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Leukotriene D_4 activates β_2-integrin adhesion in human polymorphonuclear leukocytes

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ABSTRACT: We examined the functional role and mechanisms by which activation of cysteinyl leukotriene-1 receptor ($cysLT_1R$) regulates β_2-integrin adhesion to intercellular adhesion molecule (ICAM)-1 in human polymorphonuclear leukocytes (PMNs) *in vitro*.

Human peripheral blood PMNs and eosinophils were isolated separately from the same mildly atopic donors. Surface expression of $cysLT_1R$ was identified both in PMNs and in eosinophils by immunofluorescence analysis. Total $cysLT_1R$ protein was substantially greater in eosinophils than in PMNs as determined by Western blot analysis. However, leukotriene D_4 (LTD_4) upregulated β_2-integrin adhesion of PMNs to ICAM-1 with high efficacy in a time- and concentration-dependent manner. Upregulated β_2-integrin adhesion of PMNs was related temporally and quantitatively to phosphorylation of 85-kDa cytosolic group IVa phospholipase A2 (gIVaPLA2). Augmented LTD_4-induced adhesion was blocked significantly by montelukast, a $cysLT_1R$ antagonist. Trifluoromethylketone (a gIVaPLA2 inhibitor) blocked β_2-integrin adhesion caused by LTD_4 activation, as did anti-CD18 monoclonal antibody directed against β_2-integrin on the PMN surface.

Our data demonstrate that LTD_4 causes phosphorylation of gIVaPLA2 and upregulation of β_2-integrin adhesion to ICAM-1 or ICAM-1 surrogate through $cysLT_1R$ activation. Activation of gIVaPLA2 is a critical step through which β_2-integrin adhesion is upregulated by the $cysLT_1R$ expressed on the surface membrane of human PMN.

KEYWORDS: Adhesion, cysteinyl leukotriene-1 receptor, group IVa phospholipase A2, polymorphonuclear leukocytes

Cysteinyl leukotrienes (cysLTs), prostaglandins and thromboxanes are families of pro-inflammatory mediators arising through metabolism of arachidonic acid. CysLTs are implicated in airway hyperresponsiveness in patients with asthma [1, 2]. Leukotriene D_4 (LTD_4), a cysLT, is generated extracellularly by gamma-glutamyl transpeptidase from secreted LTC_4, which is synthesised by intracellular conversion from LTA_4 by LTC_4 synthase [2, 3]. LTC_4 and LTD_4 have substantial efficacy in causing airway constriction in asthmatic subjects [1, 4, 5]. Three subclasses of cysLT receptors have been identified, $cysLT_1R$ [6–10], $cysLT_2R$ [6–10] and $cysLT_3R$ [11, 12]. Biological activities such as airway hyperresponsiveness [13], cellular adhesion [14–16], cell migration [17, 18] and mucus secretion [19] are mediated predominantly through $cysLT_1R$ activation. Whereas $cysLT_2R$ has been reported to cause vascular permeability [4, 20], blockade of $cysLT_3R$ appears to prevent hypoxic brain injury [12].

The 85-kDa cytosolic group IVa phospholipase A2 (gIVaPLA2) is a critical intracellular messenger protein for cellular adhesion and secretion of bioactive lipid mediators in human eosinophils [21, 22] and polymorphonuclear leukocytes (PMNs) [16, 23]. Activation of gIVaPLA2 causes production of arachidonate metabolites and lysophospholipids [22–26], which are essential for β_2-integrin adhesion of granulocytes.

The objective of this study was to determine whether the activation of the $cysLT_1R$, which is significantly less expressed in PMNs than eosinophils, by LTD_4 augments β_2-integrin adhesion. PMNs do not synthesise cysLTs [2, 23] so activation of this receptor on PMNs under physiological circumstances *in vivo* would require exposure to cysLTs secreted from neighbouring

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inflammatory cells, *e.g.* eosinophils and macrophages. We also examined the efficacy of LTD_4 on the surface $cysLT_1R$ of PMNs in inducing cytosolic gIVaPLA2 phosphorylation to its active state.

As in other investigators [9, 10], we have found that $cysLT_1R$ is modestly expressed in PMNs. However, the functional role of $cysLT_1R$ on PMNs remains undefined. In this study, activation with LTD_4 caused a high degree of β_2-integrin adhesion to intercellular adhesion molecule (ICAM)-1 or its surrogate protein, bovine serum albumin (BSA). This process corresponded to the time-course for gIVaPLA2 phosphorylation caused by LTD_4 activation. Inhibition of gIVaPLA2 activity by trifluoromethylketone (TFMK) significantly blocked β_2-integrin adhesion caused by exogenous LTD_4. These data are the first to demonstrate that LTD_4 causes β_2-integrin adhesion to the endothelial surface ligand, probably through gIVaPLA2 activation in human PMNs *in vitro*.

MATERIALS AND METHODS

Antibodies and reagents

Purified LTD_4 and $cysLT_1R$ polyclonal antibody (pAb) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Human PMN/eosinophil isolation kits were obtained from StemCell Technologies (Vancouver, BC, Canada). BSA fraction V was purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Recombinant human ICAM-1 was purchased from R&D Systems (Minneapolis, MN, USA). The following antibodies (Abs) were purchased as follows: anti-phosphorylated gIVaPLA2 Ab (Ser^{505}) (Cell Signaling Technology, Danvers, MA, USA); anti-total gIVaPLA2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA); goat anti-mouse Ab and goat anti-rabbit Ab (BD Biosciences, Mountain View, CA, USA); anti-CD11b (Clone Bear I) (Beckman Coulter, Fullerton, CA, USA); and anti-CD18 Ab (Ancell Immunology Research Products, Bayport, MN, USA). TFMK was purchased from Biomol (Plymouth Meeting, PA, USA) and montelukast was supplied by Merck (Rahway, NJ, USA).

Isolation of human neutrophils and eosinophils

Human PMNs and eosinophils were isolated independently from mildly atopic subjects using an isolation kit for each cell type (StemCell Technologies). The protocol used in this study is approved by the University of Chicago Institutional Review Board (Chicago, IL, USA). Atopy was defined by criteria used in the University of Chicago Asthma Research Center for the National Heart, Lung, and Blood Institute Human Cooperative Asthma Genetics projects. Informed consent was obtained from all volunteers in this study before participation. Human volunteers with symptoms or who were taking medication were excluded. The study included a total of 15 individuals aged 20–45 yrs, seven male subjects and eight female subjects. All components of the project were in compliance with the University of Chicago and US government guidelines for studies in which donors are not participating subjects.

Briefly, PMNs were isolated using the isolation kit from StemCell Technologies. Cells were washed twice and resuspended in Hanks' balanced salt solution (HBSS) buffer plus Ca^{2+}/0.2% BSA prior to counting. Cells were kept on ice until use.

Eosinophils were isolated from the same human donors using the isolation kit from StemCell Technologies. A detailed protocol for isolation of eosinophils has been described recently [27]. The purity of neutrophils and eosinophils was determined by differential counts of haematoxylin and eosin-stained cytospin preparations. Isolated PMNs and eosinophils were >98% pure and >99% viable as assessed by trypan blue exclusion dye staining [15, 16, 21–23].

Surface expression of $cysLT_1R$ and β_2-integrin (CD11b/CD18) adhesion on granulocytes

Immunofluorescence analysis

Aliquots of 5×10^5 PMNs or 5×10^5 eosinophils were incubated with $cysLT_1R$ pAb or isotype-matched control Ab at 4°C for 60 min. After two washes, the cells were incubated with an excess of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Ig) for 30 min at 4°C. The cells were washed twice, resuspended in 300 μL of 1% paraformaldehyde, and kept at 4°C until analysis. Flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA, USA). Fluorescence intensity was determined on at least 10,000 cells from each sample. Data were analysed using FlowJo (Tree Star Inc., Ashland, OR, USA), a flow cytometric analysis software.

In separate studies, surface CD11b/CD18 expression was determined on PMNs using the protocol described previously. Freshly isolated human PMNs were stained with either monoclonal antibody directed against CD11b/CD18 (Clone Bear I; Beckman Coulter, Fullerton, CA, USA) or irrelevant isotype-matched control and mean fluorescence intensity (MFI) was determined as above.

Protein determination by the Bradford assay

The protein extracts from eosinophils and PMNs were prepared as follows: the cells were washed twice in ice-cold PBS and lysed with 500 μL of lysis buffer per well (25 mM Tris; 150 mM NaCl; pH 7.2) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). After sonication, cell lysates were transferred to microcentrifuge tubes and kept on ice. Lysates were centrifuged at $13{,}800 \times g$ at 4°C for 20 min in a table centrifuge to remove cell debris. The cleared supernatants were transferred to fresh tubes and protein concentrations were determined by Bradford assay (Pierce, Rockford, IL, USA). BSA was used as standard protein control.

Immunoblotting analysis

PMNs or eosinophils were isolated from the same donor and treated samples (see below) for Western blot analysis were prepared as described previously [15, 23]. The membrane was probed with 2 $\mu g \cdot mL^{-1}$ anti-$cysLT_1R$ pAb (Cayman Chemical, Ann Arbor, MI, USA), and incubated with 1:3000 dilutions of goat anti-rabbit Ig conjugated with horseradish peroxidase. The expression of $cysLT_1R$ was analysed by an enhanced chemiluminescence system (ECL; Amersham, Arlington Heights, IL, USA). Densitometric analysis of the film was performed using a Model GS-710 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA) in transmittance mode and analysed using Bio-Rad Discovery software. Briefly, quantitative measurement of optical density in light-sensitive photographic film containing protein bands was scanned using the Model GS-710 imaging densitometer. Each trace was

corrected for background by subtracting a tracing of cysLT$_1$R protein for that blot. Optical density was expressed as arbitrary units per mm^2 (U·mm^{-2}).

In a separate study, PMNs (10^6 cells·group^{-1}) were pre-treated with either vehicle control (HBSS+0.1 g·L^{-1} calcium, pH 7.4) or with increasing concentrations of montelukast at 37°C. 10^{-7} M LTD_4 was added 30 min later at different time intervals and gIVaPLA2 phosphorylation was determined by western blot analysis. Briefly, cells were lysed and experiments were conducted as described previously. The membrane was blocked with 1% BSA in TBS-T buffer for 60 min prior to addition of 2 μg·mL^{-1} anti-phosphorylated gIVaPLA2 mAb as site Ser^{505} (Cell Signaling Technology, Beverly, MA, USA). The protein from the same blot was stripped off and probed with total gIVaPLA2 Ab to demonstrate equal loading of treated samples.

Integrin adhesion assay

A 96-microplate well plate was coated with 10 μg·mL^{-1} soluble human ICAM-1 or 50 μL of soluble human BSA, a surrogate ligand for ICAM-1 [28], dissolved in 0.05 M $NaHCO_3$ coating buffer (15 mM $NaHCO_3$, 35 mM Na_2CO_3, pH 9.2) and incubated overnight at 4°C. Treated microplate wells were washed twice with HBSS buffer prior to use. The detailed protocol is described elsewhere [15, 16, 21–23]. We have previously shown that BSA is a full surrogate of ICAM-1 [28]. Further confirmatory data are presented for this study later on.

Adhesion was assessed as residual myeloperoxidase (MPO) activity of adherent PMNs. Treated PMNs (4×10^4 cells/100 μL HBSS/0.1% gelatin) were added to soluble ICAM-1 or BSA-coated microplate wells and allowed to settle on ice for 10 min. All assays were performed in duplicate, and data were analysed by Softmax (Molecular Devices, Sunnyvale, CA, USA). No MPO activity was detected in the cell-free reaction supernatants following 30 min of incubation, confirming that MPO was not present because of spontaneous degranulation of granulocytes. Adhesion was expressed as percentage of adhesion of total adherent cells for all treated groups. The detailed protocol for adhesion assay as a function of MPO activity has been described elsewhere [15, 23].

β_2-Integrin adhesion of PMNs caused by LTD_4: blockade with montelukast or TFMK

Time-course

Isolated PMNs were activated with 10^{-7} M LTD_4 at 0, 5, 10, 20 and 30 min prior to adhesion assay. Data are expressed as percentage of adhesion for all treated groups.

Concentration-response curve

Isolated PMNs were activated with increasing concentrations of LTD_4 from 10^{-10} M to 10^{-6} M at the time when adhesion caused by LTD_4 was greatest. Adhesion assay, as a function of residual MPO activity of adherent cells, was performed as detailed previously. Data were expressed as percentage of adhesion for all treated groups.

Blockade of adhesion caused by LTD_4

PMNs were pre-treated with 10^{-10} M to 10^{-6} M montelukast or 20 μg·mL^{-1} anti-CD18 monoclonal Ab for 30 min at 37°C prior to 10^{-10} M LTD_4 or 10^{-7} M LTD_4 activation. Adhesion assay was performed as detailed previously, and data were expressed as percentage of adhesion for all treated groups.

To determine whether β_2-integrin adhesion of PMNs to endothelial counter-ligand caused by LTD_4 was mediated through activation of gIVaPLA2, PMNs were pre-treated with 3 μM or 30 μM TFMK, an inhibitor of gIVaPLA2, for 30 min prior to 10^{-7} M LTD_4 activation [21, 22]. Adhesion assay was performed as detailed previously. Cell viability was assessed for each concentration using trypan blue exclusion dye staining.

To determine whether upregulation of adhesion affected by LTD_4 was caused by upregulation of β_2-integrin, we determined the efficacy of anti-CD18 monoclonal Ab in blocking PMN binding to both soluble ICAM-1 and ICAM-1 surrogate protein coated microplate wells after activation with the optimal concentration of LTD_4 (10^{-7} M) at 20 min, the time at which maximal adhesion occurs *in vitro*.

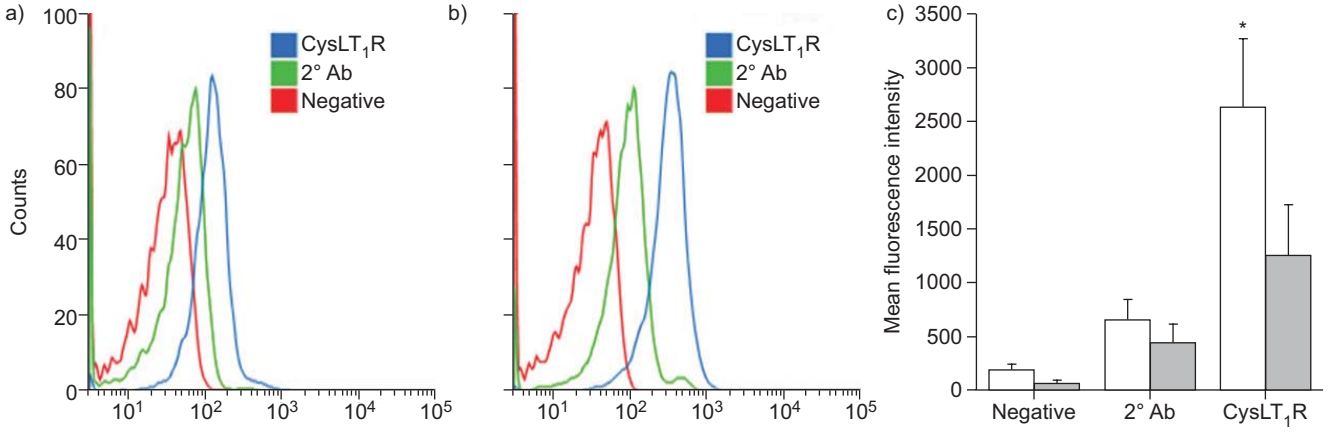

FIGURE 1. Surface expression of cysteinyl leukotriene-1 receptor (cysLT$_1$R) on a) polymorphonuclear leukocytes (PMNs) and b) eosinophils (EOS) as determined by immunofluorescence analysis using polyclonal antibody directed against cysLT$_1$R (n=5). Histograms were generated by flow cytometry using FlowJo (Tree Star Inc., Ashland, OR, USA) software analysis. Expression of cysLT$_1$R (blue line) is demonstrated by the right shift compared with cells stained with irrelevant isotype-matched control antibodies (Ab) (green line; fluorescin isothiocyanate conjugated goat anti-rabbit, 2° Ab) or unstained PMN or EOS (red line; negative, no Ab). c) Composite data (n=5 different individuals) for mean fluorescence intensity is shown for EOS (□) and PMNs (■). *: $p<0.05$ EOS *versus* PMN.

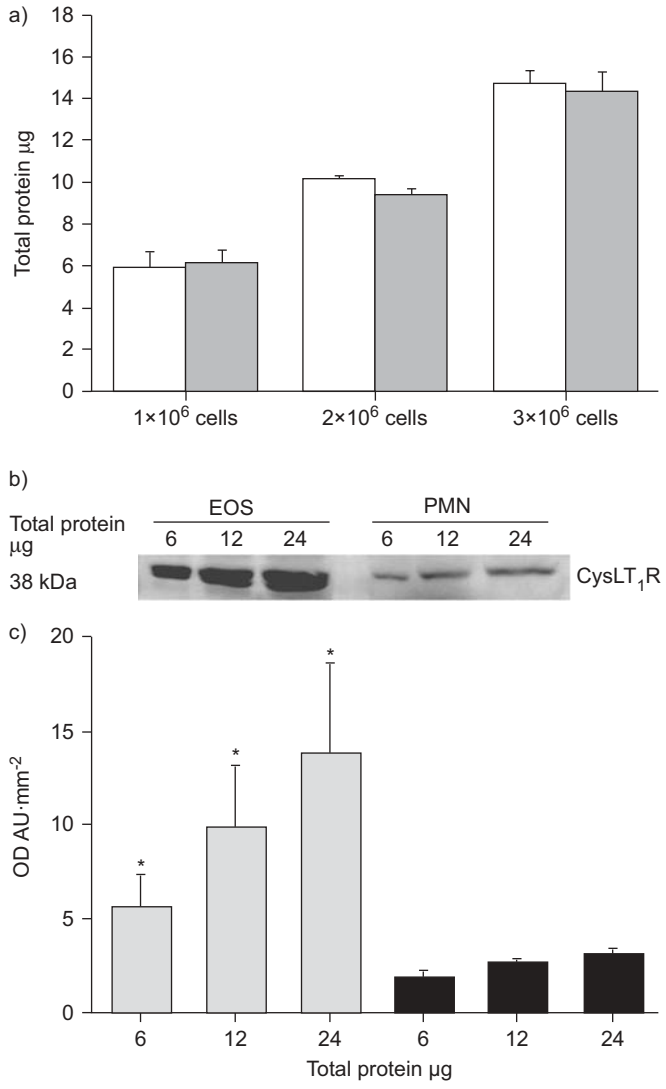

FIGURE 2. Expression of cysteinyl leukotriene-1 receptor ($cysLT_1R$) in eosinophils (EOS) and polymorphonuclear leukocytes (PMNs) by Western blot analysis. a) Total protein content extracted from isolated EOS (□) and PMNs (■) from four different individuals as assessed by the Bradford assay. b) A representative immunoblotting analysis on increasing protein content of EOS and PMNs as described in the Material and methods section. The $cysLT_1R$ protein band was detected at ~38 kDa using a molecular weight protein ladder. The full ladder is included in the supplementary material. c) Quantitation of $cysLT_1R$ expression by densitometric analysis of Western blot film (n=4 cell isolations). ■: EOS; ■: PMN. AU: arbitrary unit. *: $p<0.05$ corresponding protein content of EOS *versus* PMN.

Statistical analysis

All data are expressed as mean±SEM. Statistically significant differences among three or more groups were assessed by two-way ANOVA. Where the F-statistic indicated a difference, significance between groups of two was assessed further by the paired t-test. For concentration–response curves using the same agonist or antagonist, the paired t-test was used with Bonferroni correction for multiple comparisons on the same curve. All experiments were designed with matched controls within each experiment to enable statistical comparison as paired samples. A p-value <0.05 was considered to be statistical significant.

RESULTS

Relative expression of the cysLT$_1$R in granulocytes

Flow cytometric analysis demonstrated significant surface expression of $cysLT_1R$ on PMNs, which was less than for eosinophils in peripheral blood cells from the same individuals. Negative control is shown in green for PMNs stained with irrelevant isotype-matched control and red for unstimulated PMNs, no Ab. A representative shift to the right in surface MFI for $cysLT_1R$ (blue *versus* green histogram) was demonstrated after addition of anti-$cysLT_1R$-pAb in PMNs (fig. 1a). A shift to the right (blue histogram) in MFI is demonstrated in eosinophils from the same donors (fig. 1b). Composite data for MFI for surface $cysLT_1R$ expression on eosinophils and neutrophils isolated from five different individuals is shown in figure 1c. MFI for surface expression of $cysLT_1R$ on eosinophils was 2627±1340 after staining with pAb against $cysLT_1R$ compared with 1245±496 MFI for PMNs ($p<0.05$ *versus* eosinophils).

Total protein concentration obtained from increasing numbers of eosinophils and PMNs was comparable as assessed by the Bradford assay (fig. 2a). Expression of $cysLT_1R$ on eosinophils and PMNs increased in a protein-dependent manner as confirmed by Western blot analysis (fig. 2b). Eosinophils had significantly greater expression of 38 kDa $cysLT_1R$, than PMNs. Quantitation of the protein band was determined by densitometric analysis (fig. 2c). Optical density [5] for the 6-µg eosinophil protein band was 5.67±1.69 $U\cdot mm^{-2}$ after subtraction for background. By contrast, optical density for the 6-µg PMN protein band was 1.94±0.39 $U\cdot mm^{-2}$ ($p<0.05$ *versus* eosinophils); (fig. 2c; n=4 experiments). For eosinophils and PMNs aliquots containing 24 µg of protein, optical density was 3.17±0.28 $U\cdot mm^{-2}$ for PMN and 13.86±4.74 $U\cdot mm^{-2}$ for eosinophils ($p<0.05$).

Differential cell count analysis revealed 99% purity for both eosinophils and neutrophils after haematoxylin and eosin staining. Hence, the signal for $cysLT_1R$ on PMNs was not related to contamination of eosinophils.

Effect of LTD$_4$ on PMN adhesion

PMN adhesion to the ICAM-1 surrogate protein BSA [28], caused by LTD_4 activation, increased in a time- and concentration-related manner (fig. 3). Baseline adhesion for unstimulated PMNs binding to ICAM-1 surrogate protein was 2.79±0.72%. Maximal β_2-integrin adhesion caused by 10^{-7} M LTD_4 increased to 18.39±3.21% at 20 min ($p<0.001$; n=6); thereafter, adhesion decreased but still was significant (10.70±0.34%) at 60 min ($p<0.05$; fig. 3a). For subsequent studies, 20 min was used as the time for measurement of adhesion. Data were expressed as percentage of adhesion as assessed by measuring the residual MPO activity of adherent PMNs.

LTD_4-induced PMN β_2-integrin binding to ICAM-1 surrogate protein also increased in a concentration-dependent manner (fig. 3b). Baseline adhesion was 5.97±0.59% for unstimulated cells, no LTD_4. At 10^{-10} M LTD_4, percentage of adhesion increased to 14.15±0.44% and to 19.87±0.81% after 10^{-7} M

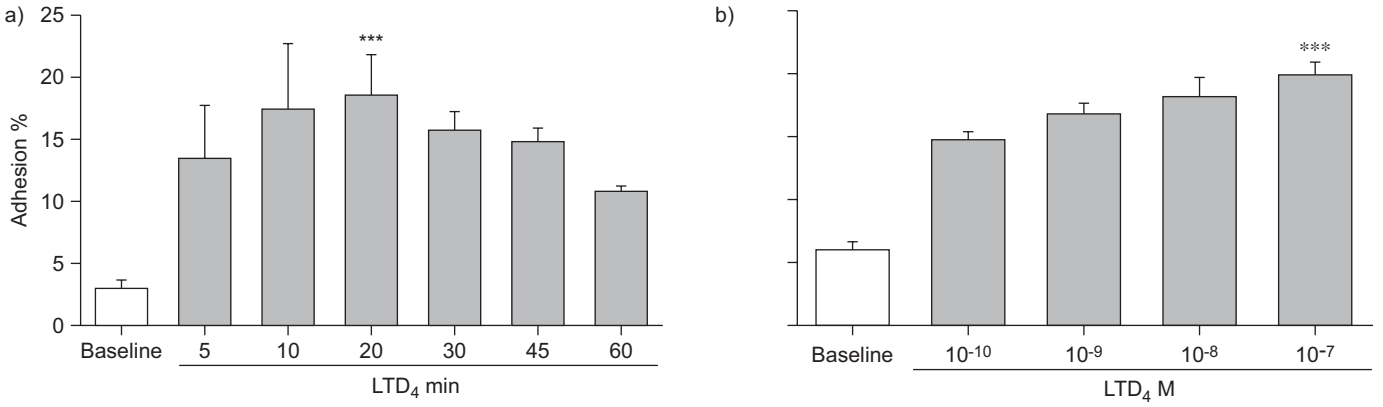

FIGURE 3. Kinetics and concentration-dependent effect of exogenous leukotriene D_4 (LTD_4) on β_2-integrin adhesion. a) Time-course for 10^{-7} M LTD_4-induced β_2-integrin adhesion on polymorphonuclear leukocytes (PMNs; n=6). b) PMNs were activated with different concentrations of LTD_4 for 20 min at 37°C (n=6). Adhesion was measured as a function of residual myeloperoxidase activity on adherent PMNs. ***: $p<0.001$ *versus* baseline value (unstimulated cells).

LTD_4 activation ($p<0.001$ *versus* unstimulated cells, no LTD_4) for 20 min (fig. 3b). For subsequent studies, 10^{-7} M LTD_4 was used to activate PMNs.

Specificity of β_2-integrin adhesion was demonstrated by pre-treatment of PMNs with anti-CD18 monoclonal Ab prior to activation with 10^{-7} M LTD_4 (fig. 4). In separate studies, adhesion of unstimulated PMNs to soluble ICAM-1-coated microplate wells was $6.78\pm1.46\%$. Adhesion elicited by 10^{-7} M LTD_4 was $16.78\pm2.91\%$ ($p<0.01$ *versus* buffer control; n=7 experiments). Pre-treatment of PMNs with anti-CD18 monoclonal Ab blocked by ~70% the increased adhesion caused by 10^{-7} M LTD_4 alone to $9.95\pm3.43\%$ ($p<0.01$ *versus* LTD_4-activated cells alone). Comparable results were obtained using the previously validated ICAM-1 surrogate, BSA [28]. Accordingly, subsequent studies were performed using the BSA surrogate only [28].

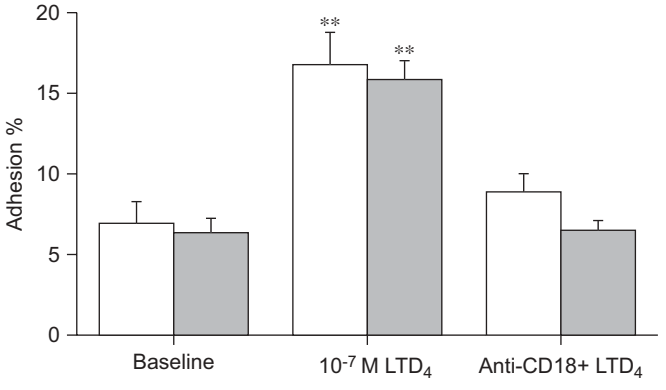

FIGURE 4. Effect of monoclonal antibody (mAb) directed against anti-CD18 on leukotriene D_4 (LTD_4)-induced β_2-integrin adhesion. Polymorphonuclear leukocytes (PMNs) were pre-incubated with anti-CD18 mAb for 30 min prior to activation with 10^{-7} M LTD_4 for 20 min at 37°C. Equivalency of PMNs binding to soluble intercellular adhesion molecule-1 (□) and bovine serum albumin (■) coated microplate wells was tested and adhesion was measured as a function of residual myeloperoxidase activity of adherent PMNs. Data are presented for seven independent experiments. **: $p<0.01$ *versus* anti-CD18 mAb.

Partial blockade of LTD_4-induced adhesion to plated ICAM-1 surrogate was inhibited with montelukast, a $cysLT_1R$ antagonist (fig. 5). Constitutive baseline adhesion of unstimulated PMNs to ICAM-1 surrogate protein was $6.33\pm0.84\%$ (fig. 5a). Adhesion decreased from $14.75\pm1.30\%$ for PMNs activated with 10^{-10} M LTD_4 alone to $9.51\pm1.80\%$ for PMN pre-treated with 10^{-6} M montelukast (n=4; $p<0.05$) (fig. 5a).

For PMNs activated with 10^{-7} M LTD_4, β_2-integrin adhesion decreased from $23.33\pm1.54\%$ to $14.07\pm1.84\%$ for PMNs pre-treated with 10^{-6} M montelukast (n=4; $p<0.05$) (fig. 5b). Thus, the maximum efficacy of the $cysLT_1R$ blockade of β_2-integrin adhesion to ICAM-1 surrogate was comparable with montelukast, for both concentrations of LTD_4.

Mechanism of blockade of β_2-integrin adhesion by $cysLT_1R$ antagonism

Adhesion caused by 10^{-7} M LTD_4 was not regulated through upregulated surface expression of CD11b/CD18 on PMNs, which remained remarkably constant under all experimental conditions (fig. 6). Constitutive expression of CD11b/CD18 was neither upregulated by 10^{-7} M LTD_4 nor downregulated by $cysLT_1R$ antagonism with concentrations of montelukast causing substantial blockade (fig. 5) of stimulated adhesion.

However, 10^{-7} M LTD_4 upregulated in time-related manner the intracellular phosphorylation of gIVaPLA2 (fig. 7). Integrin adhesion requires the phosphorylation of gIVaPLA2. Activation of PMNs with 10^{-7} M LTD_4 caused phosphorylation of gIVaPLA2 in a time-dependent manner (fig. 7a). At 20 min, gIVaPLA2 phosphorylation was greatest, the same time at which PMN adhesion to ICAM-1 surrogate protein was optimal (fig. 3a). Sample loading was confirmed by reprobing the same stripped blot with total gIVaPLA2 Ab (fig. 7b). Phosphorylated gIVaPLA2 at Ser^{505} was detected at ~110 kDa, referenced to the molecular weight protein ladder.

Blockade of adhesion caused by 10^{-7} M LTD_4 alone ($20.56\pm1.54\%$) was elicited in a concentration-dependent manner with TFMK, an inhibitor of gIVaPLA2 (fig. 8a). At 30 μM TFMK, PMN adhesion was ~70% blocked to $8.68\pm0.73\%$ ($p<0.01$ *versus* LTD_4-activated cells, no TFMK).

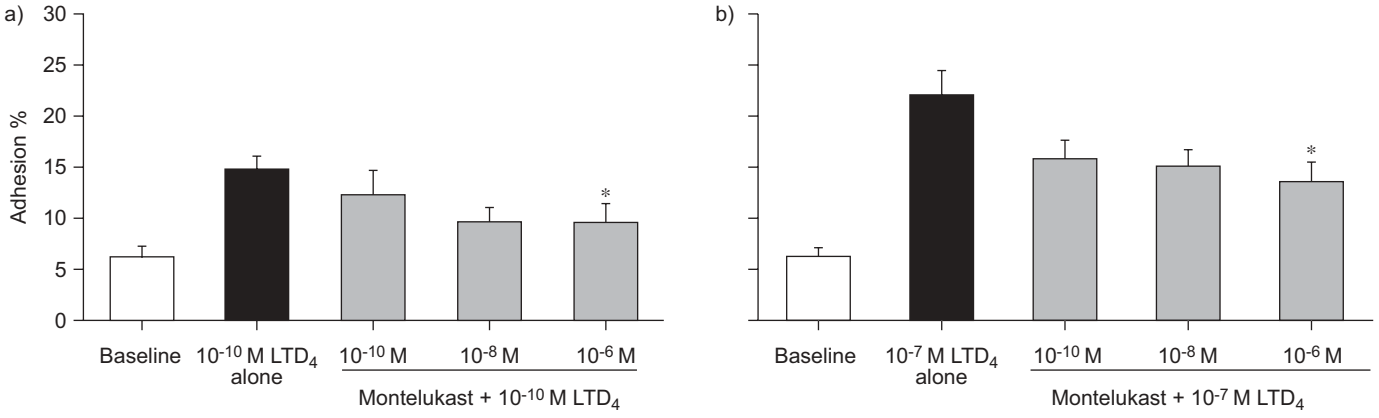

FIGURE 5. Effect of montelukast, a cysteinyl leukotriene-1 receptor antagonist, on leukotriene D_4 (LTD_4)-induced β_2-integrin adhesion. Polymorphonuclear leukocytes (PMNs) were pre-incubated with different concentrations of montelukast for 30 min prior to activation with a) 10^{-10} M LTD_4 or b) 10^{-7} M LTD_4 for an additional 20 min at 37°C. Adhesion was measured as a function of residual myeloperoxidase activity of adherent PMNs. Data are presented for four independent experiments. *: $p<0.05$ *versus* LTD_4-activated cell, no montelukast.

In separate studies, the effect of montelukast on gIVaPLA2 phosphorylation caused by LTD_4 was examined. Activation of PMNs induced phosphorylation of gIVaPLA2 and was blocked fully by 10^{-6} M montelukast (fig. 8b). Equivalent sample loading was confirmed by staining with anti-total gIVaPLA2 Ab (fig. 8b).

DISCUSSION

The objectives of this study were to assess the physiological significance of $cysLT_1R$ in the upregulation of β_2-integrin adhesion and to determine the mechanisms by which LTD_4 induces this adhesion in PMNs. We first confirmed that $cysLT_1R$ is expressed in isolated human PMNs and that the upregulated adherence caused by LTD_4 is dependent on $cysLT_1R$. A prior study using immunohistochemistry in a diverse cell population did not demonstrate the presence of the $cysLT_1R$ in PMNs [29]; however, subsequent reports [30, 31], including some by the same investigators, definitively established the presence of $cystLT_1R$ on the surface membrane of PMN. In this study, we first established the validity of our methods for quantifying the presence of this receptor on PMNs (relative to eosinophils) using purified isolates of PMNs and eosinophils from peripheral blood of the same human subjects (fig. 1). Total protein content was measured from extracts of eosinophils and PMNs to confirm the expression of $cysLT_1R$ by immunoblotting analysis. Preliminary studies demonstrated that the fraction of eosinophils (<2%) resulting from cross-contamination in these purified isolates of PMNs did not account for positive identification of $cysLT_1R$ on PMNs by either flow cytometry or Western blot analysis.

The objective of this investigation was to determine the functional role of $cysLT_1R$ on β_2-integrin adhesion in human PMNs. We observed that the total protein content of $cysLT_1R$

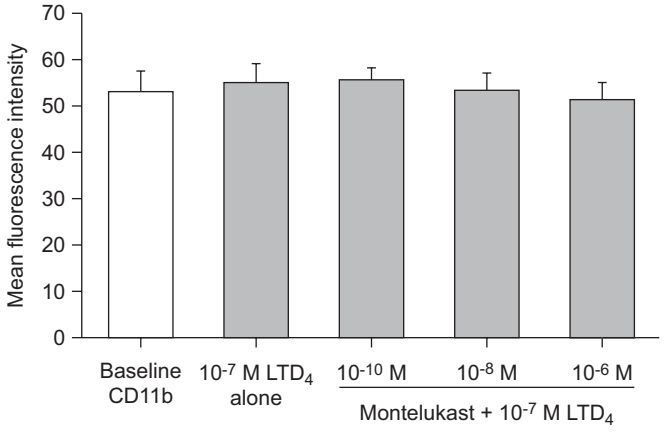

FIGURE 6. Effect of montelukast, a cysteinyl leukotriene-1 receptor antagonist, on surface CD11b/CD18 expression caused by leukotriene D_4 (LTD_4) activation. Polymorphonuclear leukocytes (PMNs) were pre-incubated with increasing concentrations of montelukast for 30 min prior to activation with buffer or 10^{-7} M LTD_4 (20 min) and immunofluorescence analysis. Treated PMNs (n=3) were stained with anti-CD11b monoclonal antibodies (the α-chain of Mac-1, which is constitutively (baseline) expressed on PMNs) for 60 min and sorted by flow cytometric analysis as described in the Material and methods section. Expression of surface CD11b on treated PMNs is calculated as mean fluorescence intensity.

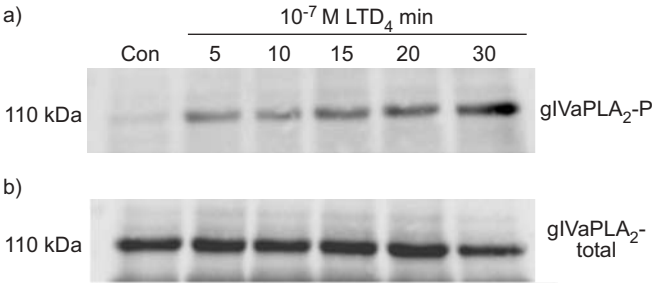

FIGURE 7. Effect of exogenous leukotriene D_4 (LTD_4) on cytosolic group IVa phospholipase A2 (gIVaPLA2) phosphorylation. a) Polymorphonuclear leukocytes (PMNs) were activated with 10^{-7} M LTD_4 at different time intervals at 37°C. Treated PMNs were lysed and subjected to 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis, followed by immunoblotting using anti-phosphorylated gIVaPLA2 at site Ser^{505}. b) Equal sample loading was confirmed by using total anti-gIVaPLA2 antibody. Phosphorylated gIVaPLA2 was detected at 110 kDa as confirmed by a molecular weight protein ladder.

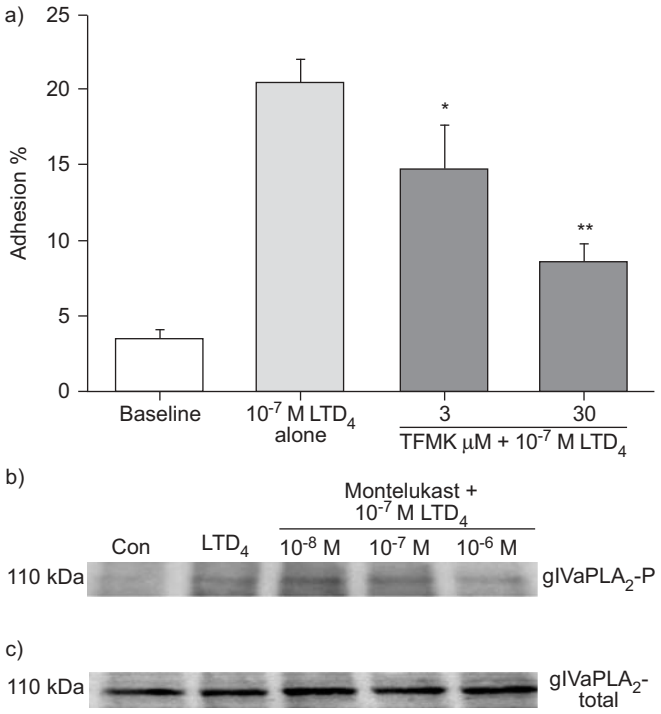

FIGURE 8. Effect of trifluomethylketone (TFMK), a cytosolic group IVa phospholipase A2 inhibitor, on leukotriene D_4 (LTD_4)-induced β_2-integrin adhesion. a) Polymorphonuclear leukocytes (PMNs) were pre-incubated with 3 μM or 30 μM TFMK for 30 min at 37°C prior to activation with 10^{-7} M LTD_4 and measurement of adhesion. *: $p<0.05$ *versus* LTD_4-activated cells, no TFMK; **: $p<0.01$ *versus* LTD_4-activated cells, no TFMK. n=4. b, c) Effect of montelukast on LTD_4-induced gIVaPLA2 phosphorylation. PMNs were pre-incubated with increasing concentrations of montelukast for 30 min prior to activation with 10^{-7} M LTD_4. Equal sample loading was confirmed by reprobing the same membrane with total gIVaPLA2 antibody. Phosphorylated gIVaPLA2 protein was detected at 110 kDa using a molecular weight protein ladder. Con: control.

in human eosinophils was substantially greater than for PMNs isolated from the same donor (fig. 2). Although $cysLT_1R$ was not highly expressed in PMNs (figs 1 and 2), we established that this receptor subtype is capable of upregulating β_2-integrin adhesion considerably in PMNs (figs 3 and 4). Blockade of PMN adhesion to ICAM-1 surrogate caused by 10^{-7} M LTD_4 was ~48% for PMNs treated with 10^{-10} M montelukast (fig. 5b). PMN adhesion caused by either 10^{-10} M or 10^{-7} M LTD_4 was comparably blocked by 10^{-6} M montelukast (fig. 5). Although statistically significant, full blockade of β_2-integrin adhesion was not achieved, even at high concentration of montelukast. The reason for this incomplete blockade was not established in this investigation. It is possible that incomplete blockade relates to the differences between the affinity of LTD_4 and montelukast for the $cysLT_1R$ or the activation of other receptor subtypes or pathways not blocked by montelukast.

We demonstrated that LTD_4 did not upregulate the surface expression of β_2-integrin adhesion and CD11b/CD18, the predominant surface adhesion molecule on PMNs causing endothelial ligation (fig. 6). Accordingly, upregulation of CD11b/CD18 does not account for augmented adhesion caused by LTD_4 activation of the $cysLT_1R$. This was expected, since human PMNs, unlike eosinophils, have high constitutive expression of β_2-integrin, even in the resting state. Nonetheless, LTD_4 caused an incremental increase in PMN adhesion to soluble ICAM-1 and BSA surrogate-coated microplate wells at very small concentrations (10^{-10} M) (fig. 3). Because of the relatively high constitutive expression of β_2-integrin (~3–8% adhesion), all data for these studies are expressed as percentage of cell adhesion for each experiment. The number of adherent cells was measured by determining the residual MPO activity of adherent cells (see Material and methods).

We further demonstrated that upregulated β_2-integrin adhesion is probably mediated through phosphorylation of gIVaPLA2 in PMNs (fig. 7). Cytosolic gIVaPLA2 phosphorylation was greatest after treatment with 10^{-7} M LTD_4 and increased activity at same time and concentrations corresponding to maximal PMN adhesion to ICAM-1 surrogate protein (figs 3 and 7). Inhibition of gIVaPLA2 activity with TFMK (fig. 8a) and montelukast (fig. 5b) also blocked β_2-integrin adhesion caused by 10^{-7} M LTD_4 in a concentration-related manner, as did monoclonal Ab directed against CD18, the functional β_2-integrin on PMNs (fig. 4). Accordingly, our data demonstrate that $cysLT_1R$ is a functional LTD_4 receptor for induction of β_2-integrin adhesion through activation of 85-kDa cytosolic gIVaPLA2, a necessary intracellular messenger protein for β_2-integrin adhesion [21–23].

It is important to consider some limitations of our *in vitro* model of cellular adhesion. Because these studies were performed using isolated cells *in vitro*, we cannot account for the far more complex *in vivo* environment in which these interactions might occur. Hence, our findings cannot at this point be extrapolated to the *in vivo* neutrophilic inflammatory disease in humans. This is particularly true, as PMNs themselves do not synthesise or secrete cysLTs. Hence, activation of the $cysLT_1R$ would have to result from LTD_4-producing neighbouring cells [1, 2, 32], *e.g.* macrophages, eosinophils, basophiles and mast cells in airway inflammatory responses. It is not possible in these studies to determine whether regional concentrations of cysLT *in vivo* are sufficient or similar to the concentrations of LTD_4 used in these studies. Nonetheless, substantial augmentation of adhesion was achieved at concentrations as small as 10^{-10} M LTD_4. Accordingly, our *in vitro* cell systems suggest one mechanism by which $cysLT_1R$ could be a functional receptor for LTD_4 in regulating β_2-integrin adhesion in PMNs in an airway inflammatory environment.

We conclude that antagonism of the $cysLT_1R$ attenuates β_2-integrin adhesion caused by LTD_4 activation *in vitro*. Montelukast substantially blocks the upregulation of PMNs β_2-integrin adhesion to ICAM-1 caused by LTD_4, but does not act directly on the surface expression of β_2-integrin adhesion (CD11b/CD18). Blockade of phosphorylation of gIVaPLA2 caused by LTD_4 with montelukast and adhesion with TFMK suggest that augmentation of β_2-integrin adhesion by LTD_4 probably results from direct stimulation of gIVaPLA2 activity through $cysLT_1R$. Accordingly, the $cysLT_1R$ may have a role in the regulation of β_2-integrin adhesion in PMNs *in vivo*. As PMNs do not synthesise cysLTs, it is possible that, under

circumstance where both eosinophils/macrophages (which make cysLTs) and PMNs are together in an inflammatory process, *e.g.* asthma and chronic obstructive pulmonary disease, PMNs may be upregulated further by close proximity to cysLTs secreted from eosinophils or macrophages.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

A statement of interest for A. Leff and the study itself can be found at www.erj.ersjournals.com/misc/statements.dtl

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