

Expression and targeting of lymphocyte function-associated antigen 1 (LFA-1) on white blood cells for treatment of allergic asthma

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ABSTRACT

Allergic asthma is a chronic respiratory disease that results from an exaggerated inflammatory response in the airways. Environment stimuli, such as pollen and HDM, cause activation and migration of inflammatory WBCs into the respiratory tract, where they cause lung damage. Migration of these WBCs is dependent on the active configuration of the β_2 integrin LFA-1. The experimental therapeutic agent LtxA specifically targets active LFA-1 and causes cell death. We investigated the association between LFA-1 and allergic asthma and hypothesized that targeting LFA-1 with LtxA could be an attractive strategy for treatment of the condition. We examined LFA-1 (CD11a) levels on PBMCs from patients with allergic asthma compared with healthy controls. Patients exhibited a significantly higher percentage of PBMCs expressing LFA-1 than healthy controls. Furthermore, the level of LFA-1 expression on patient PBMCs was greater than on healthy PBMCs. We identified a unique cellular population in patients that consisted of CD4⁺ CD11a^{hi} cells. We also evaluated LtxA in a HDM extract-induced mouse model for allergic asthma. LtxA caused resolution of disease in mice, as demonstrated by a decrease in BALF WBCs, a reduction in pulmonary inflammation and tissue remodeling, and a decrease in proinflammatory cytokines IL-4, IL-5, IL-9, IL-17F, and IL-23 α in lung tissue. LFA-1 may serve as an important marker in allergic asthma, and the elimination of activated WBCs by use of LtxA could be a viable therapeutic strategy for treating patients with this condition. *J. Leukoc. Biol.* 97: 000-000; 2015.

Abbreviations: BALF = bronchoalveolar lavage fluid, HDM = house dust mite, i.n., intranasally, LFA-1 = lymphocyte function-associated antigen 1, LtxA = leukotoxin, Leukothera, MFI = mean fluorescence intensity, PAS = Periodic Acid Schiff, qRT=PCR = quantitative RT-PCR

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

The pathology that is observed in patients with inflammatory and autoimmune diseases is mediated by an overt immune response and damage caused by immune cells migrating to the site of inflammation in response to signaling molecules, such as cytokines. One of the key molecules responsible for mediating this migration is LFA-1. LFA-1 is a β -2 integrin, consisting of an α chain (CD11a) and a β chain (CD18), and is crucial for migration of WBCs across the endothelial barrier into the surrounding tissues [1, 2]. LFA-1 undergoes an inducible conformational change from a nonadhesive, inactive state to an adhesive, active state upon activation by a stimulus [1, 3]. Active LFA-1 then binds to ICAM-1 on endothelial cells, which acts as a ligand for LFA-1. Formation of the activated LFA-1/ICAM-1 complex then leads to the migration of WBCs across the endothelial barrier. Allergic conditions and autoimmune/inflammatory diseases, such as asthma, psoriasis, multiple sclerosis, lupus, rheumatoid arthritis, Crohn's disease, ulcerative colitis, and type I diabetes, are characterized by the infiltration of highly active WBCs to the involved tissue. LFA-1 has been shown to be crucial for the initiation and perpetuation of these immune-mediated diseases [4]. Therefore, the targeting of WBCs expressing active LFA-1 is a rational therapeutic strategy with diverse clinical applications [5]. Indeed, therapeutics have been developed that target the LFA-1/ICAM-1 interaction in the form of statins, cinnamides, peptides, and mAb.

Asthma is an inflammatory disease of the airways, affects ~300 million people worldwide, and causes 250,000 annual deaths [6–8]. Approximately 34 million Americans have been diagnosed with asthma. The pathogenesis of asthma involves infiltration of activated WBCs into the airways and release of inflammatory mediators, which cause bronchial epithelium damage [8]. Allergic

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asthma is IgE mediated and involves initial exposure to the inhaled allergen and subsequent antigen presentation to Th2 lymphocytes, which secrete IL-4 and IL-13 [9]. These cytokines lead to Ig class-switching in B lymphocytes, resulting in IgE production. Subsequent binding of IgE molecules to FcεRI on mast cells and basophils results in sensitization to allergen exposure [10].

Inflammatory effects that are characteristic of asthma depend on the migration of WBCs into the airways, which is mediated by LFA-1. Various studies have shown that there is overexpression of LFA-1 on the immune cells involved in asthma with negligible contribution from other integrins [11]. In addition to its role in cellular adhesion and migration, LFA-1 acts as a costimulatory molecule for T cell sensitization [12]. Therefore, LFA-1 appears to be an ideal marker for activated immune cells, contributing to the pathogenesis of asthma. Previous studies using simvastatin to target LFA-1 in murine models of allergic asthma have shown that the drug can reduce Th2 cytokine levels [13]. In addition, mAb against CD11a (efalizumab) in randomized controlled trials of asthma patients was shown to decrease airway and sputum eosinophilia [14].

LtxA is a unique LFA-1-targeting agent that causes rapid death of WBCs expressing active LFA-1 [15–19]. LtxA is a natural protein that is produced by the oral bacterium *Aggregatibacter actinomycetemcomitans* [17, 20]. LtxA is known to target specifically WBCs expressing active LFA-1 involved in disease, while sparing healthy, resting WBCs, thus the minimization of the possibility of general immunosuppression. The exquisite specificity and activity of LtxA have formed the basis for evaluating LtxA as a targeted biotherapy for WBC diseases characterized by overexpression of LFA-1, such as hematologic malignancies and autoimmune/inflammatory diseases. Our previous studies have demonstrated significant in vitro and in vivo therapeutic efficacy of LtxA in models of leukemia, lymphoma, and psoriasis [18, 21, 22].

In this report, we examined the levels of LFA-1 on WBCs from patients with allergic asthma and demonstrate a unique cellular population that is characteristic of patients with the condition. We also show that LtxA causes significant resolution of disease in a mouse model for allergic asthma.

MATERIALS AND METHODS

LtxA purification

LtxA was purified from culture supernatants of *A. actinomycetemcomitans* strain 4500, as described previously [23, 24].

Isolation of PBMCs from blood

Whole blood was collected from 8 allergic asthma patients and 11 nonallergic healthy controls, who did not exhibit any allergies. All of the allergic asthma patients (ages 5–50) were recruited from the Emergency Room, the Adult Allergy and Immunology Clinic, or the Pediatric Pulmonary Clinic at University Hospital/New Jersey Medical School. Patients had recent evidence of reversible airway disease on spirometry and allergy to dust mite, as determined by skin testing. Patients were allowed to be on any combination of short-acting bronchodilators, inhaled corticosteroids, and long-acting bronchodilators. Patients were excluded if they were pregnant; taking omalizumab (Xolair) or other immunotherapy treatment, systemic corticosteroids, or antibiotics within 30 days; or had significant comorbidities, such as HIV, heart disease, diabetes, and cancer. The control group had no history of asthma or allergic conditions. The collection protocol was approved by the Rutgers

Institutional Review Board, and written, informed consent was obtained from all study subjects. PBMCs were isolated by use of Ficoll density gradient separation (Corning cellgro, Mediatech, Manassas, VA, USA). Viable cells were counted by use of Vi-CELL viability instrument.

Analysis of PBMCs

PBMCs (10^6 cells/ml) from patients and controls were stained with antibodies (BioLegend, San Diego, CA, USA) to the following markers: CD4 (Th cells), CD11a and CD14 (monocytes), and CD3 (T cells). PBMCs from asthma patients were also incubated overnight with buffer or LtxA (500 ng/ml) in RPMI-1640 medium. The treated PBMCs were washed with PBS and stained with Annexin V, along with the above antibodies, and analyzed through flow cytometry to measure LtxA-mediated cell death.

Animal study

Female BALB/c mice (6–8 weeks, 15–20 g; The Jackson Laboratory, Bar Harbor, ME, USA) were housed under specific pathogen-free conditions and a 12-h light/dark cycle with access to food and water. Under isoflurane anesthesia, mice were exposed to HDM extract (*Dermaphagoides pteronyssinus*; Greer Laboratories, Lenoir, NC, USA) i.n. (25 µg in 10 µl saline/nostril). Control (nonasthmatic) animals were administered an equal volume of saline alone. The frequency of exposure for HDM/saline was 5 days/week for a total of 5 weeks. Power calculations from pilot studies indicated that 4 animals/group were sufficient to detect a significant difference between control and experimental groups.

After 2 weeks, HDM-exposed mice were divided into 4 groups of 4 mice/group and treated with the following: 1) dexamethasone vehicle (saline) s.c. once daily, 5 days/week; 2) dexamethasone (1.25 mg/kg; Sigma, St. Louis, MO, USA) s.c. once daily, 5 days/week; 3) LtxA vehicle (20 mM Tris-HCl, pH 6.8, 250 mM NaCl, and 0.2 mM CaCl₂) i.p., 3 days/week; and 4) LtxA (0.5 mg/kg) i.p., 3 days/week. HDM exposure was continued throughout the 3-week treatment period.

At the end of the study, mice were euthanized by giving a fatal dose of avertin. Isolation of BALF was performed by first making a transverse incision in the trachea below the larynx and filling the space with 0.5 ml PBS. BALF from each mouse was subjected to RBC lysis for 10 min at room temperature, followed by washing twice at 400 g for 5 min and resuspending the cell pellet in PBS. Total BALF cell number was counted by use of a hemocytometer. Immunophenotypic analysis of BALF cells was performed by antibody staining and flow cytometry. For each antibody stain, 10^6 cells were first blocked with a Fc blocker (rat anti-mouse CD16/CD32; BD Biosciences, San Jose, CA, USA) for 10 min at room temperature, followed by incubation with mAb at 4°C for 30 min and analysis on an LSR II flow cytometer (BD Biosciences). Antibodies (BD Biosciences) to the following markers were used to identify the various cellular subtypes: CD3e (T cells), CD45R/B220 (B cells), Ly6G and CCR3 (neutrophils), CCR3 and Ly6G (eosinophils), and MHC II (macrophages). Relevant isotype controls were included with each experiment.

Blood from the mice was collected by ileac vein puncture immediately following the BALF isolation. Blood was collected in anticoagulant tubes and centrifuged at 400 g for 10 min. The use of animals was approved by the Rutgers Institutional Animal Care and Use Committee, and “Principles of Laboratory Animal Care” (National Society for Medical Research) was followed for the care and use of animals.

Lung tissue histology

After collecting BALF and blood, lungs were removed through dissection and stored in 10% neutral-buffered formalin at room temperature until microscopic analysis. Sections of paraffin-embedded, fixed lung tissues were stained with H&E to analyze total lung inflammation. Sections were also stained with PAS reagent to identify mucous and goblet cell hyperplasia and Sirius Red for eosinophils. The tissue preparation and examination were carried out at the New Jersey Medical School Histology Core Facility. Samples were examined by a board-certified pathologist.

Cytokine analysis

qRT-PCR was used to determine the expression levels of proinflammatory cytokines (IL-4, IL-5, IL-9, IL-17F, and IL-23 α) in the lungs of mice. Total RNA from the lung tissue was extracted with Trizol reagent (Life Technologies, Grand Island, NY, USA). Relative mRNA levels were determined by qRT-PCR. One microgram of total RNA was reverse transcribed by use of the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Amplification was carried out by use of TaqMan Fast Universal PCR Master Mix (Life Technologies). The data were normalized to GAPDH. Gene expression was calculated by use of the $\Delta\Delta$ comparative threshold method relative to naïve sample.

Statistical analysis

BALF cell counts, differential cell counts, and cytokine levels were compared by Student's *t*-test. $P \leq 0.05$ was considered significant.

RESULTS

Expression of LFA-1 on WBCs from allergic asthma patients and healthy controls

We analyzed PBMCs from the blood of 8 allergic asthma patients and 11 matched healthy controls. All of the allergic asthma patients (ages 5–50) were recruited from the Emergency Room, the Adult Allergy and Immunology Clinic, or the Pediatric Pulmonary Clinic at University Hospital/New Jersey Medical School. Patients diagnosed with asthma tested positive for an allergic reaction to HDM. We chose to examine the CD11a component of LFA-1 rather than CD18, as CD18 can be paired with other α chains, such as CD11b, CD11c, and CD11d [25]. Thus, CD11a uniquely represents LFA-1. From the total PBMC population, the percentage of CD11a-positive cells from patients was significantly higher than from the healthy controls (Fig. 1A). Patients had $95.1 \pm 3.14\%$ CD11a-positive cells, whereas healthy controls had $90.3 \pm 4.11\%$. In addition, the number of LFA-1 molecules on the surface of CD11a⁺ WBCs from allergic asthma patients (8605 ± 2519) was significantly greater than on the surface of healthy control WBCs (5089 ± 2107), as indicated by the MFI; Fig. 1B).

WBCs stained with anti-CD4 and anti-CD11a antibodies revealed a unique cellular population in allergic asthma patients that consisted of CD4⁺CD11a^{hi} cells, which were absent from the healthy control samples (Fig. 2A and B). The CD11a^{hi} population was defined as cells that had a CD11a MFI of $>10^4$. Immunophenotypic analysis revealed that this CD11a^{hi} population in asthma samples consisted primarily of CD14⁺ monocytes and CD3⁺ non-Th cells (Supplemental Fig. 1). Thus, high LFA-1 expression defines a unique cellular population that is present in patients with allergic asthma.

Effects of LtxA on PBMCs from allergic asthma patients

To determine which cells from patients are targeted by LtxA, PBMC samples were treated with LtxA for 24 h and then stained with Annexin V and analyzed by flow cytometry (Fig. 3A). Of the CD11a⁺ PBMCs, LtxA killed only the CD11a^{hi} cells and did not affect cells that had a low expression of LFA-1. Cells were killed by apoptosis (Annexin V positive) and necrosis/depletion (Fig. 3A). The cells that were depleted by LtxA express active state LFA-1, as revealed by staining with an antibody (mAb24) that recognizes LFA-1 specifically in the active conformation (Fig. 3B). The majority of these cells also stained positive for CD14, indicating that they were monocytes.

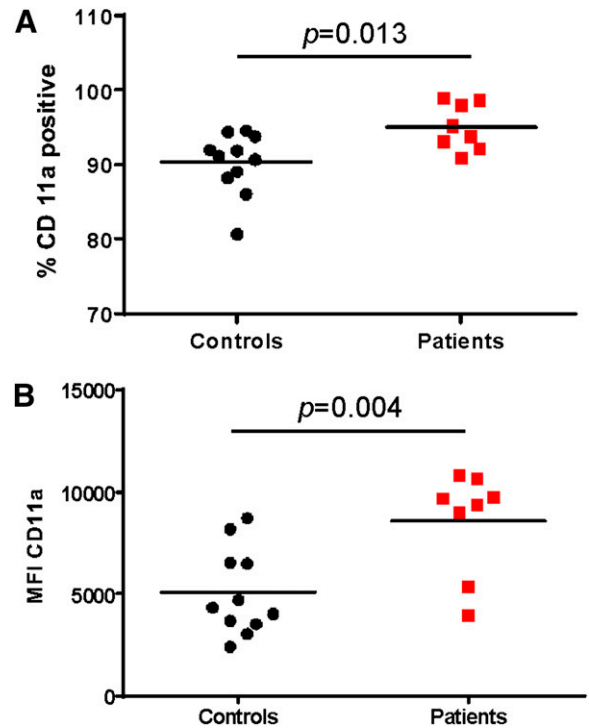


Figure 1. PBMCs from allergic asthma patients express high levels of LFA-1. PBMCs were isolated from whole blood by use of Ficoll hypaque density gradient separation and stained with antibody against CD11a. The samples were analyzed by flow cytometry. The percent of CD11a-positive cells (A) and MFI (geometric mean) of CD11a (B) were measured. The significance of the differences was determined by use of Student's *t*-test.

Evaluation of LtxA in a mouse model for allergic asthma

Given the potential role that LFA-1 plays in the pathogenesis of allergic asthma and the ability for LtxA to target specifically the LFA-1^{hi} WBCs ex vivo that are unique to allergic asthma patients, we performed an initial proof-of-principle evaluation of LtxA in a mouse model for allergic asthma. Mice were administered HDM extract or saline i.n., 5 days/week for 5 weeks. After 2 weeks of administration, HDM-exposed mice were subdivided into 4 groups of 4 mice/group and received the following treatments for an additional 3 weeks: dexamethasone vehicle, s.c., 5 days/week; dexamethasone (1.25 mg/kg), s.c., 5 days/week; LtxA vehicle, i.p., 3 days/week; and LtxA (0.5 mg/kg), i.p., 3 days/week.

At the end of the study, BALF, lung tissue, and blood were collected from all mice for further evaluation. Examination of WBCs in the BALF revealed that HDM-exposed mice, treated with the dexamethasone vehicle or LtxA vehicle, had significantly higher levels of all WBC subsets than nonasthmatic mice that were given only saline (Fig. 4A). Treatment of HDM-exposed mice with dexamethasone or LtxA caused a significant reduction in the numbers of WBCs in the BALF (Fig. 4A).

To determine if LFA-1 is involved in the migration of WBCs to the lung tissue in this animal model, we examined the levels of LFA-1 on PBMCs and BALF WBCs in two HDM-exposed mice that were treated with LtxA vehicle (Fig. 4B). We found that the migrated WBCs that were present in the BALF had significantly

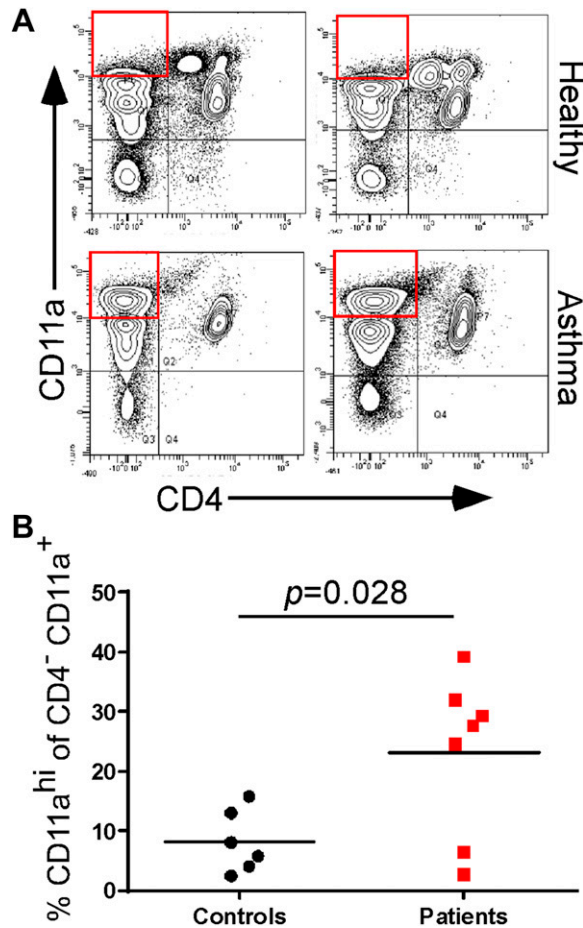


Figure 2. PBMCs from allergic asthma patients constitute a unique population. PBMCs were isolated from whole blood by use of Ficoll hypaque density gradient separation and stained with antibody against CD4 (Th2) and CD11a. The samples were analyzed by flow cytometry. (A) PBMCs from asthma patients showed a distinct population of CD4⁺CD11a⁺ cells (boxed in red) that was absent in the healthy subjects. (B) Samples were analyzed for the presence of the CD4⁺CD11a⁺ population among the CD11a-positive cells in allergic asthma patients and healthy controls. The significance of the differences was determined by use of Student's *t*-test.

higher levels of LFA-1 than on the WBCs in the peripheral blood of the same animal. This finding suggests that there is a selection for the LFA-1^{hi} subset of WBCs in the lung tissue in this mouse model for allergic asthma.

Lung tissue was sectioned and stained with H&E, PAS, or Sirius Red (Fig. 5). H&E staining revealed a large infiltration of WBCs in the lung tissue of HDM-exposed mice treated with dexamethasone vehicle or LtxA vehicle. Infiltration was not evident in saline-exposed mice. The infiltration of WBCs in HDM-exposed mice was most evident surrounding the blood vessels and bronchioles. We also observed significant goblet cell hyperplasia surrounding many of the bronchioles in the vehicle-treated controls but not in the other samples (Fig. 5A and B). Staining of polysaccharides with PAS in the lung tissue from LtxA vehicle-treated mice confirmed the presence of mucin-producing goblet cells and subepithelial accumulation of collagen. Sirius Red

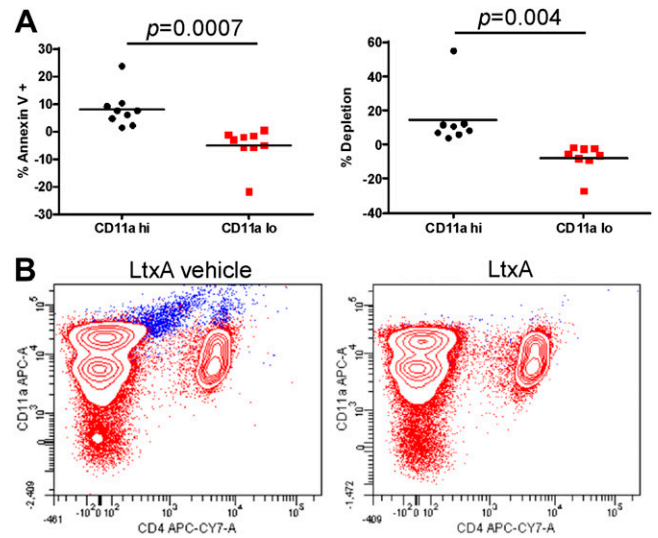


Figure 3. Preferential targeting of CD11a^{hi} cells by LtxA. PBMCs from allergic asthma patients were treated in vitro with LtxA (500 ng/mL) or buffer for 24 h. The treated PBMCs were then stained with anti-CD11a antibody and Annexin V and analyzed through flow cytometry. LtxA-mediated cytotoxicity was measured in terms of apoptosis (Annexin V positive) compared with buffer treated and depletion (buffer-treated PBMCs – LtxA-treated PBMCs). (A) LtxA preferentially killed cells with high LFA-1 (defined as CD11a-positive cells with an MFI between 10⁴ and 10⁵) and had a negligible effect on PBMCs with low LFA-1. The significance of the differences was determined by use of Student's *t*-test. (B) LtxA causes depletion of cells that express the active conformation of LFA-1 (shown in blue). PBMCs were stained with mAb24 and CD11a antibodies to identify the cells with active LFA-1. APC-A, Allophycocyanin-area; CY7, cyanine 7.

staining of sections revealed pink-staining eosinophils in the vehicle-treated mice but not in the LtxA-treated mice (Fig. 5C). Mice that were treated with dexamethasone had a reduced number of eosinophils compared with the vehicle control but still greater than LtxA-treated mice.

Proinflammatory cytokines play a crucial role in the pathogenesis of allergic asthma and other inflammatory conditions. In allergic asthma, IL-4, IL-5, IL-9, IL-17F, and IL-23 α are the primary signaling molecules involved in disease. We evaluated the levels of IL-4, IL-5, IL-9, IL-17F, and IL-23 α mRNA in the lung tissue from all mice (Fig. 6). We found that the vehicle-treated mice had significantly greater expression of the proinflammatory cytokines compared with saline-exposed mice. In addition, dexamethasone caused reduction of IL-9, IL-17F, and IL-23 α , whereas LtxA treatment caused significant reduction of all of the cytokines that we examined.

DISCUSSION

Asthma is a disease of increasing prevalence, with many patients developing resistance and intolerance to conventional therapy. To address this situation, there is a need to develop new treatments targeting novel markers. The β_2 -integrin, LFA-1, is expressed on the surfaces of WBCs and is crucial for the

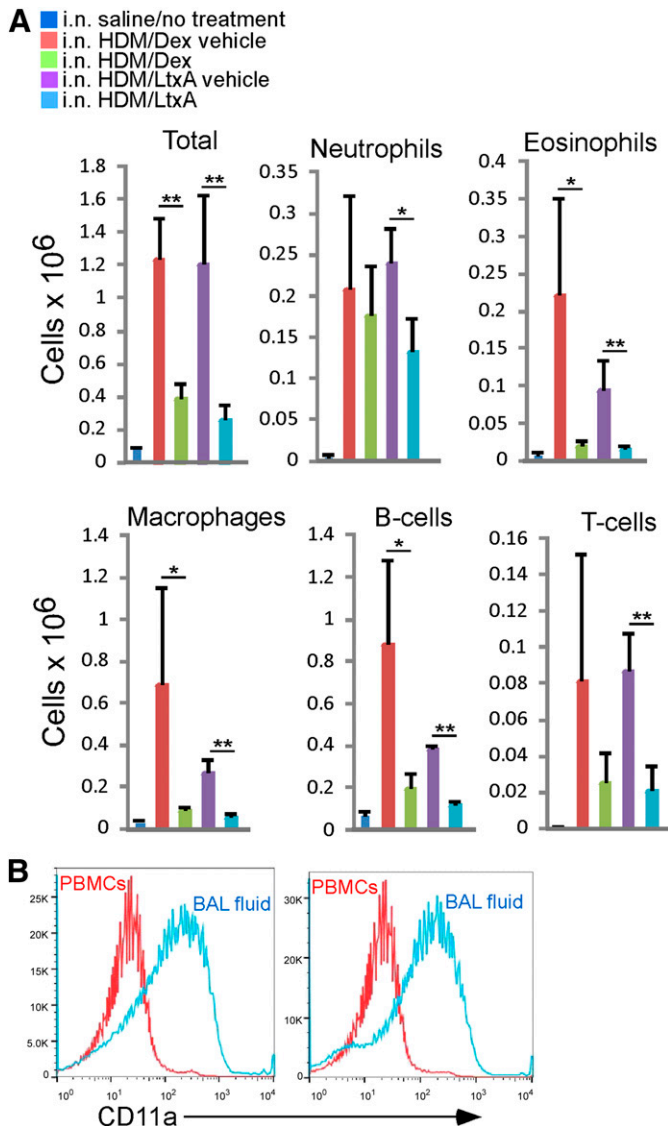


Figure 4. Effects of experimental treatment on WBCs in the BALF in a mouse model for allergic asthma. Saline-treated mice were given saline i.n., whereas all of the other groups were administered HDM extract i.n. HDM extract-exposed mice were then treated with dexamethasone or LtxA or the respective vehicles, as described in the text. (A) Cells in the BALF were stained with mAb for specific cell types and then analyzed by use of flow cytometry. Each bar represents the average from 4 mice. Error bars show SD. The significance of the differences was determined by use of Student's *t*-test (* $P \leq 0.05$; ** $P \leq 0.01$). (B) High expression of LFA-1 characterizes cells that have migrated into the airways. PBMCs and BALF WBCs from 2 HDM extract-exposed mice that were treated with LtxA vehicle were stained with anti-CD11a antibody and analyzed by use of flow cytometry.

migration of cells out of the bloodstream and into the tissue [2, 26]. LFA-1 (CD11a and CD18) has been shown to play a significant role in the pathogenesis of asthma [11, 27, 28], as well as other autoimmune and inflammatory diseases [29–31]. In asthma, various inflammatory subtypes of WBCs show up-regulation and activation of LFA-1. This activated LFA-1 then mediates the migration of these cells into the airways. Once

migrated, the inflammatory cells cause airway inflammation and remodeling, thereby leading to symptoms of asthma. The importance of LFA-1 has been demonstrated in animal models by use of mice, rats [32, 33], and rabbits [34, 35]. Human studies have also confirmed the important role that LFA-1 plays in asthma [11, 36]. More recently, Knight et al. [37] reported that CD11a polymorphisms in mice can affect Th2 cell homing and Th2-mediated allergic diseases and potentially play a role in human allergic diseases. Our findings in this report support the above paradigm.

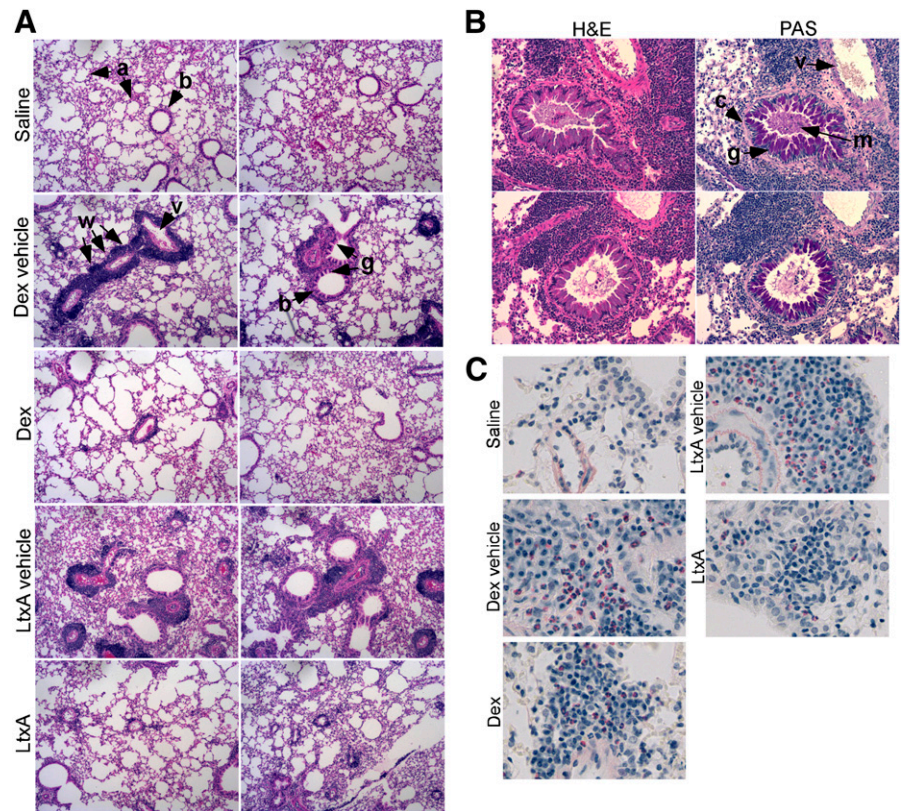
We noted that PBMCs from allergic asthma patients had higher levels of LFA-1 compared with matched healthy controls. LFA-1 could possibly be used as a diagnostic marker for the disease by acting as a differentiating factor between asthmatic and healthy PBMCs. As a higher percentage of cells expressing LFA-1 would translate to an increase in the number of inflammatory cells infiltrating the airway tissue, LFA-1 levels may also be used as a measure of disease severity. In addition, reduction in LFA-1 levels following treatment could be a potential measure of treatment efficacy. Furthermore, the CD4⁺ CD11a^{hi} cellular population could serve as a diagnostic parameter for other allergic or inflammatory diseases, as they all have a similar pathogenesis. This work lays the foundation for ongoing studies to examine larger populations of patients.

Importantly, our work demonstrates why LFA-1 may represent an important therapeutic target for the treatment of asthma and related conditions. Indeed, other agents that target LFA-1 have been found to be effective in allergic diseases. For example, use of simvastatin, a small molecule drug that can target LFA-1, and mAb against LFA-1 (efalizumab) in randomized controlled trials of asthma patients was shown to reduce airway and sputum eosinophilia [14, 38]. In clinical trials, simvastatin was able to reduce the eosinophil count and improve the late airway response but did not affect airway hyper-responsiveness or inflammatory cytokines (IL-4, IL-5) compared with the placebo. One reason could be that although simvastatin decreased the number of airway eosinophils, it increased the number of lymphocytes in the sputum. An increase in the number of Th2 cells will cause further amplification of an inflammatory response. Furthermore, only subjects with eosinophilic asthma were included in the study. Simvastatin could have had a stronger effect in preventing migration of other inflammatory subtypes, which were not included in the study.

Treatment of asthma patients with efalizumab caused a decrease in the number of inflammatory cells, as well as a decrease in the late airway response compared with placebo. It did not have any effect on the early asthmatic response. This effect of simvastatin and efalizumab in reducing airway eosinophilia but not translating into an improvement in pulmonary function may be explained by studies showing dissociation between pulmonary function and airway eosinophilia [39, 40]. Thus, simply acting on the eosinophils is not necessarily sufficient to cause resolution of asthma.

LtxA differs in its mechanism of action from simvastatin and efalizumab in several important ways. Efalizumab binds LFA-1 and blocks interactions between WBCs and endothelial cells, thereby preventing the migration of inflammatory cells. Simvastatin primarily has a cholesterol-lowering effect with a recently

Figure 5. Histologic examination of lung tissue from mice. Mouse lungs were dissected at the end of the study after collecting blood and BALF. Lung tissue was fixed in 10% formalin and embedded in paraffin. Sections of the lung were stained with (A) H&E, (B) PAS reagent to stain polysaccharides, or (C) Sirius Red to stain eosinophils, which appear pink. a, Alveoli; b, bronchiole; c, collagen; w, WBC infiltration; v, blood vessel; g, goblet cell hyperplasia; m, mucous production. Original magnification, 100× (A and B) and 400× (C).



recognized anti-inflammatory action. The mechanism of anti-inflammatory action for statins is varied, ranging from blockade of LFA-1, preventing neutrophilic migration, and release of inflammatory mediators to inducing efferocytosis (phagocytosis of apoptotic cells) [38, 41]. In contrast, LtxA causes a rapid depletion of activated inflammatory cells after binding to active LFA-1 [15, 16, 18, 22]. Simply blocking LFA-1, as efalizumab does, prevents migration of cells only while the drug is bound to the receptor, whereas depletion of activated inflammatory cells by LtxA allows for a long-term anti-inflammatory effect. Furthermore, LtxA targets all of the activated inflammatory cell types, providing a robust, anti-inflammatory effect, which is especially relevant in a heterogeneous disease, such as asthma. Similar to other biologic drugs that are used to treat inflammatory conditions, such as asthma, LtxA administered s.c. is active in experimental animals [42]. In addition, LtxA does not elicit a neutralizing response in dogs or monkeys, even after repeat dosing over several weeks (unpublished data).

In this study, we found that ex vivo treatment of PBMCs from allergic asthma patients with LtxA caused a preferential cell death of PBMCs with high LFA-1, whereas the PBMCs with low LFA-1 were not affected. This specificity of LtxA for cells expressing high LFA-1 allows the drug to target the disease-causing PBMCs, while sparing the resting PBMCs. This approach provides the benefit of resolving an immune-mediated disease without causing significant immunosuppression, which is a major drawback of the prevalent therapies for asthma and other autoimmune and inflammatory diseases that target components of the immune system nonspecifically. For example,

corticosteroids, which are the standard of care for asthma, have a generalized immunosuppressive effect, whereby they decrease migration of leukocytes and reduce the number of immune cells and production of immunomodulators and cytokines. Steroids can be well tolerated for a brief duration but chronic use often leads to multiple side-effects, such as proximal myopathy, Cushingoid habitus, hyperglycemia, diabetes, infections, and osteoporosis [43, 44]. Chemotherapy immunosuppressants, such as cyclosporine and tacrolimus, are used after organ transplants

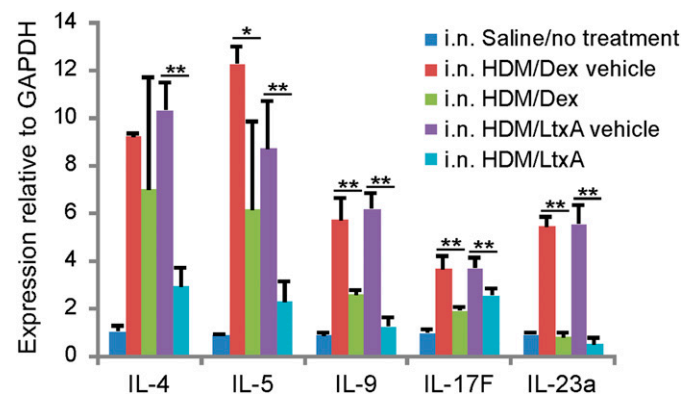


Figure 6. Effects of experimental treatment on cytokine levels in mice. Mouse lungs were dissected at the end of the study, and the tissue was processed for cytokine analysis, as described in Materials and Methods. qRT-PCR was carried out to determine the mRNA expression levels of proinflammatory cytokines in lung tissue.

and in myasthenia gravis. They act by preventing Th2 cell activation and are known to cause nephrotoxicity [45]. Targeted immune therapies, which are more similar to LtxA, include mAb, such as natalizumab and rituximab. These immune therapies do not differentiate between resting and activated immune cells and cause a more potent immunosuppression. LtxA, however, preferentially targets the activated immune cells [15, 16, 18, 22] and therefore, should show minimal immunosuppression and associated side-effects.

We tested LtxA in a validated, robust model of chronic allergic asthma [46], which is caused by chronic allergen exposure, triggering immunologic and biochemical pathways leading to airway changes, manifesting in asthmatic symptoms of wheezing and breathlessness. To reproduce this system, we used a murine model showing sustained airway eosinophilic inflammation caused by chronic exposure (5 weeks) to common allergen HDM extract. This model is characterized by airway inflammation in BALF and lung tissue, persistent Th2 response with increased cytokine production, progressive airway remodeling, and bronchial hyper-reactivity [46]. Importantly, we noted that the WBCs present in the BALF of asthmatic mice expressed significantly higher levels of LFA-1 than the peripheral WBCs in the same animals. This finding indicates that LFA-1 plays an important role in the migration of WBCs to the airways in mice and further validates the use of this model to evaluate a drug that targets LFA-1. As the saline-exposed mice did not exhibit pulmonary inflammation and therefore, did not contain inflammatory WBCs in the BALF, we were unable to compare levels of LFA-1 in these animals.

Treatment of asthmatic mice with LtxA caused significant reduction in the BALF cell count of all of the inflammatory WBC subtypes. This reduction was more than that caused by the positive-control treatment dexamethasone. Lungs of LtxA-treated mice also showed reduced inflammatory infiltrate, a decreased number of eosinophils, and negligible airway remodeling. We also found that LtxA treatment caused a significant reduction in levels of expression of IL-4, -5, -9, -17F, and -23 α in the lung.

The role of Th2 cytokines, such as IL-4, IL-5, and IL-9, has been well defined in the pathogenesis of asthma, orchestrating the initiation and amplification of the inflammatory response. The decrease of Th2 cytokine levels is also a measure of efficacy of prevalent treatments, such as steroids. IL-4 plays a role in Th2 differentiation [47], IgE synthesis [48], transmigration of inflammatory cells, and mucous secretion [49]. IL-5 is important for terminal differentiation of eosinophils [40, 50]. IL-9 is crucial for airway inflammation and airway hyper-responsiveness [51]. In addition to these Th2 cytokines, IL-17F and IL-23 α are important in asthma. IL-17F is produced by the Th17 subtype of CD4 T cells and is involved in autoimmune diseases. IL-17F causes induction of various cytokines, chemokines, and adhesion molecules and is responsible for neutrophil recruitment, airway hypersensitivity, and mucous secretion [52]. IL-23 is a novel IL-12 α family cytokine member that is involved in the maintenance and function of Th17 cells. This IL-23/Th17 axis is responsible for neutrophilic and eosinophilic recruitment in asthma [53].

In conclusion, we demonstrated that high LFA-1 expression defines a unique population of WBCs in patients with allergic asthma. We also showed that a natural bacterial protein, LtxA,

targets this specific activated population *ex vivo*. In a proof-of-principle study, LtxA also causes resolution of the disease *in vivo* by decreasing the number of inflammatory WBCs in BALF, reducing lung inflammation and remodeling, and lowering the levels of inflammatory cytokines in an allergic asthma mouse model. Given the effect of LtxA on activated WBCs and proinflammatory cytokines levels, it is feasible to test the experimental drug as an anti-inflammatory agent in other allergic diseases as well. Importantly, LtxA therapy may be an attractive alternative in patients where steroids are no longer effective or well tolerated. Ultimately, clinical studies will be required to elucidate the therapeutic potential of LtxA as a novel anti-inflammatory agent.

AUTHORSHIP

A.G. planned and carried out studies and wrote the manuscript. V.E. and L.E.G. planned and carried out studies. V.R., K.L.M., E.C., and H.A.A. planned studies and collected human blood samples. A.R.M. planned studies and edited the manuscript. C.K. planned studies and carried out experiments. S.C.K. planned studies and wrote the manuscript.

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DISCLOSURES

S.C.K. is a consultant for Actinobac Biomed and owns stock in the company.

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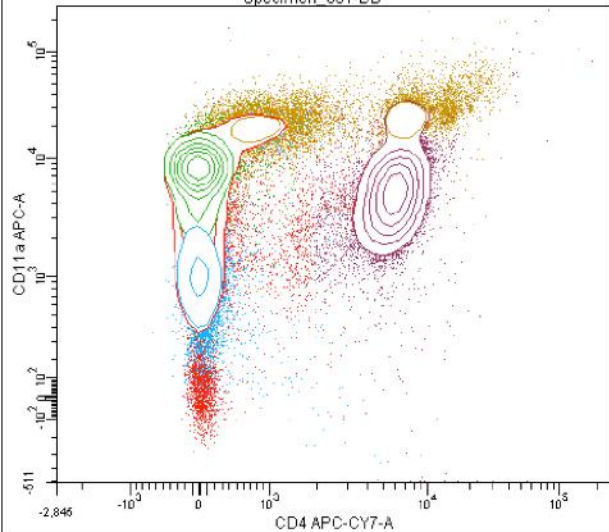
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KEY WORDS:

leukotoxin · LtxA · Leukothera · house dust mite · inflammation · CD11a

Specimen_001-DB



Specimen_001-DB

