Original article

Blockade of avidity and focal clustering of β_2-integrin by cysteinyl leukotriene antagonism attenuates eosinophil adhesion

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Background: Cysteinyl leukotriene (cysLT) antagonism attenuates migration of eosinophils into airways during immune challenge in human subjects and animal models. The intracellular signaling mechanism by which this occurs has not been elucidated.

Objective: We sought to determine the relative efficacy and mechanism by which 5-lipoxygenase (5-LO) inhibition and cysLT$_1$ receptor (cysLT$_1$R) antagonism block β_2-integrin adhesion in isolated human eosinophils *in vitro*.

Methods: Human blood eosinophils were isolated by means of immunomagnetic separation. Upregulation of CD11b expression, active conformation of CD11b, and focal clustering of β_2-integrin caused by IL-5, eotaxin-1 or leukotriene (LT) B$_4$ was assessed by means of flow cytometry and confocal microscopy. The effect and mechanism of cysLT$_1$R or 5-LO blockade on these components of β_2-integrin adhesion were determined.

Results: Montelukast, a cysLT$_1$R antagonist, and AA861, a 5-LO enzyme inhibitor, blocked (1) avidity of β_2-integrin, (2) β_2-integrin–mediated adhesion to intercellular adhesion molecule 1, and (3) focal clustering of CD11b elicited by LTB$_4$. However, adhesion caused by either IL-5 or eotaxin-1 was not attenuated for eosinophils pretreated with either montelukast or AA861.

Conclusion: Our data demonstrate that (1) LTB$_4$ causes autocrine upregulation of adhesion through secretion of cysLTs, and (2) blockade of cysLT$_1$R blocks the avidity and focal clustering of CD11b/CD18 for eosinophils activated by LTB$_4$ but not by IL-5 or eotaxin-1.

Clinical implications: Unlike cysLT-induced adhesion, adhesion caused by IL-5 or eotaxin-1 is not regulated through the cysLT$_1$R, suggesting that cysLTs have specific but limited potential to upregulate eosinophil adhesion. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Eosinophils, adhesion, cysteinyl leukotrienes, montelukast, AA861, CD11b/CD18, cytokines, chemokines, focal clustering, avidity*

Cysteinyl leukotrienes (cysLTs) are potent bioactive lipids that act through 2 structurally divergent G protein–coupled receptors, termed cysLT$_1$ receptor (cysLT$_1$R) and cysLT$_2$ receptor (CysLT$_2$R).[1,2] CysLTs (leukotriene [LT] C$_4$, LTD$_4$, and LTE$_4$) cause (1) mucus secretion,[3,4] (2) cell migration,[5,6] (3) bronchoconstriction,[4,7] and (4) tissue edema[7] *in vivo*. Recent studies in allergic mice also have demonstrated that LTD$_4$ induces inflammatory cell migration[8] and that blockade with montelukast, a specific cysLT$_1$R antagonist, attenuates eosinophilic infiltration in immune-sensitized mice.[9] To date, the functional role of the cysLT$_2$R remains incompletely defined.

LTB$_4$, a non–cysteine-containing dihydroxy leukotriene that binds to the LTB$_4$ receptor, is a potent chemoattractant for macrophages, neutrophils, and eosinophils.[10-12] Recent investigations demonstrated that deletion of genes encoding for 5-lipoxygenase (5-LO), 5-lipoxygenase activating protein (FLAP), or LTA$_4$ hydrolase caused downregulation of airway inflammation and hyperresponsiveness.[12-14] We have shown previously that eosinophil infiltration regulated by β_1/β_2-integrin adhesion of eosinophils from the lamina propria to the airway lumen of the guinea pig pouch preparation was blocked by either 5-LO inhibition or by LTB$_4$ dimethylamide (DMA), an LTB$_4$ receptor antagonist.[15] In the same studies, zafirlukast, a cysLT$_1$R antagonist, demonstrated equipotency in blocking eosinophil migration.[15] However, the mechanisms underlying the *ex vivo* and *in vitro* effects of cysLT receptor antagonism on eosinophil adhesion remain to be elucidated.

Eosinophil migration into the airways involves sequential activation of adhesion molecules on airway endothelium and inflammatory cells. There are 2 key processes for cell migration: (1) primary adhesion caused by E-, P-, and L-selectins, which initiates rolling of inflammatory cells along endothelial cell lining, and (2) secondary adhesion to integrins after cell rolling ceases, which facilitates inflammatory cell migration across endothelium into tissue. Here we describe eosinophil binding to intercellular adhesion molecule 1 (ICAM-1) surrogate protein under static conditions to elucidate the signal transduction sequence regulating cellular adhesion once cells have adhered firmly to endothelial cells.

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Supported by Merck & Company, Inc, for A.R.L. and by National Heart, Lung, and Blood Institute grant HL 085779 to A.R.L.

Disclosure of potential conflict of interest: A. R. Leff has consulting arrangements with Merck and Critical Therapeutics, has received grant support from Merck, and has served as an expert witness in matters of medical malpractice and product liability.

Received for publication April 26, 2007; revised June 8, 2007; accepted for publication July 23, 2007.

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0091-6749/$32.00

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doi:10.1016/j.jaci.2007.07.038

Abbreviations used
cysLT: Cysteinyl leukotriene
$cysLT_1R$: Cysteinyl leukotriene 1 receptor
$cysLT_2R$: Cysteinyl leukotriene 2 receptor
DMA: Dimethylamide
EPO: Eosinophil peroxidase
ERK: Extracellular signal–regulated kinase
$gIVaPLA_2$: Group IVa phospholipase A_2
ICAM-1: Intercellular adhesion molecule 1
5-LO: 5-Lipoxygenase
LT: Leukotriene
MFI: Mean fluorescence intensity
TFMK: Trifluoromethylketone

We have previously demonstrated that activation of eosinophils with cytokines (IL-5 and TNF-α), G protein–coupled receptor agonists (formyl-met-leu-phe [FMLP] and LTB_4), or chemokines (IL-8 and eotaxin-1) induces the translocation of β_2-integrin (CD11b/CD18) from secretory vesicles to the surface of plasma membrane.[16,17] We have shown that 4 steps are essential for β_2-integrin ligation after eosinophil activation: (1) surface CD11b (Mac-1)/CD18 expression must be upregulated, (2) the active conformation of β_2-integrin must be conferred,[17] (3) cytosolic group IVa phospholipase A_2 ($gIVaPLA_2$) must be translocated to the perinuclear membrane,[18,19] and (4) focal clustering/affinity of CD11b must occur.[17]

The objectives of this study were (1) to determine whether 5-LO inhibition, which blocks both LTB_4 and LTC_4 synthesis, would be more efficacious in blocking β_2-integrin adhesion of eosinophils than montelukast, which blocks only the $cysLT_1R$, and (2) to determine the mechanism of blockade of β_2-integrin adhesion caused by montelukast, a selective $cysLT_1R$ antagonist, and AA861, a 5-LO enzyme inhibitor, on eosinophil adhesion to ICAM-1 surrogate protein caused by LTB_4, IL-5, and eotaxin-1 activation.

METHODS

Reagents

Recombinant human IL-5 was purchased from PeproTech, Inc (Rocky Hill, NJ). The LTB_4-DMA and LTC_4 kit were obtained from Cayman Chemical (Ann Arbor, Mich), and IL-5 and eotaxin-1 were purchased from R&D Systems (Minneapolis, Minn). Anti-CD11b mAb (Bear I) was purchased from Immunotech (Westbrook, Me), and CBRM1/5 mAb against activated CD11b was from eBioscience (San Diego, Calif). Montelukast sodium ([R-(E)-1-[[[1-[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl) phenyl]propyl]thio]methyl]cyclopropaneacetic acid, monosodium salt]) was provided by Merck and Co, Inc (Rathway, NJ). AA861 (2,3,5-trimethyl-6-[12-hydroxy-5,10-dodecadiynyl]-1,4-benzoquinone) was obtained from Biomol (Plymouth Meeting, Pa).

Eosinophil purification

Eosinophils were isolated from peripheral blood obtained from mildly atopic nonsmoking volunteers. Atopy was defined by means of criteria used in The University of Chicago Asthma Research Center for the National Heart, Lung, and Blood Institute Human Cooperative Asthma Genetics projects. Briefly, blood was anticoagulated with 1:1000 heparin solutions, diluted with calcium-free HBSS, and layered onto 1.077 g/mL Ficoll-Paque solution (GE Healthcare Bioscience AB, Piscataway, NJ).[20] The cell pellet containing granulocytes and erythrocytes was lysed with HBSS containing 0.2% BSA. The total number of cells was counted by using a Coulter counter (Beckman Coulter, Hialeah, Fla) and a mixture of mAbs directed against cell-surface antigens on human hematopoietic cells combined with secondary labeling with a colloidal suspension of magnetic dextran iron particles (Stem Cell Technologies, Vancouver, Canada) were added to selectively extract neutrophils and other unwanted cells.[21,22] Thirty minutes later, the cell suspension was transferred to a type C magnetic separation column held within a 0.6-Tesla MACS magnet (Miltenyi Biotech, Auburn, Calif). The purity of eosinophils was determined by using differential counts of hematoxylin and eosin–stained cytospin preparations. Eosinophil viability was assessed by using trypan blue exclusion dye.

Adhesion assay: Measurement of residual eosinophil peroxidase activity

We have established previously that plated BSA is a full surrogate for ICAM-1 for integrin binding *in vitro*.[23] Briefly, eosinophils (10^4 cells) were preincubated with either montelukast (10^{-8} mol/L to 10^{-6} mol/L) or AA861 (10^{-8} mol/L to 10^{-6} mol/L) for 15 minutes before addition to BSA-coated microplate wells and activation with buffer control, 10^{-7} mol/L LTB_4 (5 minutes), 10 ng/mL IL-5 (15 minutes), and 100 ng/mL eotaxin-1 (15 minutes) at 37°C. At the end of the activation period, nonadherent cells were washed 3× with HBSS buffer/0.1% gelatin, and 100 μL of HBSS/0.1% gelatin was added to the reaction wells. Serial dilutions of the original cell suspension (10^6 cells/10 mL) were added to the noncoated microplate wells to generate a standard curve, and experiments were conducted as follows. A mixture of 1 mM hydrogen peroxide, 1 mM *o*-phenylenediamine, and 0.1% Triton X-100 in Tris buffer, pH 8.0, was used as a substrate to analyze cell adhesion, and the reaction was stopped by means of addition of 4 mol/L sulfuric acid. All assays were performed in duplicate. The detection of eosinophil peroxidase (EPO) by using this method was linear between concentrations of 10^3 to 10^4 cells/well, as measured by using a standard curve. No residual EPO was detected in a cell-free reaction supernatant, indicating that EPO was not released because of spontaneous cell degranulation. The absorbance was measured at 490 nm in a Thermomax microplate reader (Molecular Devices, Menlo Park, Calif).

Analysis of surface CD11b expression by means of immunofluorescence flow cytometry and confocal microscopy

Eosinophils (5×10^5 cells) were preincubated with either 10^{-8} mol/L to 10^{-6} mol/L montelukast or 10^{-8} mol/L to 10^{-6} mol/L AA861 for 15 minutes before activation with buffer control, 10^{-7} mol/L LTB_4 (5 minutes), 10 ng/mL IL-5 (15 minutes), and 100 ng/mL eotaxin-1 (15 minutes) at 4°C in PBS solution containing 2% BSA. Treated cells were washed and incubated with 10 μg/mL mAb against CD11b (surface expression), CBRM1/5 (avidity), or equivalent concentrations of irrelevant IgG1 control antibody for another 30 minutes at 4°C. Cells were washed and counterstained with 1:50 fluorescein isothiocyanate–conjugated secondary mAb for an additional 30 minutes. Thereafter, the treated cells were fixed with 1% paraformaldehyde in PBS, and fluorescence intensity was determined on at least 5000 cells from each sample, as acquired with a FACscan flow cytometer (Becton Dickinson, Mountain View, Calif). For quantitative evaluation, the CD11b populations were

gated manually, and the percentage of CD11b-positive cells was determined by using Cell Quest software (Becton Dickinson Immunocytometry Systems).

Treated cytoslides were stained with CD11b mAb to confirm the distribution of CD11b (focal clustering, and fluorescence was analyzed with an Axiovert confocal microscope (Zeiss, Oberkochen, Germany) equipped with an external argon-krypton laser at 488 nm.

Determination of cysLT secretion by means of enzyme immunosorbent assay

The concentration of LTC_4 secreted during eosinophil activation was assayed by means of competitive enzyme immunosorbent assay (Cayman Chemical, Ann Arbor, Mich) with a method that we have reported previously.[21,24] Eosinophils were treated as above (see adhesion assay). All samples were run in duplicate, and LTC_4 secretion was expressed in picograms per 10^6 cells.

Western blot analysis: Phosphorylation of extracellular signal–regulated kinase 1/2

Eosinophils (10^6 cells per group) were pretreated with 10^{-8} mol/L to 10^{-6} mol/L montelukast or 10^{-8} mol/L to 10^{-6} mol/L AA861 for 15 minutes before activation with buffer control, 10^{-7} mol/L LTB_4, 10 ng/mL IL-5, and 100 ng/mL eotaxin-1, and phosphorylation of extracellular signal–regulated kinase (ERK) 1/2 was analyzed as follows. The cell pellets were lysed in a disruption buffer (20 mmol/L Tris-HCl, 30 mmol/L $Na_4P_2O_7$, 50 mmol/L sodium fluoride, 40 mmol/L NaCl, 5 mmol/L EDTA, and 1% Nonidet-40, protease inhibitors tablet) and centrifuged at 12,000 rpm for 30 seconds to remove nuclear and cellular debris. SDS-PAGE loading buffer was added to the collected supernatant, boiled for 5 minutes, and then stored at −70°C. Prepared samples were subjected to 10% SDS-PAGE, as described previously.[22,25] The resolved proteins were transferred onto a polyvinylidene fluoride membrane by using a semidry apparatus system. After blockade with 1% BSA, the membrane was incubated with antibodies specific for phosphorylated ERK-1/2 (Cell Signaling Technology, Beverly, Mass) overnight at 4°C. The protein of interest was visualized with an enhanced chemiluminescence system (Amersham, Arlington Heights, Ill).

Statistical analysis

All measurements were expressed as means ± SEMs. Variation between 2 groups was tested by using the Student *t* test. Variation among more than 2 groups was tested by using ANOVA, followed by the Fisher protected least significant difference. A *P* value of less than .05 was accepted as significant.

RESULTS

Effect of LTB_4, IL-5, and eotaxin-1 on cell surface and conformational change of CD11b expression: Blockade by montelukast and AA861

We first determined the inhibitory effect of montelukast and AA861 on stimulated surface expression of CD11b in eosinophils (Fig 1, left; n = 4 separate donors). Eosinophils treated with the isotype-matched control had a mean fluorescence intensity (MFI) of 2.4 ± 0.13; baseline CD11b expression was 15.6 ± 2.1 MFI for nonstimulated cells stained with mAb to CD11b integrin.

Comparable upregulation of CD11b expression in eosinophils was caused by LTB_4, IL-5, and eotaxin-1. LTB_4 activation increased the surface CD11b expression to 27.9 ± 2.4 MFI ($P < .05$; Fig 1, *A*), IL-5 activation increased surface expression to 26.74 ± 2.2 MFI (Fig 1, *B*), and eotaxin-1 activation increased surface expression to 31.2 ± 5.2 (Fig 1, *C*; $P < .02$ vs baseline CD11b expression; P = not significant vs LTB_4-activated cells). Neither montelukast nor AA861 blocked upregulation of surface CD11b expression caused by LTB_4, IL-5, or eotaxin-1, as assessed by means of flow cytometric analysis.

We next determined whether blockade of $cysLT_1R$ or 5-LO would block the activated conformational change of CD11b, as assessed by using the selective mAb CBRM-1/5 (Fig 1, right). By using the aliquots of isolated eosinophils from the same individuals (n = 5 donors), LTB_4 was more effective in upregulating the active conformational change of CD11b (Fig 1, *D*) than IL-5 (Fig 1, *E*) or eotaxin-1 (Fig 1, *F*). Baseline (unstimulated) CD11b expression was 16.2 ± 2.4 MFI and increased to 82.7 ± 11.8 MFI for cells activated with LTB_4 ($P < .01$ vs baseline), to 44.7 ± 9.9 MFI in eosinophils activated by IL-5 ($P < .05$ vs baseline), and to 58.2 ± 12.1 MFI for cells activated by eotaxin-1 ($P < .05$ vs baseline). Montelukast (10^{-6} mol/L) or AA861 (10^{-6} mol/L) blocked the active conformational change of the β_2-integrin adhesion molecule caused by LTB_4 to 45.9 ± 7.6 MFI ($P < .05$ vs activated cells) and 32.8 ± 6.9 MFI ($P < .02$ vs activated cells), respectively (Fig 1, *D*). By contrast, increased avidity of CD11b caused by IL-5 or eotaxin-1 was not blocked by either $cysLT_1R$ antagonism or 5-LO inhibition (Fig 1, E and *F*).

Effect of montelukast and AA861 on β_2-integrin–mediated adhesion

We next examined the inhibitory effect of montelukast and AA861 on β_2-integrin adhesion caused by LTB_4, IL-5, or eotaxin-1 (Fig 2, n = 6 donors). β_2-Integrin adhesion to microplate wells coated with ICAM-1 surrogate protein was 23.5% ± 1.2% after 10^{-7} mol/L LTB_4 versus 5.21% ± 1.1% for buffer-activated cells (Fig 2, left; $P > .01$). Pretreatment with montelukast blocked LTB_4-induced adhesion in a concentration-dependent manner (Fig 2, *A*). Stimulated adhesion decreased to 19.1% ± 3.0% at 10^{-10} mol/L montelukast (P = not significant vs LTB_4-activated cells), to 17.1% ± 0.58% at 10^{-8} mol/L montelukast ($P < .05$ vs LTB_4-activated cells), and to 11.6% ± 0.93% at 10^{-6} mol/L montelukast ($P < .02$ vs LTB_4-activated cells). Similarly, AA861 caused a progressive inhibition of adhesion caused by LTB_4 (Fig 2, *B*); at 10^{-6} mol/L AA861, adhesion was attenuated to 13.3% ± 1.6% ($P < .02$ vs LTB_4-activated cells).

Activation of cytosolic $gIVaPLA_2$ is an essential step for cell adhesion and subsequent migration.[15,17] Cells were treated with trifluoromethylketone (TFMK), a $gIVaPLA_2$ inhibitor, or LTB_4-DMA, an LTB_4 receptor antagonist, before LTB_4 activation to test the regulatory role and specificity of this enzyme in induction of cellular adhesion by LTB_4. Both TFMK and LTB_4-DMA fully attenuated eosinophil binding to ICAM-1 surrogate (Fig 2, *C*; n = 6 donors).

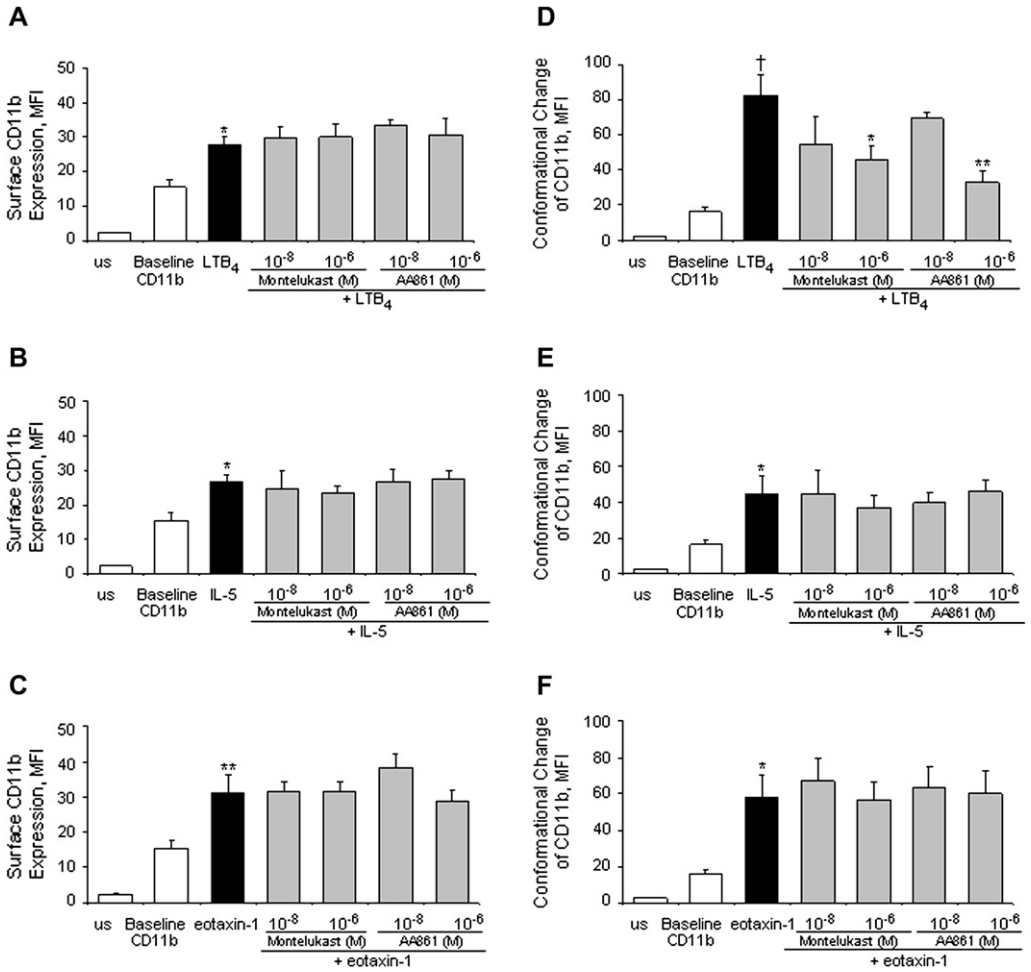

FIG 1. Effect of montelukast or AA861 on surface CD11b expression **(A-C)** or avidity of CD11b **(D-F)** caused by 10^{-7} mol/L LTB_4, 10 ng/mL IL-5, or 100 ng/mL eotaxin-1 (n = 4 donors). *$P < .05$ versus baseline CD11b or LTB_4-activated cells (Fig 1, *D*); **$P < .02$ versus baseline CD11b or LTB_4-activated cells (Fig 1, *D*); †$P < .01$ versus baseline CD11b.

In aliquots of isolated eosinophils taken from the same donors (n = 6 donors), IL-5 elicited 26.8% ± 0.42% adhesion ($P < .01$ vs unstimulated cells; Fig 2, right) and eotaxin-1 elicited 22.9% ± 1.65% adhesion ($P < .01$ vs unstimulated cells; Fig 2, right). Adhesion was unchanged after pretreatment with montelukast or AA861 at any concentration tested. By contrast, TFMK substantially blocked the stimulated adhesion, suggesting that, like LTB_4-activated cells, $gIVaPLA_2$ activation is necessary for IL-5– and eotaxin-1–induced adhesion independent of $cysLT_1R$ activation.

Effect of montelukast and AA861 on focal clustering of CD11b

A representative focal clustering of CD11b on eosinophils (n = 3 donors) was observed for cells activated with LTB_4 (Fig 3, *A*), IL-5 (Fig 3, *B*), or eotaxin-1 (Fig 3, *C*).

Pretreatment with 10^{-6} mol/L montelukast (Fig 3, *D*) or 10^{-6} mol/L AA861 (Fig 3, *G*) fully blocked the focal clustering of CD11b caused by LTB_4. By contrast, focal clustering of CD11b caused by IL-5 or eotaxin-1 was not blocked in eosinophils treated with 10^{-6} mol/L montelukast (Fig 3, *E* and *F*) or 10^{-6} mol/L AA861 (Fig 3, *H* and *I*). Absence of CD11b was shown for cells stained with IgG1, an isotype-matched control (Fig 3, *J*), whereas dispersed nonclustered cytoplasmic CD11b was demonstrated for unstimulated cells stained with mAb directed against CD11b (Fig 3, *K*).

Effect of LTD_4 on eosinophil β_2-integrin–mediated adhesion

Experiments were performed to determine the effect of exogenous LTD_4 on eosinophil β_2-integrin binding to ICAM-1 to test the possibility that LTD_4 has an autoregulatory role in β_2-integrin adhesion. LTD_4 caused increased β_2-integrin adhesion, which was blocked comparably by either montelukast or AA861. Basal adhesion for unstimulated cells was 4.54% ± 0.161%, increased to 8.45% ± 1.0% for cells activated with 10^{-8} mol/L LTD_4 (P = not significant), and further increased to 18.72% ± 3.17%

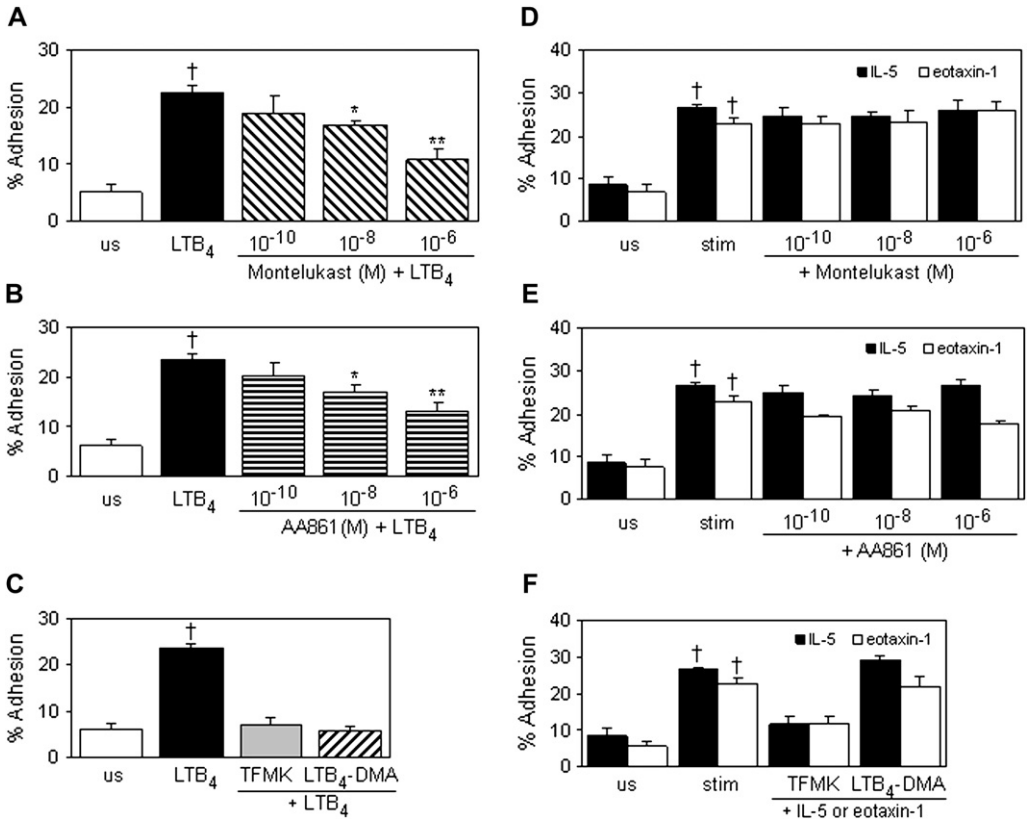

FIG 2. Effect of montelukast or AA861 on β_2-integrin–mediated adhesion. Eosinophils (n = 6 donors) were pretreated with montelukast, AA861, TFMK, or LTB_4-DMA before LTB_4 **(A-C)**, IL-5 **(D-F)**, or eotaxin-1 **(D-F)** activation. †$P < .01$ versus unstimulated cells *(us)* versus TFMK versus LTB_4-DMA; *$P < .05$ versus LTB_4-activated cells; **$P < .02$ vs LTB_4-activated cells and †$P < .01$ versus unstimulated cells versus TFMK.

in response to 10^{-6} mol/L LTD_4 ($P < .01$, Fig 4). Montelukast and AA861 at equimolar concentrations blocked the adhesion caused by 10^{-6} mol/L LTD_4 by 50%; by contrast, blockade of LTB_4 receptor with LTB_4–DMA had no effect on activated cells.

To confirm further the autocrine effect of LTD_4 on adhesion, we also determined whether LTB_4, IL-5, or eotaxin-1 causes synthesis of LTC_4 in isolated eosinophils. Secretion of LTC_4 increased from 18.1 ± 11.4 pg/10^6 cells to 297.8 ± 109.6 pg/10^6 cells after LTB_4 activation ($P < .01$; Fig 4, *B*). However, neither IL-5 nor eotaxin-1 elicited LTC_4 secretion. These data suggest that LTB_4 causes release of cysLT, which might amplify the eosinophil adhesion in an autocrine manner.

Effect of montelukast and AA861 on ERK-1/2 phosphorylation

We next examined whether montelukast or AA861 blocks ERK-1/2 activation caused by LTB_4 (Fig 5, *A*), IL-5 (Fig 5, *B*), or eotaxin-1 (Fig 5, *C*). We have shown previously that ERK-1/2 activation is an essential step in gIVaPLA$_2$ phosphorylation, which is necessary for integrin adhesion. Eosinophils (n = 3 donors) first were pretreated with buffer control, montelukast, or AA861 for 15 minutes before cell activation. Maximum ERK-1/2 activation occurred at 2 minutes for all activated cells. However, stimulated ERK-1/2 phosphorylation was unchanged for eosinophils treated with either montelukast or AA861 (Fig 5).

DISCUSSION

In this investigation we examined the role of cysLT signaling in LTB_4-, IL-5–, or eotaxin-1–induced (1) upregulation of surface CD11b expression (Fig 1, left), (2) conformational change of CD11b (Fig 1, right), (3) β_2-integrin adhesion to ICAM-1 surrogate protein (Fig 2), and (4) focal clustering of CD11b in isolated human eosinophils (Fig 3). Our data demonstrate that activation by LTB_4, IL-5, and eotaxin-1 increases the avidity and affinity of CD11b to ICAM-1 surrogate protein. Blockade with montelukast, a $cysLT_1R$ antagonist, or AA861, a 5-LO inhibitor, attenuated avidity, focal clustering, and hence adhesion caused by LTB_4. Neither montelukast nor AA861 blocked any of these responses in eosinophils activated by IL-5 or eotaxin-1. However, downstream gIVaPLA$_2$ inhibition fully blocked the β_2-integrin mediated adhesion elicited by LTB_4, IL-5, and eotaxin-1 (Fig 2, *C* and *F*). We also observed that LTB_4 caused release

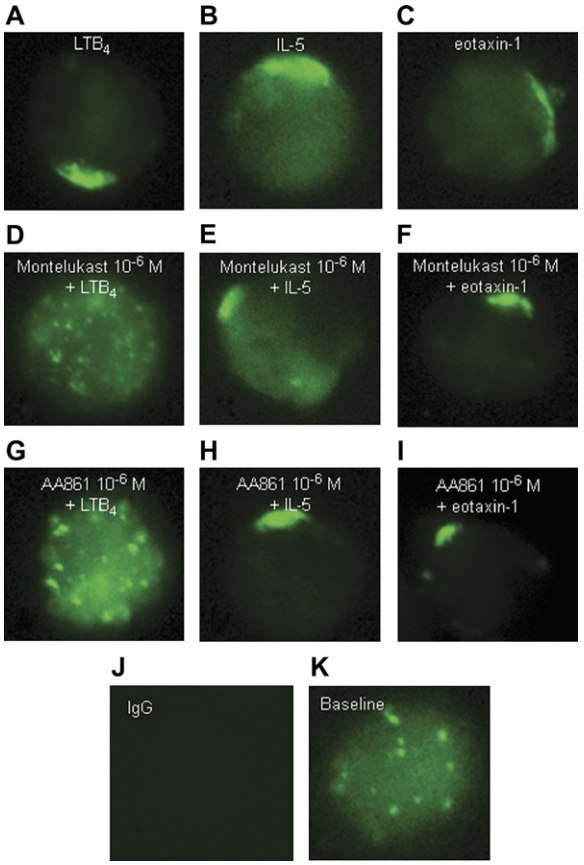

FIG 3. Effect of montelukast or AA861 on stimulated focal clustering of CD11b. Eosinophils (n = 3 donors) were preincubated with montelukast **(D-F)** or AA861 **(G-I)** for 30 minutes before activation with LTB_4 **(A)**, IL-5 **(B)**, or eotaxin-1 **(C)**. **J**, Naive eosinophils were stained with IgG1 antibody. **K**, baseline CD11b was stained with anti-CD11b mAb, as described in the Methods section.

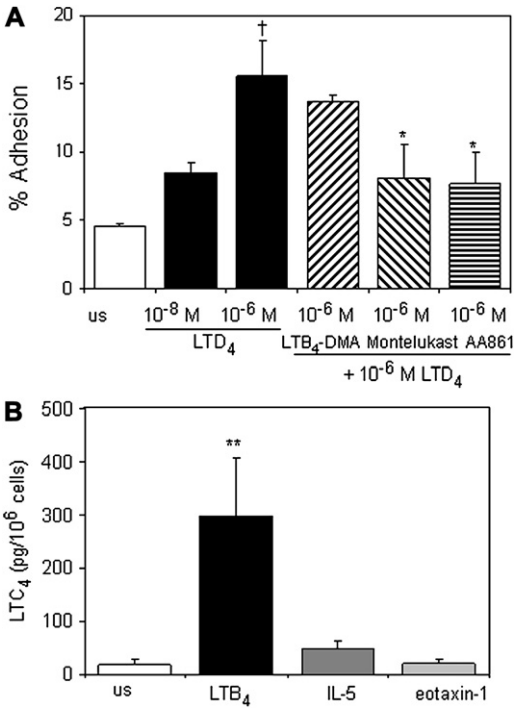

FIG 4. A, Effect of montelukast, AA861, or LTB_4-DMA on β_2-integrin adhesion caused by exogenous LTD_4. Data represent means ± SEMs (n = 3 donors). †$P < .01$ versus unstimulated cells *(us)*; *$P < .05$ versus LTD_4. **B**, Effect of LTB_4, IL-5, or eotaxin-1 on LTC_4 synthesis (n = 6 donors). **$P < .02$ versus unstimulated cells *(us)* versus IL-5 versus eotaxin-1.

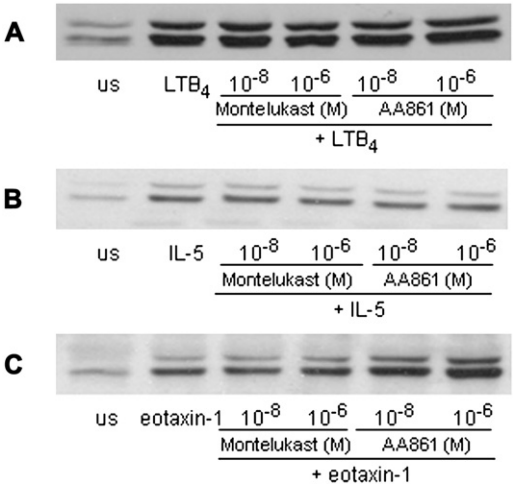

FIG 5. Effect of montelukast or AA861 on ERK-1/2 phosphorylation. Eosinophils were pretreated with increasing concentrations of montelukast or AA861 before LTB_4 **(A)**, IL-5 **(B)**, or eotaxin-1 **(C)** activation and determination of ERK-1/2 phosphorylation. Immunoblotting was performed with a specific mAb for phosphorylated ERK-1/2, as described in the Methods section.

of cysLTs in activated eosinophils; neither IL-5 nor eotaxin-1 caused secretion of LTC_4 (Fig 4, *B*). Our article is the first demonstration of the mechanism by which LTB_4 upregulates β_2-integrin–mediated adhesion: through the $cysLT_1R$ to cause cysLT-dependent focal clustering of CD11b and active conformational change of this integrin.

To test our hypothesis of whether cysLT secretion caused by endogenous LTB_4 upregulates β_2-integrin adhesion, we further examined the effect of exogenous LTD_4 of the cysLT receptor blockade on β_2-integrin adhesion (Fig 4, *A*). Exogenous LTD_4 caused increased adhesion, which was blocked by both montelukast and AA861. Because blockade of the LTB_4 receptor had no effect on LTD_4-induced adhesion (Fig 4, *A*), our findings suggest that production of cysLTs by eosinophils could result from autocrine activation on β_2-integrin–mediated adhesion. A postulated schema by which cysLT synthetic and signal pathways regulate integrin adhesion is suggested in Fig 6. The figure demonstrates our findings that activation of $cysLT_1R$ alone causes upregulation of β_2-integrin adhesion and that either montelukast or AA861 can block adhesion caused by cysLT synthesis.

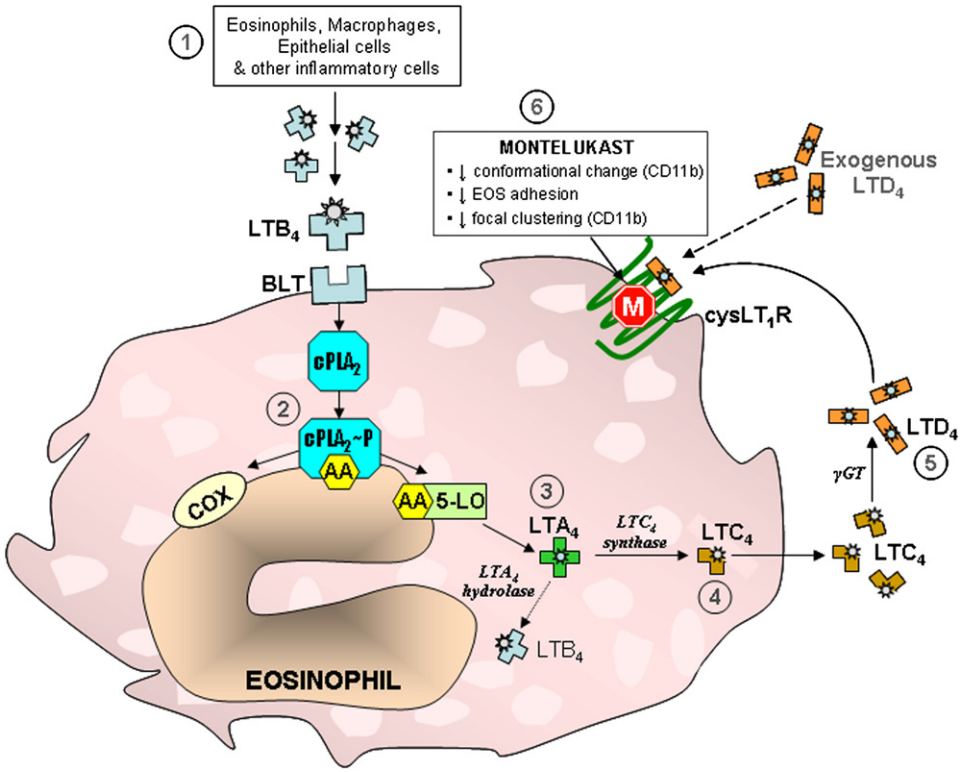

FIG 6. Postulated schema of cysLT synthetic and signaling pathways regulating integrin adhesion. *1*, Activation of inflammatory cells causes LTB_4 synthesis, binds to the LTB_4 receptor, and causes subsequent activation of glVaPLA$_2$. *2*, GIVaPLA$_2$ hydrolyzes membrane phospholipids to produce lysophospholipids and free fatty acids, including arachidonic acid. *3*, Conversion of LTA_4 to LTB_4 and cysLTC$_4$ occurs. LTC_4 is actively transported into the blood, where it is converted to LTD_4 *(4)* and binds to cysLT$_1$R *(5)* on eosinophils. *6*, Montelukast, a cysLT$_1$R antagonist, blocks conformational change of CD11b (Fig 1, *right*), eosinophil adhesion to ICAM-1 (Fig 2), and focal clustering of CD11b (Fig 3). Eosinophils independently produce cysLTs for autocrine or paracrine cell activation, resulting in an amplification of inflammation. *EOS*, Eosinophils.

It is important to consider some limitations to our *in vitro* model. These investigations were carried out *in vitro* by using activated eosinophil binding to ICAM-1 surrogate protein that might be pathophysiologically unrelated to events that initiate eosinophil migration during allergen challenge or human asthma. The eosinophils isolated from mildly atopic donors then are presumed to remain in a quiescent state until further activated to upregulate adhesion. It is necessary to study signal transduction in isolated cells in the static state to elucidate signal transduction of β_2-integrin alone. Regulation of adhesion by selectins, which cause cell rolling before firm adhesion caused by integrins, was by intention excluded to achieve this focus. Thus data obtained from these studies cannot be extrapolated to the *in vivo* human asthmatic state or to the specific behavior of eosinophils during transmigration in allergic or asthmatic conditions. However, our data do suggest one mechanism by which cysLT regulates integrin adhesion.

We conclude that blockade of 5-LO activity or the cysLT$_1$R comparably prevents assumption of the active conformational change and focal clustering of CD11b/CD18, which are essential for β_2-integrin adhesion. By contrast, blockade of cysLT synthesis or the cysLT$_1$R does not block eosinophil adhesion caused by IL-5 or eotaxin-1. Thus, cysLTs appear to have both a specific and limited role in the global regulation of eosinophil adhesion *in vitro*.

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