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LeukotrieneB4 receptor 1 is Differentially Expressed on Peripheral T Cells of Steroid-Sensitive and -Resistant Asthmatics

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Introduction

T lymphocytes are central to the induction and maintenance of the allergic inflammatory response with emphasis on the role of CD4⁺ T cells and their ability to produce Th2 cytokines such as IL-4, IL-5, and IL-13 (1). CD8⁺ T cells have also been demonstrated in the bronchoalveolar lavage (BAL) fluid and sputum of asthmatics (2,3) and in lung tissue of fatal asthma (4), but their specific role in asthma pathogenesis has been somewhat controversial. Both enhancing (5) and protective (6) roles for CD8⁺ T cells have been described

Among the many mediators released during an asthmatic response, leukotriene levels, including leukotriene B4 (LTB4) were increased in the BAL fluid and breath condensates of asthmatics (7) and are potent mediators of asthma (8,9). LTB4 is an important regulator of neutrophil chemotaxis to the lung (10). We and others showed the importance of LTB4 interacting with its high affinity receptor, BLT1, in the accumulation of T cells in the lung (11–14). We found in a mouse model of experimental asthma that BLT1-expressing effector memory CD8⁺ T cells were a potent source of IL-13 and required for the development of lung allergic responses (15). Moreover, these CD8⁺BLT1⁺ T cells were more resistant to corticosteroids than CD4⁺ T cells and corticosteroids enhanced their activation and effector function by upregulating BLT1 expression through increased IL-2 receptor expression (16). In asthmatics, the numbers of CD8⁺BLT1⁺ T cells were increased in the tissue and bronchoalveolar (BAL) fluid and numbers of CD8⁺BLT1⁺ T cells producing IL-13 correlated with diminished lung function (17,18). In severe asthmatics, comparison of

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Author Contributions: EHC carried out most of the studies, while YJ and KT helped perform some of the assays, DYML, ERS, AD, and RJM were involved in obtaining samples from patients and controls, EWG supervised the project, design and data analysis. All contributed to drafting of the manuscript.

transcriptome analyses showed large changes in circulating CD8+ but not CD4+ T cells compared to patients with non-severe asthma or controls (19).

To further define potential differences in peripheral blood CD4+ and CD8+ T cells that may contribute to asthma pathogenesis, we compared responses of these T cell subsets from steroid-sensitive (SS) and steroid-resistant (SR) asthmatics. The results identified important differences in the responses of CD4+ and CD8+ T cells to cell activation and between SS and SR asthmatics in the induction of BLT1 expression, steroid sensitivity and cytokine production in these subsets.

Methods

Subjects

Subjects with a diagnosis of asthma according to American Thoracic Society criteria were selected. To qualify for study, baseline FEV₁ was less than or equal to 80% predicted. All subjects were non-smokers (no smoking for >1 year with a total of less than 10 pack/year) and had not experienced an upper respiratory tract infection for at least 6 weeks prior to enrollment. Asthmatic patients had a PC₂₀ of 8 mg/ml or lower if steroid-naïve or 16 mg/ml if on an inhaled corticosteroid (ICS); patients otherwise demonstrated a 12% or greater improvement in FEV₁ after albuterol. After baseline characterization, all subjects received prednisolone (40 mg) orally for 7 days and were categorized as having SS asthma if the FEV₁ value improved by 10% or greater or as having SR asthma if the FEV₁ improved by less than 10%. For controls, healthy adults with no history of atopic or respiratory disease were enrolled. None of the subjects received systemic corticosteroid therapy for at least 6 weeks before study. This study was approved by the Institutional Review Board of National Jewish Health (Denver, CO).

Human peripheral blood mononuclear cell (PBMC) purification, isolation of CD4+ and CD8+ T cells, and culture procedures

Human PBMCs were isolated from heparinized venous blood by density gradient centrifugation and CD4+ and CD8+ T cells were isolated by negative selection using the magnetic bead human CD4+ T cell isolation kit II and human CD8+ T cell isolation kit II (Miltenyi Biotec, Auburn, CA), respectively. Isolated CD4+ and CD8+ T cells (>95% purity) were stimulated in culture with anti-human CD3 mAb (2 µg/mL, BD Pharmingen, San Jose, CA)/anti-human CD28 mAb (2 µg/mL, BD Pharmingen) and human recombinant IL-2 (100 U/mL, Peprotech, Rocky Hill, NJ) in the presence or absence of 100 nM dexamethasone (Dex, Sigma-Aldrich, St. Louis, MO). Cells were cultured for 8 days at 37°C and 5% CO₂ in 12-well culture plates (Becton Dickinson, Franklin Lakes, NJ) at 1×10⁶ cells/mL per well. Medium containing IL-2, with or without Dex, was changed every other day and analysis of BLT1 surface expression was carried out at day 0 and day 8 of culture, the day of peak BLT1 expression (16). At all time points, cell viability remained >90% as assessed by trypan blue dye exclusion.

Flow cytometry

For staining of BLT1, cells were blocked with 10% human IgG for 15 min at 4°C. FITC conjugated anti-hLTB4 receptor (BLT1) mAb (AbD Serotec, Raleigh, NC) or isotype control was added and incubated for 30 min at 4°C and washed three times. BLT1 surface receptor staining was analyzed by flow cytometry using FlowJo software (Tree Star, Inc., Ashland, OR). Allophycocyanin-conjugated anti-CD3, PerCP-conjugated CD4, PE-conjugated, and PerCP Cy5.5-conjugated CD8 were used for staining of CD4+ and CD8+ T cells.

Intracellular Ca²⁺ monitoring

Activation of CD8⁺ T cells following ligation of BLT1 by LTB4 was monitored by changes in intracellular Ca²⁺ concentrations. Isolated CD8⁺ T cells (5×10^6 cells/mL) were incubated for 45 min at 37°C with 5 μM indo-1 acetoxymethyl ester (Invitrogen, Carlsbad, CA). Cells were resuspended and subjected to analysis by flow cytometry (LSR II; Becton Dickinson, Franklin Lakes, NJ) with FlowJo software (16). After a baseline was established, cells were stimulated with LTB4 (100 nM, 1000 nM, Sigma-Aldrich) and intracellular Ca²⁺ concentrations were determined by measurement of the median fluorescence ratio of indo-1 acetoxymethyl ester at 390 nm/490 nm emission.

Cytokine levels in cell culture supernatant fluid

Equal numbers of isolated CD4⁺ and CD8⁺ T cells were stimulated in culture with anti-CD3/anti-CD28 in the presence of IL-2 for 48 hrs. Culture supernates were harvested for analysis of cytokine levels (IL-4, IL-5, IL-10, IL-13, and IFN-γ) by ELISA (R&D systems, Minneapolis, MN). The detection limits were 8.0 pg/mL for IFN-γ, 10 pg/mL for IL-4, 3.0 pg/mL for IL-5, 3.9 pg/mL for IL-10, and 32 pg/mL for IL-13.

Statistical analysis

Patient clinical variables were analyzed using descriptive statistics, including proportions or means where appropriate. Data were expressed as means (\pm SEM or SD). The paired *t* test was used to compare functional responses in the presence or absence of Dex from the same donors. Between group (control, SS, and SR asthma) comparisons were performed using one-way ANOVA. *P* values of 0.05 or less were considered significant. Analysis for statistically significant differences for ELISA data was performed using the unpaired 2-tailed *t* test and the nonparametric Mann-Whitney test, respectively. Graph Pad Prism, version 4.01 (San Diego, CA) was used for all statistical calculations.

Results

Demographics of enrolled subjects

A total of 19 subjects, including 9 asthmatics and 10 healthy controls, were enrolled (Table 1). Patients in the asthma group fulfilled the American Thoracic Society definition of asthma (>12% bronchodilator response) and were atopic as demonstrated by multiple positive immediate skin prick responses to allergens. Serum IgE levels were highest and lung function the lowest in SR asthmatics when compared to controls ($P < 0.05$). The percentage of total lymphocytes in BAL fluid expressing both CD8 and BLT1 and those expressing CD8, BLT1, and IL-13 were higher in asthmatics than in controls with the highest percentages in SR asthmatics (Table 1).

BLT1 expression on isolated CD4⁺ and CD8⁺ T cells

BLT1 expression on isolated, live CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells was analyzed at baseline and in the presence or absence of Dex in controls, SS and SR asthmatics. When CD4⁺ T cells from each group were compared, the percentages of BLT1-positive cells at baseline was low and there was only a modest increase following activation of the CD4⁺ T cells with anti-CD3/anti-CD28 (Fig. 1). Addition of Dex to the cultures had little effect on the percentages of BLT1 in CD4⁺ T cells. ($P > 0.05$).

In contrast, when isolated CD8⁺ T cells were examined, the following was shown: in all groups, activation with anti-CD3/anti-CD28 resulted in an increase in the percent of CD8⁺ T cells expressing BLT1. The increases were higher in isolated CD8⁺ T cells than in isolated CD4⁺ T cells after stimulation (Fig. 2). The increases in BLT1-staining CD8⁺ T cells was

higher in asthmatics than in controls ($P<0.05$) and these increases were higher in CD8+ T cells from SR than in CD8+ T cells from SS asthmatics ($P<0.05$). In SS asthmatics (and controls), the percentages of BLT1-expressing CD8+ T cells on day 8 was lower when cells were cultured in the presence of corticosteroid unlike SR CD8+ T cells, where numbers were maintained ($P<0.05$). Individual responses in CD4+ and CD8+ T cells from controls and SS and SR asthmatics are shown in Supplemental Figure 1.

Ca²⁺ influx in isolated CD8+ T cells

To determine the functional activity of the upregulated expression of BLT1 on the activated CD8+ T cells, we determined the effect of adding LTB₄ to CD8+ T cells and monitored intracellular Ca²⁺ concentrations. When activated CD8+ T cells with upregulated BLT1 expression were exposed to two concentrations of LTB₄, intracellular Ca²⁺ concentrations increased in a dose-dependent manner (Fig. 3). Activated CD8+ T cells expressing BLT1 from all groups showed similar increases in intracellular Ca²⁺ concentrations (data not shown).

Sensitivity to corticosteroids

To further delineate the differences in CD4+ and CD8+ T cells in SS and SR asthmatics, isolated subsets were stimulated and expanded with anti-CD3/anti-CD28 and IL-2 in the presence or absence of Dex. At the end of the culture period (day 8), cell counts were performed to determine the extent of expansion. When CD4+ T cells were stimulated in the presence of Dex, cell numbers on day 8 were significantly lower than in cultures not containing Dex. In cultures of CD8+ T cells, the attenuation of anti-CD3/anti-CD28 and IL-2-induced expansion was lowest (Table 2) and the expansion of CD8+ T cells in the SR group was reduced to the lowest extent.

Cytokine levels in cell culture supernatant fluid

We previously showed that in both mice and in asthmatics, CD8+ T cells were a source of the pro-allergic cytokine IL-13 (15,18). We compared cytokine production in CD4+ and CD8+ T cells after culture for 48 hrs in the presence of anti-CD3/anti-CD28 and IL-2. Levels of IL-13 were higher in supernates from cultured CD8+ T cells than from cultures of CD4+ T cells, whereas IL-10 levels were higher in CD4+ T cells from controls and SS asthmatics than from cultures of CD4+ T cells from SR asthmatics ($P<0.05$) (Fig. 4). In cultures of both CD4+ and CD8+ T cells, levels of IFN- γ were lowest from cells of SR asthmatics ($P<0.05$). IL-4 levels were at the level of detection and did not differ among the groups, nor did levels of IL-5 (data not shown).

Discussion

The contributions of CD4+ T helper cells in the development of allergic airway inflammation are well established (1). A growing body of evidence suggests that CD8+ T cells may also have the capacity to trigger allergic inflammatory processes in the lungs that contribute to clinically relevant immunopathological processes (1,5,18,20,21). A cross-sectional relationship between CD8+ T cells and asthma outcomes has been observed in fatal asthma (21). Studies examining a potential role for CD8+ T cells in asthma have noted a positive correlation between CD8+ T cell numbers, disease severity and the decline in lung function (5,18). We compared the phenotype and activation outcomes of CD4+ and CD8+ T cells in two subsets of asthmatics defined as SS or SR. The percentages of cells which expressed BLT1, the high affinity receptor for the lipid mediator LTB₄, was higher in asthmatics than in non-asthmatics and in SR asthmatics, the highest percentages of BLT1-expressing cells were detected among activated CD8+ T cells. This increase in BLT1-expressing cells in SR asthmatic CD8+ T cells was maintained in the presence of

corticosteroid and was functional as determined by the intracellular Ca^{2+} response to ligation of the receptor by LTB₄. CD8⁺ T cells produced the highest levels of the cytokine IL-13, important in asthma and mucus production. SR asthmatic CD4⁺ T cells produced the lowest levels of the anti-inflammatory cytokine IL-10 and IFN- γ levels were the lowest in cultures of SR asthmatic CD4⁺ and CD8⁺ T cells. Importantly, CD8⁺ T cell expansion, especially in SR asthmatics, was relatively resistant to corticosteroid compared to CD4⁺ T cells.

Although LTB₄ was initially described as a chemoattractant for myeloid leukocytes (23), it was shown that LTB₄ could bind to a small proportion of human peripheral blood T cells (24). Consistent with these findings, LTB₄ was shown to induce chemotaxis of human peripheral blood lymphocytes (25). Elevated levels of LTB₄ have been reported in various allergic diseases and these levels have been related to disease activity and response to treatment. LTB₄ levels were increased in the sputum, plasma, and bronchoalveolar lavage (BAL) fluid of asthmatic patients but not of healthy subjects (7,26). Many studies have shown that LTB₄, although perhaps not a primary mediator of allergic disease, may be important in specific conditions such as severe persistent asthma (26). Both BAL LTB₄ levels (8) and bronchial wall CD8⁺ T cell numbers have correlated with disease severity (5), supporting a potential link between these previously assumed independent findings.

Corticosteroids effectively suppress inflammatory responses through repression of many immune genes and are therefore widely used for the treatment of various allergic diseases. Th2 cytokine-producing CD4⁺ T cells and eosinophils play important roles in the pathogenesis of allergic diseases, and numbers of these cells, but not CD8⁺ T cells, were dramatically decreased in peripheral blood and inflamed bronchial tissues of patients with asthma after initiation of corticosteroid treatment (27). Although considered to be immunosuppressive and anti-inflammatory, several lines of investigation have now demonstrated that corticosteroids may increase immune/inflammatory responses and even enhance adaptive immune responses (28). As confirmed here, human (29) CD8⁺ T cells were shown to be relatively steroid-resistant compared to CD4⁺ T cells, similar to findings in mice (16). It has been well-established that a proportion of asthmatic patients suffer a decline in lung function despite high doses of inhaled or oral corticosteroid treatment and have been labeled as steroid-insensitive or steroid-refractory (30). Since the pathway leading to the generation of lipid mediators is activated in many inflammatory diseases, but is resistant to corticosteroid treatment (8), the LTB₄-BLT1 axis in steroid-insensitive CD8⁺ T cells may be a major factor in steroid-refractory asthma.

In the present study, we confirmed this notion in a small group of SS and SR asthmatics and demonstrated, despite small numbers of patients, significant differences between CD4⁺ and CD8⁺ T cells. Activation with anti-CD3/anti-CD28 resulted in the expansion of BLT1-expressing CD8⁺ T cells in all groups, in contrast to CD4⁺ T cells. The numbers of BLT1+ CD8⁺ T cells were higher in asthmatics than in the controls and the increases were higher in CD8⁺ T cells from SR than from SS asthmatics. Expansion of activated CD4⁺ T cells with anti-CD3/anti-CD28 and IL-2 was significantly reduced on day 8 in the presence of Dex, whereas the effects of Dex on expansion of activated CD8⁺ T cells was lower and the smallest effects were seen in the CD8⁺ T cells from SR compared to SS asthmatics.

Although much of the previous work in asthma has focused on the role of CD4⁺ Th2 cells and their capacity for pro-allergic cytokine production, including IL-13, we previously showed that both mouse (15) and asthmatic (18) CD8⁺ T cells were a potent source of IL-13. When cytokine levels in cultures of CD4⁺ and CD8⁺ T cells activated with anti-CD3/anti-CD28 were assessed, levels of IL-13 were higher in CD8⁺ T cells than in CD4⁺ T cells. In contrast, levels of the anti-inflammatory cytokine, IL-10, were higher in CD4⁺ T cells

from controls and SS asthmatics compared to CD4+ T cells from SR asthmatics. This inverse relationship between IL-13 and IL-10, increased IL-13 and decreased IL-10 levels in BAL fluid, has correlated with disease severity (31,32). Corticosteroids may exert some of their beneficial effects through the induction of IL-10 production (31,33). Levels of IFN- γ , which may downregulate Th2 cytokine production and asthmatic responses, were also shown to be lowest in both CD4+ and CD8+ T cells from SR asthmatics.

These studies extend previous findings implicating an important role for CD8+ T cells in a subset of asthmatics. The data presented identify impressive differences between CD4+ and CD8+ T cells and in SS and SR asthmatics. In asthmatics, numbers of BLT1+ CD8+ T cells were higher and demonstrated relative corticosteroid insensitivity and capacity for IL-13 production. These differences appeared enhanced in CD8+ T cells from SR asthmatics. Severe asthma has been defined as abnormal lung function despite treatment with high doses of corticosteroids (34). Because of differences in baseline lung function in the SR asthmatics in addition to differences in steroid sensitivity, it is difficult to distinguish at the present time if the differences in CD8+ T cell responses are related in part to disease severity as well as steroid responsiveness. Nonetheless, the findings identify the need for development of newer therapeutic approaches that target these pathogenic CD8+ T cells if we are to alter the course of severe asthma, especially when corticosteroids do not achieve asthma control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Eun Hee Chung is currently affiliated with the Asthma Atopy Allergy Center, National Medical Center, Seoul, South Korea.

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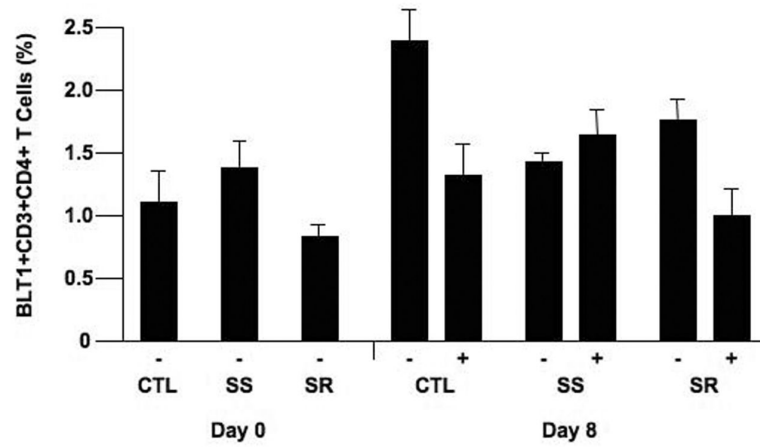


Figure 1. BLT1 expression on stimulated CD4+ T cells. Isolated CD4+ T cells were stimulated with anti-CD3/anti-CD28 for 8 days and BLT1 expression was assessed by flow cytometry. There were no significant differences between any of the groups.

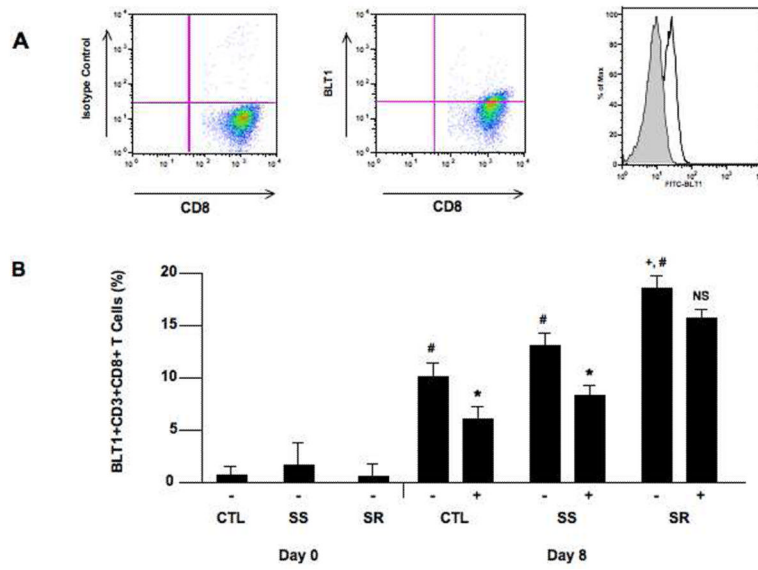


Figure 2.

BLT1 expression on stimulated CD8+ T cells. Isolated CD8+ T cells were stimulated with anti-CD3/anti-CD28 for 8 days and BLT1 expression was assessed by flow cytometry. A) Representative plots are illustrated. Representative data showing isotype control staining (left panel) and BLT1 expression (center panel). The right panel shows the flow analysis of BLT1 staining and the discrimination between the FITC-isotype control and FITC-BLT1 monoclonal antibody staining. B) BLT1 expression on unstimulated and stimulated CD8+ T cells in the presence or absence of dexamethasone (Dex). #P<0.05 comparing unstimulated to stimulated group. *P<0.05 comparing steroid treated to non-treated group. +P<0.05 comparing SR to CTL and SS groups. -: no Dex, +: includes Dex.

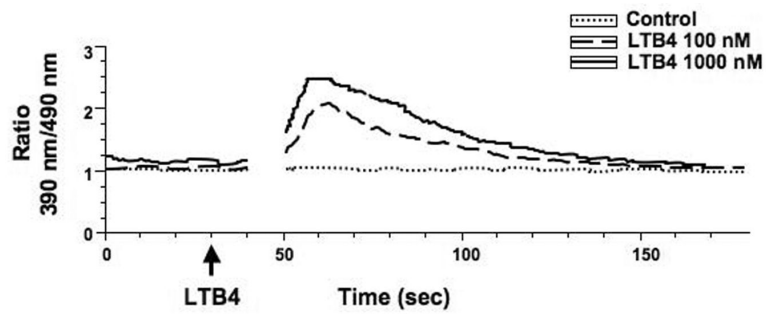


Figure 3. Addition of LTB4 to stimulated CD8+ T cells results in increased intracellular Ca^{2+} concentrations. Isolated CD8+ T cells stimulated with anti-CD3/anti-CD28 for 8 days were incubated with LTB4 (100 or 1,000 nM) and intracellular Ca^{2+} concentrations were monitored comparing the median fluorescence ratio of indo-1 to indo-2 as described in Methods.

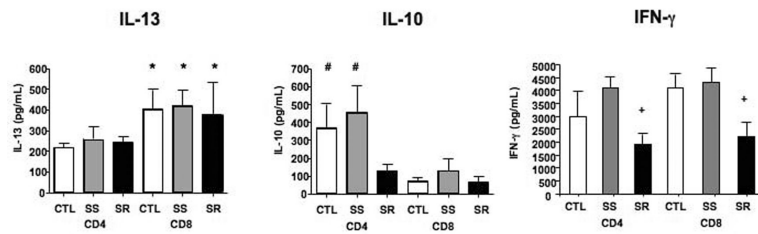


Figure 4.

Cytokine production from cultures of CD4+ and CD8+ T cells. Isolated CD4+ and CD8+ T cells from CTL, SS, and SR patients were stimulated with anti-CD3/anti-CD28 for 48 hours and culture supernates were harvested and assayed by ELISA. * $P < 0.05$ compared to CD4+ T cells. # $P < 0.05$ compared to SR CD4+ T cells and CD8+ T cells. + $P < 0.05$ comparing SR cells to CTL or SS cells.

Table 1

Patient Demographics and Pulmonary Function

Group Parameter	Control (CTL)	Steroid-Sensitive (SS)	Steroid-Resistant (SR)	P value
No. of subjects	10	5	4	
Age (yrs) (mean±SD)	32.7±11.0	30.6±6.5	35.5±9.9	
Sex (M/F)	5:5	3:2	2:2	
Race	Cauc (8), Asian (2)	Cauc (4), Hisp(1)	Cauc (2), Hisp(1), AFAM(1)	
Average positive skin prick tests (range)	0	5.6+ (4+~9+)	5.0+ (1+~10+)	
Serum total IgE (IU/mL) (range)	76 (2~377)	177 (86~409)	333 (56~589)*	<0.05*
Prebronchodilator FEV1 % predicted (mean±SD)	95.1±10.2	75.8±8.2*	65.8±8.2*	<0.05*
Postbronchodilator	104.4±10.6	89.8±9.9	79.5±13.0	<0.05*
FVC % predicted (mean±SD)	99.2±3.9	90.8±10.1	88.5±19.8	NS
Postbronchodilator	99.2±5.9	97.4±12.5	97.8±26.1	NS
FEV1/FVC	0.82±0.03	0.70±0.05	0.63±0.06	<0.05*
Prebronchodilator				
Postbronchodilator	0.86±0.05	0.78±0.08	0.69±0.08	<0.05*
% Lymphocytes in BAL expressing:				
BLT1 ⁺ CD8 ⁺	13.97±1.6	25.32±3.4	34.2±5.1	<0.05*
BLT1 ⁺ CD8 ⁺ IL-13 ⁺	4.78±1.2	13.4±3.7	16.5±2.3	<0.05*

Cauc: Caucasian, Hisp: Hispanic, AFAM: African American.

* Compared to controls.

Table 2

Expansion of CD4+ and CD8+ T Cells and the Effect of DEX Treatment

Group	CD4 ⁺ T cells		CD8 ⁺ T cells	
	Total cell count (x10 ⁶) (mean ±SD)	% Reduction of Cell Expansion	Total cell count (x10 ⁶) (mean ±SD)	% Reduction of Cell Expansion
CTL Dex(-)	24.4±11.7		31.7±14.8	
CTL Dex(+)	8.5±8.6 [*]	65%	16.0±8.2 [*]	50%
SS Dex(-)	38.4±22.1		44.1±26.0	
SS Dex(+)	7.8±4.4 [*]	80%	25.5±15.5 [*]	60%
SR Dex(-)	16.3±9.0		21.4±5.5	
SR Dex(+)	3.3±1.9 [*]	80%	17.6±5.3 ^{NS}	19% [#]

NS: Not significant.

^{*} P<0.05 compared to non-DEX-treated group.[#] P<0.05 compared to CTL and SS groups.