy

donors were always simultaneously assayed and taken as controls. In these cells, after allergen challenge, no differences of CD62L values were detected in either % of positive cells (Fig. 2a) or MFI (Figs 2b and 3). Similarly, when cells were incubated with allergens to which the patients were not sensitized, no CD62L down-regulation was detected in either % of positive cells (Fig. 2c) or MFI (Fig. 2d). Nevertheless, in both of these preparations, CD62-L-down-regulation was

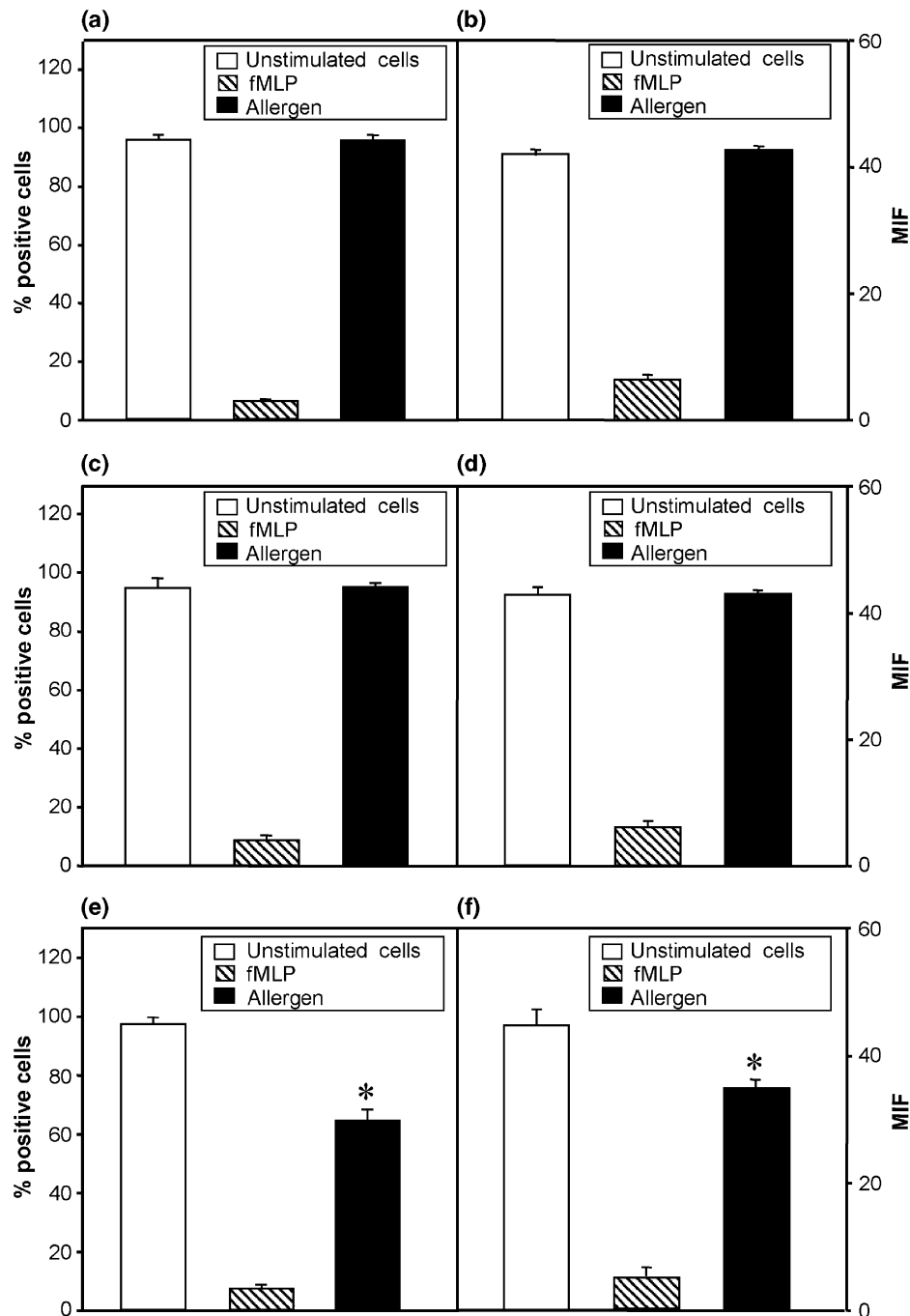


Fig. 2. Specificity of allergen response by the surface expression of L-selectin (CD62L). After allergen challenge of neutrophils from healthy donors ($n = 10$), no difference of CD62L values was detected either in % of positive cells (a) or in mean fluorescence intensity (MFI) (b). When cells were incubated with allergen to which the patients were not sensitized ($n = 10$), no CD62L down-regulation was detected either in % of positive cells (c) or in MFI (d). In immunotherapy (IT) asthmatic group ($n = 10$), the CD62L down-modulation was attenuated significantly ($P < 0.001$) with respect to the non-IT asthmatic group (e,f). In all cases, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (100 nM)-stimulated cells were used as positive control. Dose of allergen used was 10 μ g/mL.

observed when neutrophils were treated with fMLP, a potent inducer of this process. In the IT asthmatic group, the CD62L down-modulation was attenuated significantly ($P < 0.001$) with respect to that in the non-IT asthmatic group (Figs 2e,f).

The presence of specific IgE from the neutrophil cell surface was examined. Only patients with positive SPTs and serum specific IgE to the studied allergens showed positive specific IgE from the neutrophil cell surface. None of the SPT and serum-specific-IgE negatives to these allergens (patients and controls) had positive specific IgE from the neutrophil cell surface. The levels of specific IgE from the cell surface varied from 8.4 to 41.2 ng/mL. After *in vitro* allergen challenge of neutrophils, only patients with specific IgE from the neutrophil cell surface positive to this allergen demonstrated down-regulation of the CD62L (data not shown). There was a correlation between the amount of specific IgE eluted from the cell surface and the amount of L-selectin shedding from the surface of neutrophils after allergen challenge ($R = 0.844$, $P < 0.001$). None of the patients or controls showed specific IgG to allergens in the supernatant of immunoglobulins eluted from the neutrophil cell surface.

Down-modulation of L-selectin on neutrophils after mAb anti-FcεRI, -FcεRII (CD23) and -galectin-3 challenge of cells from allergic patients

Experiments were designed to analyse which IgE receptor was involved in the surface expression of CD62L. Figure 3 illustrates that when neutrophils from allergic patients were incubated with mAbs against FcεRI, FcεRII/CD23 and galectin-3 (5 µg/mL), there was a similarly extensive down-modulation of L-selectin on the neutrophil surface in all the three cases.

Specific allergen initiates L-selectin shedding

As indicated previously, L-selectin shedding is known to be a major mechanism by which L-selectin expression on the plasma membrane is regulated by different factors. Therefore, to determine whether the observed down-regulation effect of allergen on L-selectin was exerted through a shedding mechanism, sL-selectin levels in supernatants from allergen-treated cultures were analysed. The results are shown in Fig. 4. When neutrophils from allergic patients were incubated with 10 µg/mL of allergen for 30 min, levels of sL-selectin in the culture supernatant were higher than when neutrophils were left untreated (Fig. 4b). These results are consistent with the shedding of L-selectin from the surface of the neutrophils in the same cell preparation (Fig. 4a).

Down-modulation of L-selectin surface expression by allergen is dependent on the activity of protein kinase C, phosphatidylinositol 3-kinase and phospholipase A₂

The mechanism by which the allergen negatively modulates L-selectin surface levels was examined by performing studies with different inhibitors, in order to analyse some signalling pathways confluent on the activation of PKC. As shown in Fig. 5a, the presence of calphostin C prior to addition of the allergen attenuated the shedding of CD62L. Calphostin C at 1 µM inhibited the effect of the allergen by about 80%. However, at higher doses of calphostin C, a reversion of

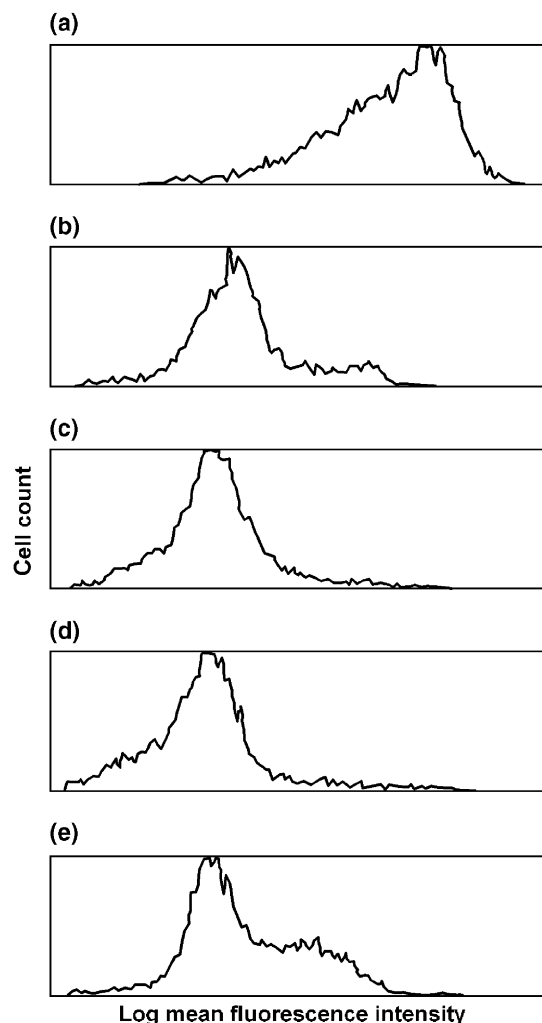


Fig. 3. Representative flow cytometry experiment, showing the down-modulation of L-selectin expression by neutrophils treated with allergen and monoclonal antibodies (mAbs) anti-IgE receptors. Neutrophils from allergic patients ($n = 5$) were stimulated with 10 µg/mL of allergen, 5 µg/mL of mAb anti-CD23, 5 µg/mL of mAb anti-galectin-3, and 5 µg/mL of mAb anti-FcεRI for 30 min at 37 °C. The figure shows the cytograms of control cells (a), cells treated with allergen (b), with anti-FcεRII (CD23) (c), with anti-galectin-3 (d) and with anti-FcεRI (e).

allergen action was found. Thus, present data on the allergen-induced shedding of CD62L are in agreement with previous suggestions on the role of protein kinase(s) activation in the shedding of CD62L [11]. A similar effect was observed after the inhibition of PLA₂ with manoalide (Fig. 5b) [36]. Notably, the sensitivity to this inhibitor was very high, since at 1 µM there was a total cancellation of the allergen's effect on CD62L expression. Present data suggest that the sequence of allergen-induced events includes the release of some lipid by the action of PLA₂, which in turn activates PKC, a lipid/Ca²⁺-dependent enzyme. The participation of PLA₂ activity in the modulatory effect of the allergen seems evident, since manoalide practically abolished the spontaneous shedding detected in untreated control cells, presumably by an endogenous activation of PLA₂. This possibility is reinforced by the fact that other pathways eliciting an increase in Ca²⁺ levels through a PI₃-dependent mechanism have been implicated in CD62L shedding. In this context, the inhibition

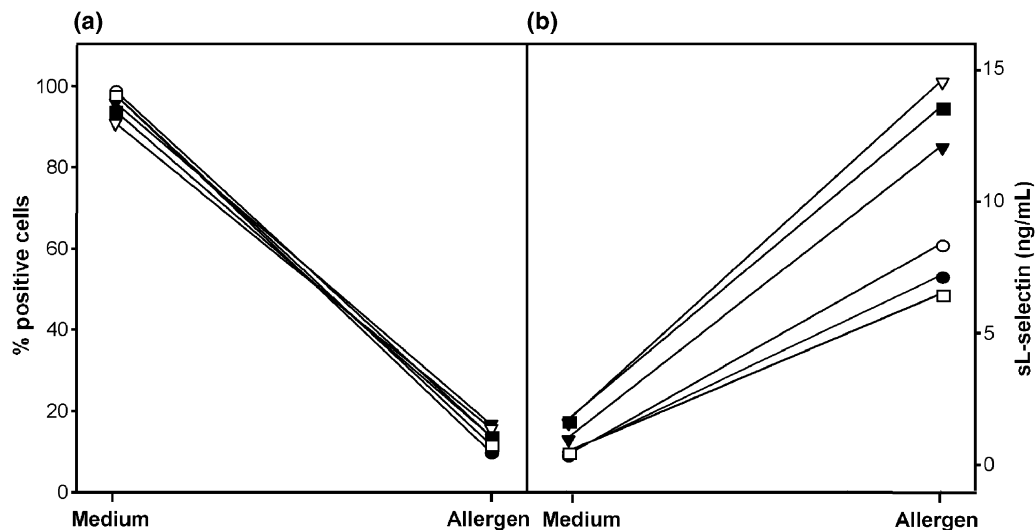


Fig. 4. L-selectin shedding upon allergen stimulation. Neutrophils from allergic patients ($n = 6$) were stimulated with $10 \mu\text{g/mL}$ of allergen for 30 min at 37°C . Shed L-selectin (sL-selectin) levels in culture supernatant were determined in replicate wells using a sandwich ELISA. (a) Down-modulation of L-selectin on neutrophils. (b) Levels of sL-selectin in the same patients.

of PI 3-kinase by wortmannin partially cancelled the allergen-induced down-regulation of CD62L (Fig. 5c). Taken together, these data demonstrate that L-selectin down-modulation from the neutrophil cell surface can be triggered via specific intracellular signalling pathways which could act in concert to activate PKC. Other inhibitory compounds, such as herbimycin A and erbstatin (phosphotyrosine kinase inhibitors) [37, 38], okadaic acid (phosphatase 1A and 2A inhibitor) [39], ET-18-OCH₃ (phosphatidylinositol phospholipase C inhibitor) [40] and D609 (phosphatidylcholine phospholipase C inhibitor) [41], did not alter the allergen-induced shedding of CD62L (data not shown). These results suggest that the corresponding signalling on which they act is not involved in the allergen's modulatory effect of CD62L expression.

Effect of anti-allergic therapeutic modalities in allergen down-modulation of L-selectin surface expression

As shown in Fig. 2, immunotherapy negatively modulates the IgE-dependent down-modulation of L-selectin surface expression on neutrophils from atopic subjects that had received allergen immunotherapy (IT asthmatic group). Pre-incubation (120 min) with the glucocorticosteroid budesonide resulted in a concentration-dependent attenuation of the IgE-dependent down-regulation of L-selectin (Fig. 6) (similar results were obtained with dexamethasone, data not shown). The effect of budesonide was not specific to allergen-induced L-selectin shedding, and did it inhibit fMLP-dependent shedding (data not shown). Other allergic therapeutic modalities such as antihistamines (terfenadine, loratadine and cetirizine) or nedocromil sodium did not affect the IgE-dependent shedding of L-selectin (data not shown).

Discussion

Cell functions can be directly controlled by modulating cell-surface receptor expression. The experiments described here

provide the first evidence that neutrophils from allergic patients modulate expression of their cell-surface adhesion-molecule receptors by an IgE-dependent mechanism. First, the activation *in vitro* of neutrophils appears to be highly allergen-specific. In this context, the allergen down-regulates the L-selectin in cells from sensitized patients, but allergens other than those which produce clinical symptoms do not modulate the surface expression of L-selectin. Furthermore, allergens were ineffective on neutrophils from healthy donors. These data closely agree with previous reports showing that the stimulation of neutrophils with activating or chemo-attractant factors such as phorbol 12-myristate acetate (PMA), C5a, fMLP, tumour necrosis factor alpha (TNF- α), leokotriene (LT)B₄, lipopolysaccharide, or IL-1 causes a rapid decrease in the number of surface L-selectin molecules [10, 42, 43]. Presumably, the mechanism of the CD62 down-modulation by allergens is IgE-dependent, because only patients with specific IgE from the neutrophil cell surface positive to these allergens demonstrated down-regulation of the CD62L. The event that initiates hypersensitivity is the binding of antigen to IgE on the cell surface. Cells are activated by cross-linking of Fc ϵ R molecules, which is thought to occur by binding of multivalent antigens to the attached IgE molecules. Experimentally, antigen binding can be mimicked by anti-Fc ϵ R antibodies. Like other immunoglobulins, IgE has separate regions that are specialized to react with antigens (Fab) and with a variety of biologic effector systems (Fc). Reaction of antigen with the Fab region initiates any allosteric signal that is transmitted to the Fc region, and hence to the receptor to which the IgE is bound [44]. Instead, the antigen-induced activation of the cell appears to be initiated solely by the aggregation of the cell-bound IgE that occurs as a consequence of the latter's interaction with a multivalent antigen. Indeed, any manoeuvre that leads to aggregation of Fc regions of the IgE is effective in initiating activation. Aggregation of the Fc regions leads to aggregation of the receptors to which they are bound. It is this aggregation of the receptors that constitutes the critical event. Thus, antibodies to the receptor

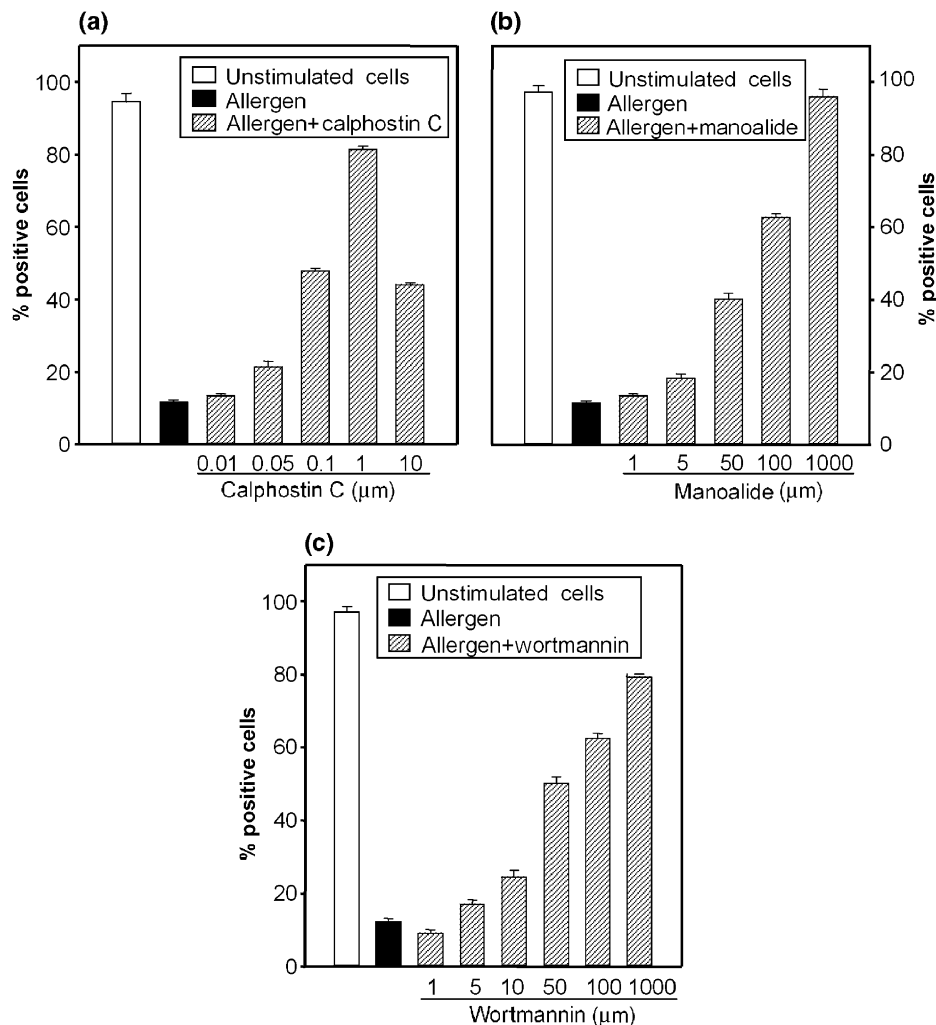


Fig. 5. Down-modulation of L-selectin surface expression by allergen is dependent on protein kinase C, (PKC), phosphatidylinositol (PI) 3-kinase and phospholipase A_2 activity. Human neutrophils from allergic patients ($10^6/\text{mL}$) were incubated with allergen ($10 \mu\text{g}/\text{mL}$) at 37°C for 30 min. Effect of calphostin C ($n = 5$) (a), manoalide ($n = 5$) (b), and wortmannin ($n = 5$) (c). n , number of patients.

can stimulate activation because they can cluster the receptor [45, 46]. Triggering of the three kinds of IgE receptor of different cells, including neutrophils, by antibodies against these three IgE receptors has been shown to activate the cells, modulate some of their functions and enhance production of various mediators [17, 19, 21]. In an attempt to discover which IgE receptor is involved in the down-modulation of CD62L, we challenged the neutrophils with mAb to Fc ϵ RI, Fc ϵ RII (CD23) and galectin-3 receptors. We have shown in this paper that the three receptors of IgE are involved in this phenomenon. As far as we are aware, this is the first time that the possible relationship of the three IgE receptors in neutrophil function has been investigated. Previously only the relationship of Fc ϵ RI with the liberation of IL-8 by these cells has been demonstrated [17]. The physiological significance of these findings could be that extravasation and activation of neutrophil granulocytes are essential during the inflammatory response. Leukocyte extravasation into inflamed areas involves complex interaction of leukocytes with endothelium through regulated expression of surface adhesion molecules. Of these, L-selectin appears to play a key role during the initial attachment of circulating leukocytes to

endothelium. It is assumed that the shedding of L-selectin is one of the prerequisites for cell migration, permitting leukocyte extravasation [47–49]. Thus, the IgE-dependent mechanism might lead to direct neutrophil extravasation into local inflammatory sites in allergic pathological situations. Furthermore, granulocytes recruited to the lung in allergic patients show a significantly decreased CD62L expression, compared with their peripheral blood counterparts [50, 51, 52]. The observed optimal dose in the allergen-dependent activation of neutrophils from allergic patients is $10 \mu\text{g}/\text{mL}$; this finding closely agrees with our previous results [25, 26, 33]. The observed kinetics of CD62L shedding are similar to those previously reported [10, 42, 43], since after 30 min only 10–15% of CD62L was found on the surface of neutrophils, as compared with untreated cells. As is illustrated in Figs 1 and 2, levels of the down-modulation of CD62L as measured by % of positive cells were similar to those by MFI, and they are in agreement with previous reports [3, 28].

When the neutrophils from allergic patients were stimulated with allergen, the down-regulation of CD62L led to an increase of sCD62L in the supernatant of neutrophils. This result is in agreement with the observed effect of other

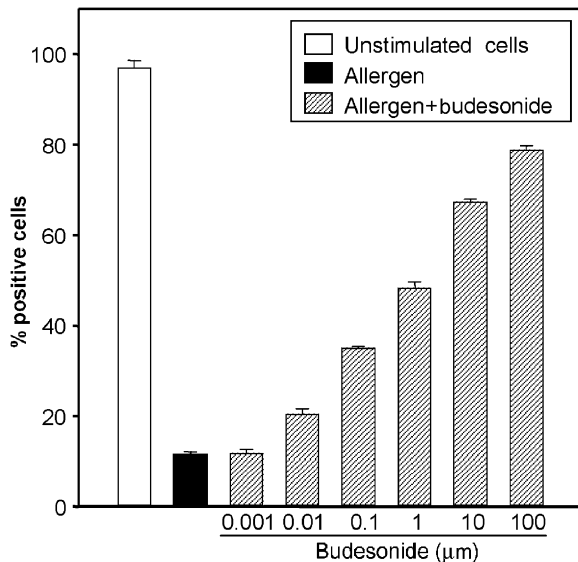


Fig. 6. Effect of budesonide on L-selectin expression challenged with allergen. Neutrophils ($10^6/\text{mL}$) were pre-incubated for 120 min at 37°C and then challenged with $10\text{ }\mu\text{g/mL}$ allergen for 30 min. Data are mean $\pm$ SE of six experiments with different donor ($n = 5$) cell preparations (similar results were obtained with dexamethasone, data not shown).

activating factors, such as fMLP, IL8, PMA, GM-CSF, TNF and LPS, which have also been shown to affect cell-surface L-selectin expression [3–6, 10, 11]. We describe here that when neutrophils were stimulated with allergen, the cell-surface down-modulation of L-selectin in the same cell preparation directly correlated with an increase of sL-selectin in the culture supernatant (Fig. 4).

The mechanism by which the allergen elicits CD62L shedding remains to be clarified. Several notable differences were observed between the regulation of L-selectin surface density by the allergen and that by other stimulating factors (e.g. platelet-activating factor (PAF), PMA and anti-Leu mAb), suggesting that these stimuli operate through distinct pathways to control L-selectin expression. PLA_2 from neutrophils has been shown to be activated by different stimulating factors [53, 54]. Therefore, we have investigated whether the down-regulation of CD62L could be mediated by a mechanism related to PLA_2 . In fact, manoilide was found to inhibit the shedding of CD62L from neutrophils when they were challenged with allergen (Fig. 5b). However, it has previously been shown that other stimuli seem to act through alternative metabolic signalling pathways, since Ca^{2+} ionophore A23187- and scalaradial (a PLA_2 inhibitor)-treated human neutrophils exerted no effect on the shedding of the extracellular domain of L-selectin [36].

Wortmannin actively contributes to the regulation of several complex regulatory processes in human neutrophils, including agonist-induced respiratory burst and adhesion of these cells in response to LPS [55, 56]. Wortmannin also suppresses IgE-mediated histamine release [57] and, at nM concentrations similar to those used in the present work, acts as a selective inhibitor of PI 3-kinase [28, 55]. In our case, wortmannin inhibited in a dose-dependent fashion the allergen-mediated down-regulation of L-selectin on neutrophils from allergic patients. Thus, present data indicate that PI 3-kinase activity could mediate the down-regulation of

CD62L on neutrophils from allergic patients after activation with allergen.

Previous reports had shown that stimulation of lymphocytes with activators of PKC, such as PMA or 1-oleoyl 2-acetyl-glycerol, results in a nearly complete loss of surface expression of CD62L, whereas pretreatment of the cells with the PKC inhibitors H-7 or staurosporine prevents the loss of CD62L [11]. In sharp contrast, an anti-Leu 13 mAb-induced L-selectin down-modulation was demonstrated to involve a PKC-independent pathway [58]. We have demonstrated here that allergen down-regulates L-selectin surface expression via a calphostin-C-sensitive pathway, suggesting that a PKC-dependent mechanism is involved.

The phosphotyrosine inhibitor herbimycin A blocked loss of L-selectin induced by anti-CD3 from CD4^+ T cells. In contrast, and in agreement with our findings, herbimycin A did not affect the loss of L-selectin either from CD8^+ cells stimulated with anti-CD3 MoAb, or from neutrophils treated with nonsteroidal anti-inflammatory drugs [59, 60]. Another phosphotyrosine kinase inhibitor, erbstatin, in disagreement with our findings, reduced the hypertonicity-stimulated shedding of L-selectin [61]. As far as we know, other inhibitory compounds, such as okadaic acid (phosphatase 1A and 2A inhibitor) [39], ET-18-OCH₃ (phosphatidylinositol phospholipase C inhibitor) [40] and D609 (phosphatidylcholine phospholipase C inhibitor) [41], have not been used to investigate the shedding of L-selectin from any cell. In our case, although these inhibitors affect some functions in neutrophils [62–64], they did not alter the allergen-induced shedding of CD62L. Taken together, these results suggest that the phosphotyrosine kinase, phosphatase 1A and 2A and phosphatidylinositol phospholipase C and phosphatidylcholine phospholipase C intracellular signalling pathways were not involved in the IgE-dependent effect of CD62L expression.

Allergen immunotherapy negatively modulates the IgE-dependent down-modulation of L-selectin surface expression on neutrophils from atopic subjects who had received allergen immunotherapy (IT asthmatic group). This finding is in agreement with our previous report on IgE-mediated down-regulation of L-selectin on lymphocytes from asthmatic patients [65] and with respect to the allergen immunotherapy that modulates certain functions of neutrophils, such as their chemotactic activity and the release of myeloperoxidase [66, 67]. Preincubation with the two glucocorticosteroids, budesonide and dexamethasone, resulted in a concentration-dependent attenuation of the IgE-dependent down-regulation of L-selectin (Fig. 6). These observations are consistent with previous studies demonstrating that dexamethasone attenuated PAF-induced down-regulation of L-selectin [68]. In contrast, other allergic therapeutic modalities such as antihistamines (terfenadine, loratadine and cetirizine) or nedocromil sodium did not affect the IgE-dependent shedding of L-selectin, although all these drugs affect several functions in neutrophils [69, 70].

In summary, we present evidence that specific allergens were able to regulate the expression of L-selectin on neutrophils from allergic patients, that the three receptors of IgE are involved in this phenomenon, and that IgE mediates CD62L down-regulation probably through PKC-, PI 3-kinase-, and PLA_2 -dependent pathways. The phospho-

tyrosine kinase, phosphatase 1A and 2A, and phosphatidylinositol phospholipase C and phosphatidylcholine phospholipase C intracellular signalling pathways were not involved in the IgE-dependent effect of CD62L expression. Specific allergen immunotherapy and glucocorticoids modulate IgE-dependent CD62L down-regulation, whereas other allergic therapeutic modalities such as antihistamines (terfenadine, loratadine and cetirizine) or nedocromil sodium did not affect the IgE-dependent shedding of L-selectin. These observations reveal a novel mechanism, which could affect neutrophil adhesion to endothelial cells and their migration into allergic sites.

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