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$_1$R) regulates β$_2$-integrin adhesion to 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$_1$R was identified both in PMNs and eosinophils by immunofluorescence analysis. Total cysLT$_1$R protein was substantially greater in eosinophils than in PMNs as determined by Western blot analysis. However, LTD$_4$ upregulated β$_2$-integrin adhesion of PMNs to ICAM-1 with high efficacy in 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 A$_2$ (gIVaPLA$_2$). Augmented LTD$_4$-induced adhesion was blocked significantly by montelukast, a cysLT$_1$R antagonist. Trifluoromethylketone (TFMK, a gIVaPLA$_2$ inhibitor) blocked β$_2$-integrin adhesion caused by LTD$_4$ activation, as did anti-CD18 mAb directed against β$_2$-integrin on the PMN-surface. Our data demonstrate that LTD$_4$ causes a) phosphorylation of gIVaPLA$_2$ and b) upregulation of β$_2$-integrin adhesion to ICAM-1 or ICAM-1-surrogate through cysLT$_1$R activation. Activation of gIVaPLA$_2$ is a critical step through which β$_2$-integrin adhesion is upregulated by the cysLT$_1$R expressed on the surface membrane of human PMN.

KEYWORDS: Adhesion, cysteinyl leukotriene-1 receptor, group IVa phospholipase A$_2$, polymorphonuclear leukocytes

INTRODUCTION

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]. LTD_4, a cysLT, is generated extracellularly by gamma-glutamyl transpeptidase from secreted LTC_4, which is synthesized 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. While $cysLT_2R$ has been reported to cause vascular permeability [4, 20], blockade of $cysLT_3R$ appears to prevent hypoxic brain injury [12].

The 85 kDa cytosolic $gIVaPLA_2$ is a critical intracellular messenger protein for cellular adhesion and secretion of bioactive lipid mediators in human eosinophils [21, 22] and PMNs [16, 23]. Activation of $gIVaPLA_2$ 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 if 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 synthesize cysLTs [2, 23] so activation of this receptor on PMNs under physiological circumstances in vivo would require exposure to cysLTs secreted from neighbouring inflammatory cells, e.g. eosinophils and macrophages. We

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also examined the efficacy of LTD_4 on the surface $cysLT_1R$ of PMN in inducing cytosolic $gIVaPLA_2$ phosphorylation to its active state.

Like 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 ICAM-1 or its surrogate protein, BSA. This process corresponded to the time-course for $gIVaPLA_2$ phosphorylation caused by LTD_4 activation. Inhibition of $gIVaPLA_2$ activity by TFMK blocked **significantly** β_2-integrin adhesion caused by exogenous LTD_4. These data are the first demonstration of that LTD_4 causes β_2-integrin adhesion to endothelial surface ligand, likely through $gIVaPLA_2$ 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 Co. (Ann Arbor, MI). Human polymorphonuclear leukocytes (PMNs)/eosinophils isolation kits were from StemCell Technologies (Vancouver, BC). BSA fraction V was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Recombinant Human ICAM-1 was from R & D Systems (Minneapolis, MN). Abs were purchased: anti-phosphorylated $gIVaPLA_2$ Ab (Ser^{505}) (Cell Signaling Technology, Danvers, MA); anti-total $gIVaPLA_2$ Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); goat anti-mouse Ab and goat anti-rabbit Ab (BD Biosciences, Mountain View, CA); anti-CD11b [Clone Bear I] (Beckman Coulter, Fullerton, CA); and anti-CD18 Ab (Ancell Immunology Research Products, Bayport, MN). Trifluoromethylketone (TFMK) from Biomol (Plymouth Meeting, PA); Montelukast was supplied by Merck and Co. (Rahway, NJ).

Isolation of human neutrophils and eosinophils

Human polymorphonuclear leukocytes (PMNs) and eosinophils were isolated independently from mildly atopic subjects using an isolation kit for each cell type (StemCell Technologies, Vancouver, BC). The protocol used in this study is approved by the University of Chicago Institutional Review Board. 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 taking medication were excluded. The study included a total of 15 individuals age 20 to 45 years, 7 male subjects and 8 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 (Vancouver, BC). Cells were washed twice and resuspended in HBSS buffer + Ca^{++}/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 (Vancouver, BC). A detailed protocol for isolation of eosinophils has been described recently [27]. The purity of neutrophils and eosinophils was determined by differential counts of H & E 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$_1$R and β$_2$-integrin (CD11b/CD18) adhesion on granulocytes

A. Immunofluorescence analysis.

Aliquots of 5×10^5 PMNs or 5×10^5 eosinophils were incubated with cysLT$_1$R polyclonal antibody (pAb) or isotype-matched control Ab at 4 °C for 60 min. After 2 washes, the cells were incubated with an excess of fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit immunoglobulin 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). Fluorescence intensity was determined on at least 10,000 cells from each sample. Data was analysed using FlowJo, a flow cytometric analysis software.

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

B. 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 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 13,000 rpm 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, USA). Bovine serum albumin was used as standard protein control.

C. 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 μg/ml anti-cysLT$_1$R pAb (Cayman Chemical Co.; Ann Arbor, MI) and incubated with 1:3000 dilutions of goat anti-rabbit Ig conjugated with HRP. The

expression of cysLT$_1$R was analysed by an enhanced chemiluminescence system (ECL; Amersham, Arlington Heights, IL). Densitometric analysis of the film was performed using a Model GS-710 imaging densitometer (Bio-Rad Laboratories, Hercules, CA) in transmittance mode and analysed using Bio-Rad Discovery software. **Briefly, quantitative measurement of optical density in light-sensitive photographic film containing protein band was scanned using the above device. Each trace was corrected for background by subtracting a tracing of cysLT$_1$R protein for that blot. Optical density (OD) was expressed as arbitrary units per millimeter square (U/mm^2).**

In a separate study, PMNs (10^6 cells/group) were pretreated with either vehicle control **(HBSS + 0.1 g/L Calcium, pH 7.4) or with increasing concentrations of montelukast at 37 °C.** Thirty min later, 10^{-7} M LTD$_4$ was added at different time intervals and gIVaPLA$_2$ phosphorylation was determined by Western blot analysis. Briefly, cells were lysed and experiments were conducted as above. The membrane was blocked with 1% BSA in TBS-T buffer for 60 min prior to addition of 2 μg/ml anti-phosphorylated gIVaPLA$_2$ mAb as site Ser[505] (Cell Signaling Technology, Beverly, MA). The protein from the same blot was stripped off and probed with total gIVaPLA$_2$ Ab to demonstrate equal loading of treated samples.

Integrin-Adhesion assay

A 96-microplate well plate was coated with 10 μg/ml 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$_2$CO$_3$, pH 9.2) and incubated

overnight at 4 °C. Treated microplate wells were washed 2X with HBSS buffer prior to use. The detailed protocol is described elsewhere [15, 16, 21-23]. We have shown previously that BSA is a full surrogate of ICAM-1 [28]. Further confirmatory data are presented for this study (see Results).

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). No MPO activity was detected in the cell-free reaction supernatants following 30 min incubation, confirming that MPO was not present because of spontaneous degranulation of granulocytes. Adhesion was **expressed as % 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

A. Time-course.

Isolated PMNs were activated with 10^{-7} M LTD$_4$ at 0 min, 5 min, 10 min, 20 min and 30 min prior to adhesion assay (see above Adhesion assay). Data are **expressed as % adhesion for all treated groups.**

B. 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 above. Data were **expressed %** adhesion for all treated groups.

C. Blockade of adhesion caused by LTD$_4$.

PMNs were pretreated with 10^{-10} M to 10^{-6} M montelukast or 20 μg/ml anti-CD18 mAb 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 above, and data were **expressed as % 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 gIVaPLA$_2$, PMNs were pretreated with 3 μM or 30 μM trifluoromethylketone (TFMK), an inhibitor of gIVaPLA$_2$, for 30 min prior to 10^{-7} M LTD$_4$ activation [21, 22]. Adhesion assay was performed as above. Cell viability was assessed for each concentration using trypan blue exclusion dye staining.

To determine that the upregulation of adhesion **affected** by LTD$_4$ was caused by upregulation of β$_2$-integrin, we determined the efficacy of anti-CD18 mAb 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.

Data analysis

All data are expressed as mean ± SEM. Statistically significant differences among 3 or more groups were assessed by 2-way ANOVA. Where the F-statistic indicated a difference, significance between groups of 2 was assessed further by

Student's t-test. For concentration-response curves using the same agonist or antagonist, paired Student's 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. Statistical significance was claimed where $p < 0.05$.

RESULTS

Relative Expression of the $cysLT_1R$ 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 mean fluorescence intensity (MFI) for $cysLT_1R$ (blue vs green histogram) was demonstrated after addition of anti-$cysLT_1R$-polyclonal antibody (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 5 different individuals is shown in fig. 1c. MFI for surface expression of $cysLT_1R$ on eosinophils was 2627 ± 1340 after staining with pAb against $cysLT_1R$ compared to 1245 ± 496 MFI for PMNs ($p < 0.05$ vs eosinophils).**

Total protein concentration obtained from increasing numbers of eosinophils and PMNs was comparable as assessed by Bradford assay (fig. 2a). Expression of $cysLT_1R$ on eosinophils and PMNs increased in 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 (OD) [5] for the 6 μg eosinophil protein band was 5.67 ± 1.69 units (U)/mm^2 after subtraction for background. By contrast, OD for the 6 μg PMN protein band was 1.94 ± 0.39 U/mm^2 for ($p < 0.05$ vs eosinophils); (fig. 2c; n = 4 experiments). For eosinophils and PMNs

aliquots containing 24 μg protein, OD was 3.17 ± 0.28 U/mm^2 for PMN and 13.86 ± 4.74 U/mm^2 for eosinophils ($p < 0.05$).

Differential cell count analysis revealed 99% purity for both eosinophils and neutrophils after H & E staining. Hence, the signal for $cysLT_1R$ on PMNs was not related to minimal contamination of eosinophils, if any.

Effect of leukotriene D_4 on PMN adhesion

PMN adhesion to the ICAM-1 surrogate protein, BSA [28], caused by LTD_4 activation increased in time- and concentration-related manner (fig. 3). Baseline adhesion for unstimulated PMNs binding to ICAM-1-surrogate protein was $2.79 \pm 0.72\%$. Maximal β_2-integrin adhesion caused by 10^{-7} M LTD_4 increased to **$18.39 \pm 3.21\%$** at 20 min ($p < 0.001$; n = 6); thereafter, adhesion decreased but still was significant (**$10.70 \pm 0.34\%$**) 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 % 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 concentration-dependent manner **(fig. 3b).** Baseline adhesion was $5.97 \pm 0.59\%$ for unstimulated cells, no LTD_4. At 10^{-10} M LTD_4, % adhesion increased to **$14.15 \pm 0.44\%$** and further to **$19.87 \pm 0.81\%$** after 10^{-7} M LTD_4 activation ($p < 0.001$ vs 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 pretreatment of PMNs with anti-CD18 mAb 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 $6.78 \pm$ 1.46%. Adhesion elicited by 10^{-7} M LTD$_4$ was **16.78 ± 2.91%** (p < 0.01 vs buffer control; n = 7 experiments). Pretreatment of PMNs with anti-CD18 mAb blocked by ~70% the increased adhesion caused by 10^{-7} M LTD$_4$ alone to **9.95 ± 3.43%** (p < 0.01 vs 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].

Partial blockade of LTD$_4$-induced adhesion to plated ICAM-1 surrogate was inhibited with montelukast, a cysLT$_1$R antagonist (fig. 5). Constitutive baseline adhesion of unstimulated PMNs to ICAM-1 surrogate protein was $6.33 \pm 0.84\%$ (fig. 5a) Adhesion decreased from **14.75 ± 1.30%** for PMNs activated with 10^{-10} M LTD$_4$ alone to **9.51 ± 1.80%** for PMN pretreated with 10^{-6} M montelukast (n = 4; p < 0.05) (fig. 5a).

For PMN activated with 10^{-7} M LTD$_4$, β_2-integrin adhesion decreased from **23.33 ±1.54% to 14.07 ± 1.84%** for PMNs pretreated with 10^{-6} M montelukast (n = 4; p < 0.05) (fig. 5b). Thus, the maximum efficacy of the cysLT$_1$R 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$_1$R 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$_1$R antagonism with

concentrations of montelukast causing substantial blockade (see fig. 5) of stimulated adhesion.

However, 10^{-7} M LTD$_4$ upregulated in time-related manner the intracellular phosphorylation of gIVaPLA$_2$, the process by which this enzyme is activated (fig. 7) [22]. Integrin adhesion requires the phosphorylation of gIVaPLA$_2$ (see Discussion). **Activation of PMNs with 10^{-7} M LTD$_4$ caused phosphorylation of gIVaPLA$_2$ in time-dependent manner (fig. 7a). At 20 min, gIVaPLA$_2$ phosphorylation was greatest, the same time at which PMN adhesion to ICAM-1 surrogate protein was optimal (see fig. 3a). Sample loading was confirmed by re-probing the stipped same blot with total gIVaPLA$_2$ Ab (fig. 7b). Phosphorylated gIVaPLA$_2$ at Ser^{505} was detected at ~110 kDa, referenced to molecular weight protein ladder.**

Blockade of adhesion caused by 10^{-7} M LTD$_4$ alone (**20.56 ± 1.54%**) was elicited in concentration-dependent manner with trifluoromethylketone (TFMK), an inhibitor of gIVaPLA$_2$ (fig. 8a). At 30 μM TFMK, PMN adhesion was ~70% blocked to **8.68 ± 0.73%** (p < 0.01 vs LTD$_4$-activated cells, no TFMK).

In separate studies, effect of montelukast on gIVaPLA$_2$ phosphorylation caused by LTD$_4$ was examined. Activation of PMNs induced phosphorylation of gIVaPLA$_2$ was blocked fully by 10^{-6} M montelukast (fig. 8b, above). Equivalent sample loading was confirmed by staining with anti-total gIVaPLA$_2$ Ab (fig. 8b, below).

DISCUSSION

The objectives of this study were a) to assess the physiological significance of cysLT$_1$R in the upregulation of β_2-integrin adhesion and b) to determine the mechanisms by which LTD$_4$ induces this adhesion in PMNs. We first confirmed that cysLT$_1$R is expressed in isolated human PMNs and that the upregulated adherence caused by LTD$_4$ is dependent on cysLT$_1$R. A prior report using immunohistochemistry in a diverse cell population did not demonstrate the presence of the cysLT$_1$R in PMNs [29]; however, subsequent reports [30, 31] including some of the same investigators definitively established the presence of cystLT$_1$R 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$_1$R 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$_1$R on PMNs by either flow cytometry or Western blot analysis.

The objective of this investigation was to determine the functional role of cysLT$_1$R on β_2-integrin adhesion in human PMNs. We observed that the total protein content of cysLT$_1$R in human eosinophils was substantially greater than for PMNs isolated from the same donor (fig. 2). Although cysLT$_1$R was not highly expressed in PMNs (figs. 1 and 2), we established that this receptor subtype is capable of upregulating considerably β_2-integrin adhesion 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 blocked comparably 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.

In further studies, we demonstrated that LTD_4 did not upregulate the surface expression of β_2-integrin adhesion, 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 % cell adhesion** for each experiment. **The number of adherent cells was measured by determining the residual myeloperoxidase activity of adherent cells (see Methods).**

We further demonstrated that upregulated β_2-integrin adhesion is likely mediated through phosphorylation of $gIVaPLA_2$ in PMNs (fig. 7). **Cytosolic $gIVaPLA_2$ 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 (compare figs, 3 and 7). Inhibition of gIVaPLA$_2$ activity with TFMK **(fig. 8a) and montelukast (fig. 5b)** also blocked β_2-integrin adhesion caused by 10^{-7} M LTD$_4$ in concentration-related manner, as did mAb directed against CD18, the functional β_2-integrin on PMNs (fig. 4). Accordingly, our data demonstrate that cysLT$_1$R is a functional LTD$_4$ receptor for induction of β_2-integrin adhesion through activation of 85 kDa cytosolic gIVaPLA$_2$, 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, because PMNs do not themselves synthesize or secrete cysLTs. Hence activation of the cysLT$_1$R would have to result from LTD$_4$-producing neighbouring cells [1, 2, 32], e.g. macrophages, eosinophils, basophils, and mast cells in airway inflammatory responses. It is not possible in these studies to determine if regional concentrations of cysLT in vivo are sufficient or comparable 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$_1$R 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 blocks substantially 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 $gIVaPLA_2$ caused by LTD_4 with montelukast and** adhesion with TFMK suggest that augmentation of β_2-integrin adhesion by LTD_4 likely results from direct stimulation of $gIVaPLA_2$ 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 synthesize 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 COPD, PMNs may be upregulated further by close proximity to cysLTs secreted from eosinophils or macrophages.**

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FIGURE 1. Surface expression of cysteinyl leukotriene-1 receptor (cysLT$_1$R) on a) polymorphonuclear leukocytes (PMN) and b) eosinophils (EOS) as determined by immunofluorescence analysis using polyclonal antibody (pAb) directed against cysLT$_1$R (n = 5 subjects). Histograms were generated by flow cytometry using FlowJo software analysis. Expression of cysLT$_1$R (blue line) is demonstrated by the right shift compared to cells stained with irrelevant isotype matched-control Ab (green line; FITC conjugated goat anti-rabbit, 2º Ab) or unstained PMN or EOS (red line; negative, no mAb). **c) Composite data (n = 5 different individuals) for mean fluorescence intensity (MFI) is shown for eosinophils (EOS; gray bar) and polymorphonuclear leukocytes (PMN; black bar).** [*]**p < 0.05 EOS vs PMN.**
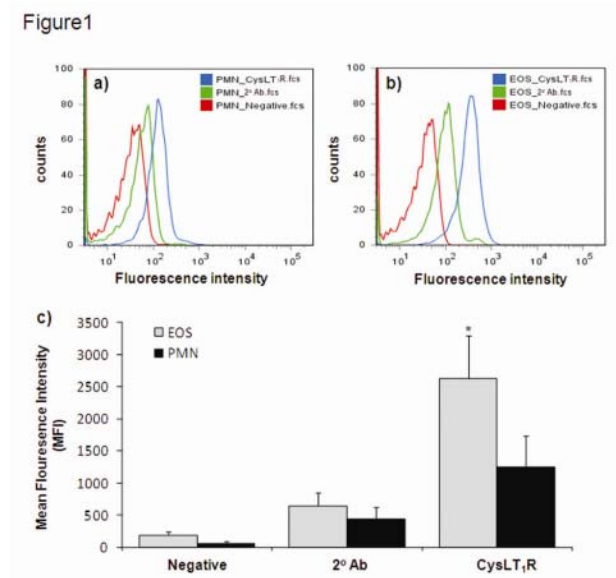
Figure1

FIGURE 2. Expression of cysteinyl leukotriene-1 receptor (cysLT$_1$R) in EOS and PMN by Western blot analysis. **a) Total protein content (μg) extracted from isolated**

eosinophils (EOS) and polymorphonuclear leukocytes (PMN) from 4 different individuals as assessed by Bradford assay. b) A representative immunoblotting analysis on increasing **protein content** of eosinophils (EOS) and polymorphonuclear leukocytes (PMN) as described in the Methods section. **The cysLT$_1$R protein band was detected at ~38 kDa using a molecular weight protein ladder.** Full ladder is included in the online depository of the European Respiratory Journal. **c)** Quantitation of cysLT$_1$R expression by densitometric analysis of Western blot film (n = 4 cell isolations) *p < 0.05 corresponding **protein content** of EOS vs PMN. Optical density (OD) are expressed in units/mm^3.

Figure 2
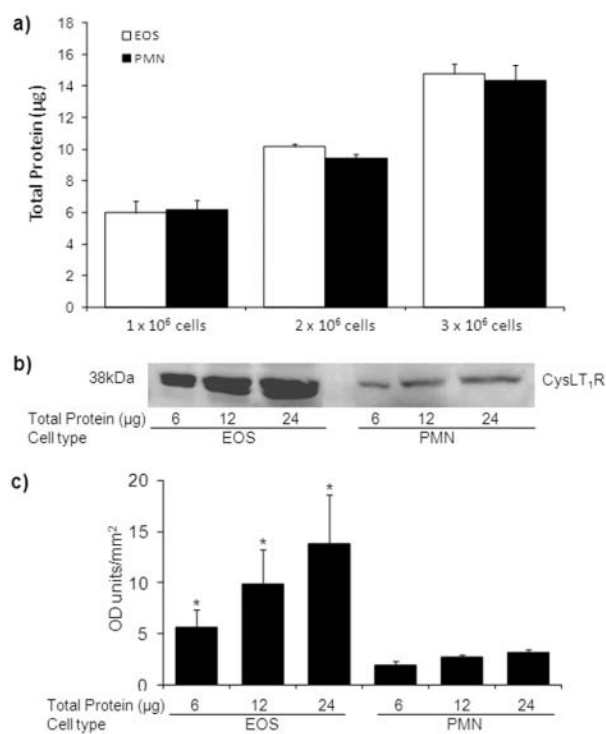
FIGURE 3. Kinetics and concentration-dependent effect of exogenous LTD$_4$ on β$_2$-integrin adhesion. a) Time-course for 10^{-7} M LTD$_4$-induced β$_2$-integrin adhesion on

polymorphonuclear leukocytes (PMNs; n = 6). b) PMNs were activated with different concentrations of LTD_4 for 20 mins at 37 °C (n = 6). Adhesion was measured as a function of residual myeloperoxidase activity on adherent PMNs. Data, **mean ± SEM,** are expressed as % adhesion **of total adherent cells.** *p < 0.001 vs baseline value (unstimulated cells).
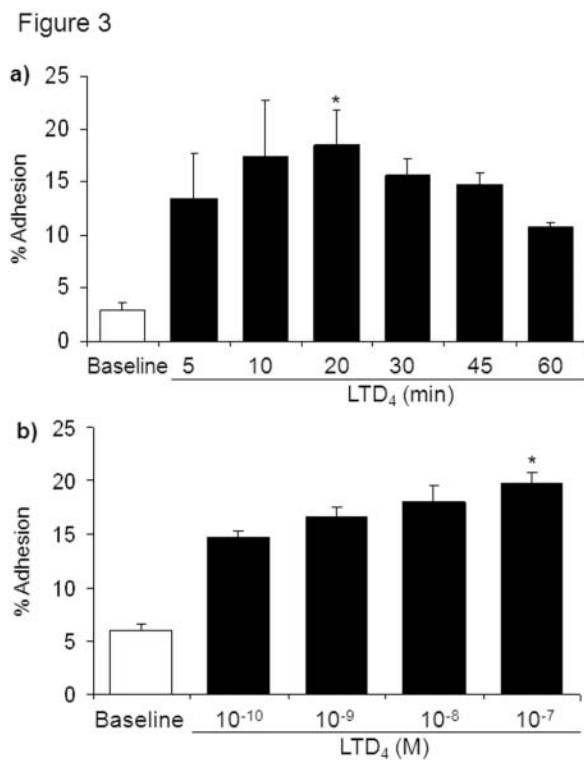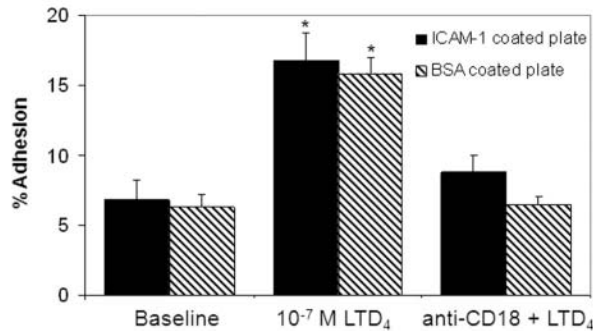
Figure 3

FIGURE 4. Effect of mAb directed against anti-CD18 on LTD_4-induced β_2-integrin adhesion. Polymorphonuclear leukocytes (PMNs) were preincubated with anti-CD18 mAb for 30 min prior to activation with 10^{-7} M LTD_4 for 20 min at 37 °C. Equivalency of PMN binding to soluble ICAM-1 and BSA-coated microplate wells were tested and adhesion was measured as a function of residual myeloperoxidase activity of adherent

PMNs. Data, **mean ± SEM,** are expressed as % adhesion for 7 independent experiments. *p < 0.01 vs anti-CD18 mAb.
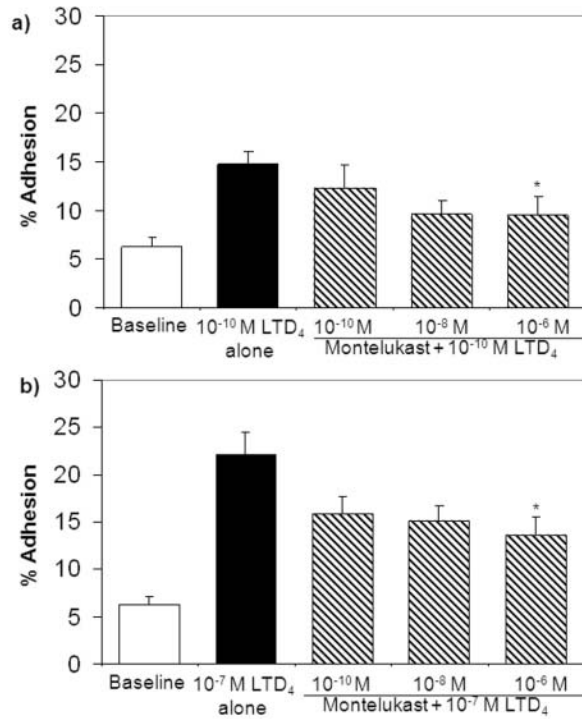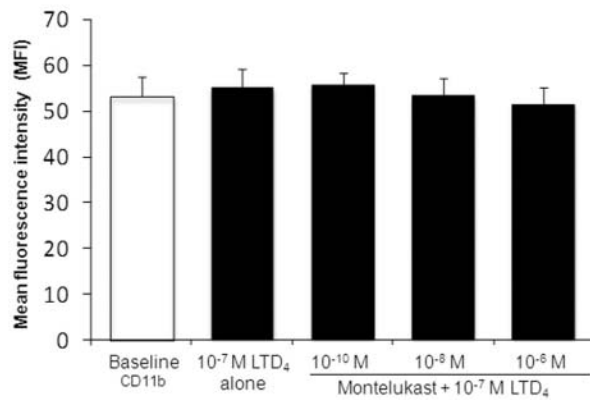

Figure 4

FIGURE 5. Effect of montelukast, a cysLT$_1$R antagonist, on 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 additional 20 min at 37°C. Adhesion was measured as a function of residual myeloperoxidase activity of adherent PMNs. Data are expressed as % adhesion and as mean ± SEM for 4 independent experiments. *p < 0.05 vs LTD$_4$-activated cell, no montelukast.

Figure 5

a)

% Adhesion

Baseline | 10^{-10} M LTD$_4$ alone | 10^{-10} M | 10^{-8} M | 10^{-6} M
Montelukast + 10^{-10} M LTD$_4$

b)

% Adhesion

Baseline | 10^{-7} M LTD$_4$ alone | 10^{-10} M | 10^{-8} M | 10^{-6} M
Montelukast + 10^{-7} M LTD$_4$

FIGURE 6. Effect of montelukast, a cysLT$_1$R antagonist, on surface CD11b/CD18 expression caused by LTD$_4$ activation. Polymorphonuclear leukocytes (PMNs) were preincubated 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 mAb [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 Methods section. **Expression of surface CD11b on treated PMNs is calculated as mean fluorescence intensity (MFI)**

Figure 6

FIGURE 7. Effect of exogenous LTD$_4$ on cytosolic group IVa phospholipase A$_2$ (gIVaPLA$_2$) 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% SDS-PAGE, followed by immunoblotting using anti-phosphorylated gIVaPLA$_2$ at site Ser^{505}. b) Equal sample loading was confirmed by using total anti-gIVaPLA$_2$ Ab. **Phosphorylated gIVaPLA$_2$ was detected at 110 kDa as confirmed by a molecular weight protein ladder.**

Figure 7
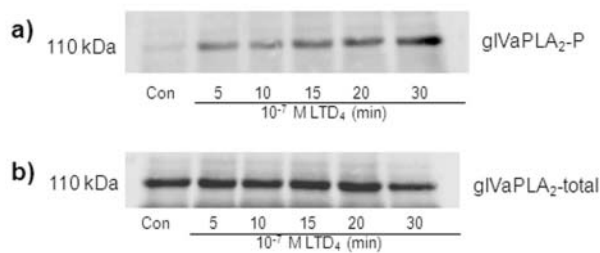
FIGURE 8. Effect of trifluomethylketone (TFMK), a cytosolic group IVa phospholipase A$_2$ inhibitor, on LTD$_4$-induced β$_2$-integrin adhesion. **a)** Polymorphonuclear leukocytes

(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 vs LTD$_4$-activated cells, no TFMK; **p < 0.01 vs LTD$_4$-activated cells, no TFMK. Data (mean ± SEM; n = 4) are expressed as % adhesion of total adherent cells. **b) Effect of montelukast on LTD$_4$-induced gIVaPLA$_2$ phosphorylation. PMNs were preincubated 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 gIVaPLA$_2$ Ab. Phosphorylated gIVaPLA$_2$ protein was detected at 110 kDa using a moleculat weight protein ladder.**

Figure 8