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Original Article

Effects of leukotriene D4 on adenoidal T cells in children with obstructive sleep apnea syndrome

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Abstract: Purpose: This study aims to determine whether leukotriene D4 (LTD4) can promote T cell proliferation in adenoid tissues *via* activation of CysLT1 receptors in children with OSAS. Methods: $CD4^+$ and $CD8^+$ T cell proliferation was assessed by flow cytometry in adenoid mononuclear cells (AdMCs) stimulated with LTD4 from children with OSAS. The activation of mitogen-activated protein kinase pathways and their effects on the proliferation of $CD4^+$ and $CD8^+$ T cells in AdMCs were observed by western blotting. Results: LTD4 increased the proliferation rates of both phytohemagglutinin (PHA)-stimulated $CD4^+$ T cells (15.5±8.4% in the PHA group *vs.* 24.8±6.3% in the PHA+LTD4 group; n=27; P<0.001) and $CD8^+$ T cells (17.2±5.9% in the PHA group *vs.* 23.5±5.2% in the PHA+LTD4 group; n=27; P<0.05) in AdMCs. LTD4 (10^{-4} mmol) significantly increased the phosphorylation of extracellular signal-regulated kinase (ERK1/2) and p38, but not c-Jun N-terminal kinases (JNK). The ERK1/2 inhibitor PD98059 significantly inhibited the proliferation of $CD4^+$ and $CD8^+$ T cells in LTD4-stimulated AdMCs. Conclusion: LTD4 regulates the proliferation of $CD4^+$ and $CD8^+$ T cells in PHA-stimulated AdMCs *via* upregulation of the ERK1/2 pathway. This finding indicates that CysLT1 receptors play a regulatory role in the pathogenesis of OSAS in children.

Keywords: LTD4, MAPKs, proliferation, adenoid, OSAS

Introduction

Obstructive sleep apnea syndrome (OSAS) is a common pediatric otolaryngologic disease that occurs in children of all ages, particularly in 2-8 year-old children [1-4]. OSAS in children is a breathing disorder during sleep characterized by prolonged partial upper airway obstruction and/or intermittent complete obstruction (obstructive apnea) that disrupts normal ventilation during sleep and normal sleep patterns [5]. OSAS seriously affects a child's health if left untreated.

The etiology of OSAS in children is complex. Several studies have suggested that a combination of structural and neuromuscular abnormalities needs to be present for OSAS to occur [6]. The most common form of pharyngeal narrowing in children is associated with hypertrophy of the tonsils and adenoids. However, the process underlying this benign hyperplasia and hypertrophy of lymphatic tissues remains unknown. Epidemiological studies have suggested that some inflammatory factors may be involved [7]. Therefore, some investigators [8] have suggested that the pathogenesis of OSA in children is not only due to an imbalance in the mechanical strength, but also due to the participation of inflammatory factors.

Cysteinyl leukotrienes (CysLTs) are a family of potent inflammatory lipid mediators synthesized from the arachidonic acid metabolism/lipoxygenase pathway. The family includes leukotriene C4 (LTC4), leukotriene D4 (LTD4) and leukotriene E4 (LTE4); and all of which are potent biological mediators associated with the pathophysiology of inflammatory diseases [9]. Recent studies have found that CysLTs are associated with systemic and local inflammation in children with OSAS [10]. Kaditis *et al.* [11] suggested that LT1-R within the tonsillar extrafollicular areas is expressed by T lymphocytes, which are the dominant inflammatory cells present in the pharyngeal mucosa of adults with obstructive sleep apnea.

In a study reported by Dayyat *et al.* [12], LT pathways mediated intrinsic proliferative and inflammatory signaling pathways in adenotonsillar tissues from children with OSA. Nevertheless, the effects of LT pathways on T lymphocytes in adenoids have not been consistently documented by other studies. In order to clarify the role of these pathways, we determined whether LTD4 could mediate T cell proliferation in adenoids *via* activation of the CysLT1 receptor in children with OSAS.

Materials and methods

Subjects

The study was approved by the Human Research Committee of Chongqing Medical University-Affiliated Children's Hospital, and informed consent was obtained from parents/guardians before the children participated into the study. Furthermore, child's consent was also obtained from patients >7 years old. Consecutive children undergoing adenoidectomy for OSAS were identified prior to surgery, and were recruited for the study. The diagnosis of OSAS was established by overnight polysomnography in a sleep laboratory, which required an apnea-hypopnea index ≥5 of total sleep time [13]. Exclusion criteria were cardiovascular, neuromuscular, or genetic disorders; acute or chronic inflammatory disorders; respiratory tract infections (e.g. bronchiolitis); or asthma, allergic rhinitis, history of allergies, and/or having received corticosteroid or LT-modifier therapy within one year prior to surgery.

Adenoid mononuclear cell preparation

Surgically removed adenoids from children with OSAS were immediately placed in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) plus antibiotics, and were sent to our laboratory within 10 minutes after excision. The tissues were thoroughly washed with phosphate-buffered saline (PBS) under aseptic conditions. The central parts of the specimens were cut into small pieces and gently homogenized with a syringe plunger through a 70-μm mesh screen to obtain a mixed cell suspension.

Adenoid mononuclear cells (AdMCs) were isolated from the mixed cell suspension by density gradient centrifugation over Lymphoprep® (Nycomed Pharma, Oslo, Norway). Then, cells were suspended in $4×10^6$/ml in RPMI 1640 medium containing 5% fetal calf serum (FCS) and 2 mM of L-glutamine. Cells with at least 95% viability were used for further analyses.

Flow cytometry specimen preparation and cell proliferation assay

AdMCs were thoroughly washed with PBS plus antibiotics, and were suspended at a cell density of $5×10^6$ cell/ml in the aforementioned RPMI 1640 medium. Then, cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) in an incubator with 5% CO_2 at 37°C for 10 minutes. The reaction was terminated with precooled standard FBS. The specimens were washed with RPMI 1640 twice and transferred into 96-well round bottom plates at a concentration of $5×10^6$ viable cells/well with a final volume of 200 μl in RPMI 1640. Then, cells were incubated either alone or stimulated with 10 μg/ml of 6-phosphonohexanoic acid (AP6, one type of PHA, Sigma-Aldrich, USA) or PHA plus $1×10^{-4}$ mmol/l of LTD4 (purchased from Cayman Pharma, Neratovice, Czech Republic) for 72 hours before their proliferation was examined. Proliferation rates of the T cell subsets were measured by flow cytometry. The following monoclonal antibodies (BD Biosciences/BD Pharmingen, San José, CA) were used for the surface marker analysis of AdMCs: anti-CD3-PERCP-CY5.5, anti-CD4-PE-Cy7, and anti-CD8-APC. Data were analyzed using by flow cytometry (BD FACSDiva Software).

Immunohistochemistry

Double indirect immunofluorescence studies were performed in adenoid tissues. Adenoid tissues were fixed in 10% buffered formalin and embedded in paraffin. Specimens were cut into 40-μm slices using a microtome. After deparaffinization and hydration, antigen retrieval was performed by heating the samples in 0.01 mol/l of citrate buffer solution (pH=6) for 20 minutes in a microwave oven. After antigen retrieval, the sections were incubated in 0.3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase, and blocked with 5% normal goat serum. Subsequently, the sections were incubated overnight at 4°C with anti-LT1-R antibody (1:150 dilution; Cayman Chemicals, USA) and fluorescent anti-rabbit

Table 1. Demographic and Polysomnographic Characteristics in 27 Children With OSA Undergoing adenoidectomy ($\overline{x}\pm s$)

Characteristics	OSA (n=27)
Age, yr (range)	6.1±0.7
Gender, % female	38.2
BMI (z score)	0.75±1.13
AHI (/h TST)	12.5±1.2
Nadir Sao_2, %	85±1.9
Arousal index (/h TST)	18.9±2.7

AHI: Apnea-Hypopnea Index; BMI: body mass index; Sao_2: arterial oxygen saturation; TST: total sleep time.

secondary antibodies (1:400 dilution; Invitrogen Corporation, Carlsbad, CA) at 37°C for 45 minutes. After washing with PBS, the sections were incubated with anti-CD3 antibody (1:40 dilution; Abcam, USA) at 4°C for 24 hours and fluorescent anti-mouse secondary antibodies (1:400 dilution; Invitrogen Corporation, Carlsbad, CA) at 37°C for 45 minutes. The sections were thoroughly washed with PBS after each step. Negative controls were prepared by omitting either the primary or secondary antibodies for both receptors. A confocal laser scanning microscope and a digital camera were used to detect immunoreactivity.

Western blotting

After treatment with LTD4 and antagonists, AdMCs were collected and lysed in a Cell and Tissue Protein Extraction Solution (Kangchen Biotechnology, Shanghai, China). Then, protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce Chemicals, Rockford, IL). Samples (40 μg of protein) were solubilized in sample buffer by boiling for 10 minutes, fractionated on 12% sodium dodecyl sulfate-polyacrylamide gels using electrophoresis (Invitrogen), and transferred onto nitrocellulose membranes. After that, the membranes were washed with Tris-buffered saline (TBS) supplemented with 0.1% Tween 20 and blocked with 5% nonfat dry milk in TBS solution. The primary antibodies used included polyclonal antibodies against extracellular signal regulated kinase (ERK) 1/2, p-ERK1/2, c-Jun N-terminal kinases (JNK), p-JNK (1:1,000 dilution; Cell Signaling Technology, Beverly, MA, USA), p38 and p-p38 (1:1,000 dilution; Abcam, USA); and were applied overnight at 4°C. After washing three times with TBS solution supplemented with 0.05% Tween 20 (TBST), the membranes were incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for one hour with gentle shaking. Intensities of the protein bands were quantified using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Sunnyvale, CA).

Results

Study population

A total of 27 children undergoing adenoidectomy for OSAS were recruited for this study. The diagnosis of OSAS required an apnea-hypopnea index ≥5, as detected by overnight polysomnography. Demographic characteristics and major overnight polysomnographic findings for the 27 participants are presented in **Table 1**.

Adenoid immunohistochemistry

The present study reproduced the findings of Kaditis and Dayyat, who determined cell types that harbored cysLT1 receptors in a population with different genetic backgrounds and environmental influences. Therefore, fluorescent double-labeling was conducted to assess five adenoid tissue specimens in this study, and whether the cysLT1 receptors were expressed in $CD3^+$ T cells was determined by confocal laser scanning microscopy. LT1-R was observed to be expressed by $CD3^+$ lymphocytes (**Figure 1**).

Cell proliferation assay

In order to assess the effects of LTD4 on the proliferation of $CD4^+$ and $CD8^+$ T cells in AdMCs, AdMCs were stimulated with PHA and/or LTD4 for 72 hours. Significant differences were noted between the two groups. LTD4 increased the proliferation rates of both $CD4^+$ T cells (15.5±8.4% in the PHA group *vs.* 24.8±6.3% in the PHA+LTD4 group, n=27, P<0.001) and $CD8^+$ T cells (17.2±5.9% in the PHA group *vs.* 23.5±5.2% in the PHA+LTD4 group, n=27, P<0.05) in the AdMC culture system. However, no concentration-dependent effect of LTD4 was observed. The addition of a leukotriene receptor antagonist, montelukast (25 nmol/l), to the AdMC culture system elicited reductions in cell proliferation (**Figures 2** and **3**).

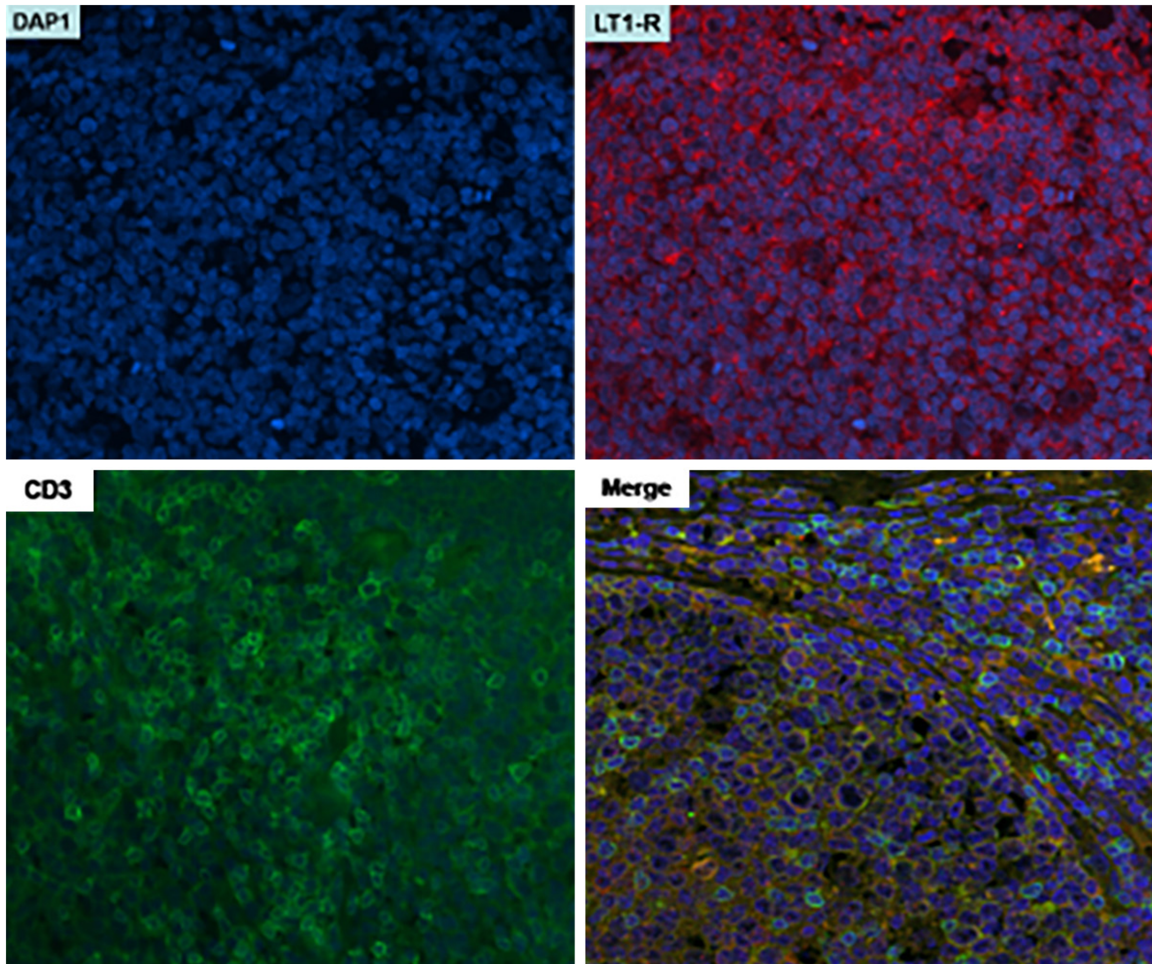

Figure 1. Top left: Fluorescent DAPI staining of one adenoid of a patient who had adenoidectomy for OSAS. Top right and bottom left (same tissue section): Immunoreactivity for LT1-R (red color) and CD3 (green color) is abundant. Merging the three pictures (bottom right) reveals that LT1-R is expressed by a subset of $CD3^+$ adenoid lymphocytes (orange/yellow color).

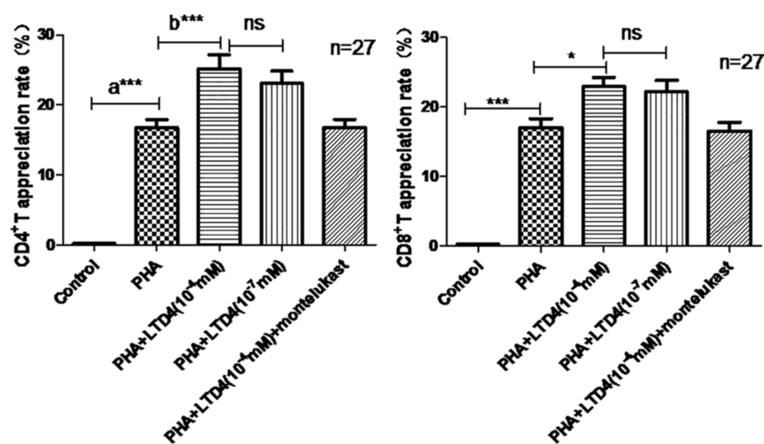

Figure 2. Effect of LTD4 on proliferation of $CD4^+$ T and $CD8^+$ T cell in PHA-stimulated AdMC (a***P<0.001 vs. Control group; b***P<0.001 vs. PHA+LTD4 (10^{-4} mM) group; ***P<0.001 vs. Control group; *P<0.05 vs. PHA+LTD4 (10^{-4} mM) group.

ERK1/2 activation and effects

In order to study the involvement of mitogen-activated protein kinase (MAPK) pathways in the proliferation of AdMCs, MAPK activation was observed in LTD4-stimulated AdMCs. LTD4 (10^{-4} mmol for 15 minutes, 30 minutes, one hour, or 1.5 hours) significantly increased the phosphorylation of ERK1/2, with its peak level observed at one hour after exposure (**Figure 4A**). Increased EKR1/2 phosphorylation was blocked by treat-

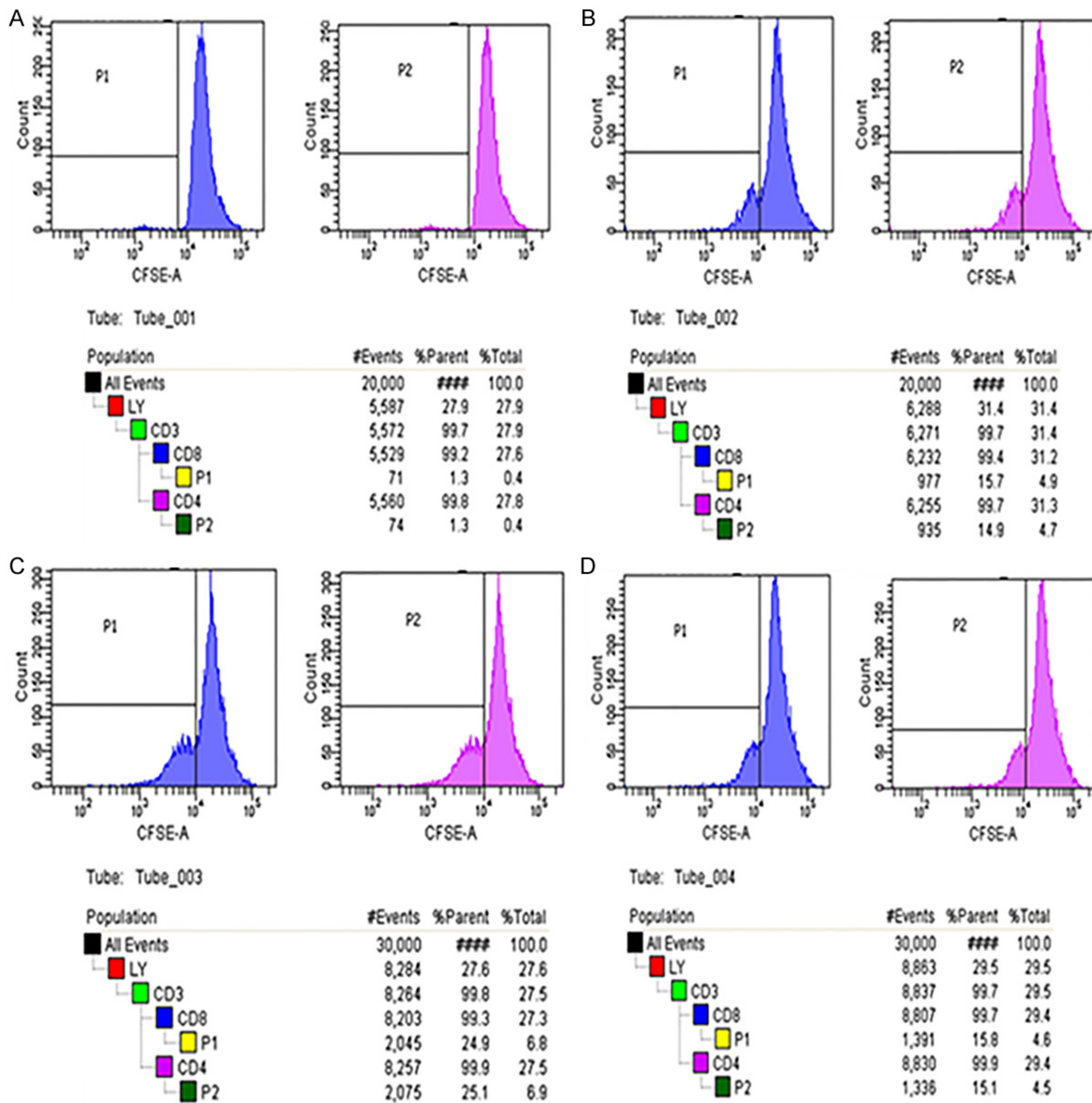

Figure 3. Promotion of LTD4 on proliferation of CD4+ T and CD8+ T cell in PHA-stimulated AdMC (CFSE labeling) (A: Control group; B: PHA group; C: PAH+LTD4 group; D: PAH+LTD4+ montelukast group. Blue: CD8+ T. Red: CD4+ T). The proliferation rates of CD4+ T cells is 14.9% and of CD8+ T cells is 15.7% in PHA group. The proliferation rates of CD4+ T cells is 25.1% and of CD8+ T cells is 24.9% in PAH+LTD4 group. The proliferation rates of CD4+ T cells is 15.1% and of CD8+ T cells is 15.8% in PAH+LTD4+ montelukast group.

ment with 25 nM of montelukast or 20 μM of PD98059, an ERK1/2 inhibitor (**Figure 4B**). The ERK1/2 inhibitor significantly inhibited the proliferation of both CD4+ and CD8+ T cells in LTD4-induced AdMCs (**Figure 5**). Moreover, exposure to LTD4 (10^{-4} mmol for five minutes, 15 minutes, 30 minutes, one hour, or two hours) significantly increased the phosphorylation of p38, which reached a peak at one hour after exposure (**Figure 4C**). In addition, increased p38 phosphorylation was blocked by treatment with 25 nM of montelukast or 30 μM of SB20-3580, a p38 inhibitor (**Figure 4D**). However, this p38 inhibitor did not significantly inhibit the proliferation of CD4+ and CD8+ T cells in LTD4-stimulated AdMCs. In addition, LTD4 did not affect the phosphorylation of JNK (not shown).

Discussion

This study clearly demonstrates that CysLT1 receptors are expressed in adenoid tissues. Moreover, we discovered that the LTD4-stimulated proliferation of AdMCs is mediated by

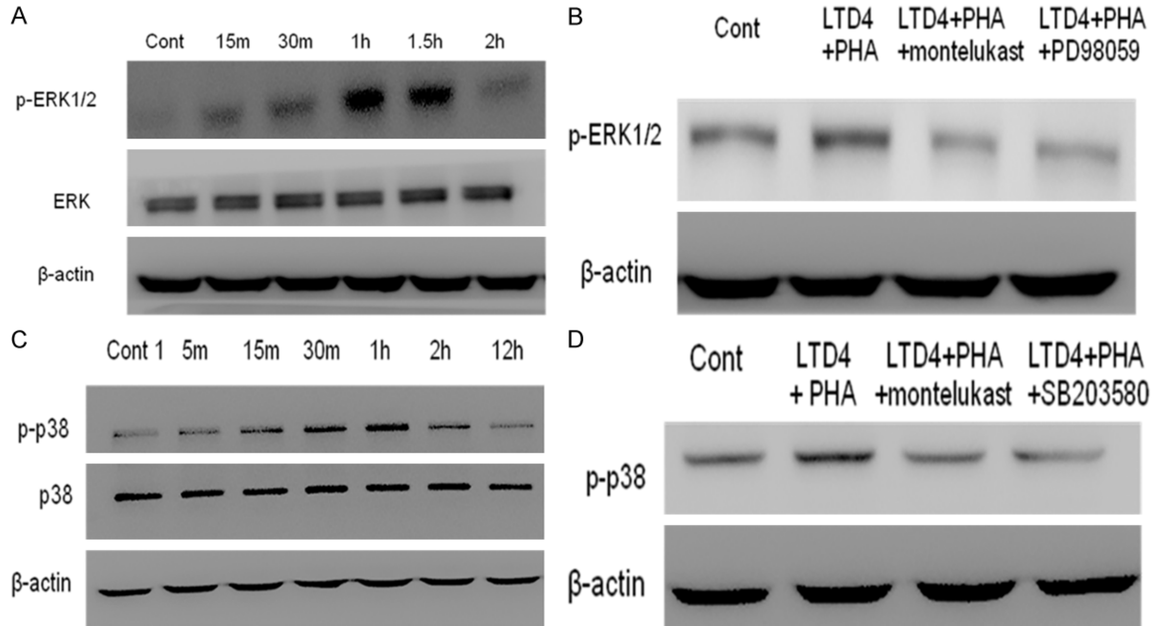

Figure 4. Activation of ERK1/2 and p38 induced by LTD4 in AdMC. A and C: Effect of LTD4 on p-ERK1/2 and p-p38 proteins in AdMC at different time points. *P<0.05, **P<0.01 and ***P<0.001 vs. Control group (n=5). B: LTD4-induced ERK1/2 phosphorylation was blocked by montelukast (Mon, 25 nM) and PD98059 (20 μM). D: LTD4-induced p-p38 phosphorylation was blocked by montelukast (Mon, 25 nM) and SB203580 (30 μM). a***P<0.001 vs. Control group; b***P<0.001 vs. LTD4+PHA+montelukast group, #P<0.001 vs. LTD4+PHA+PD98059 group or LTD4+PHA+SB group, (n=5).

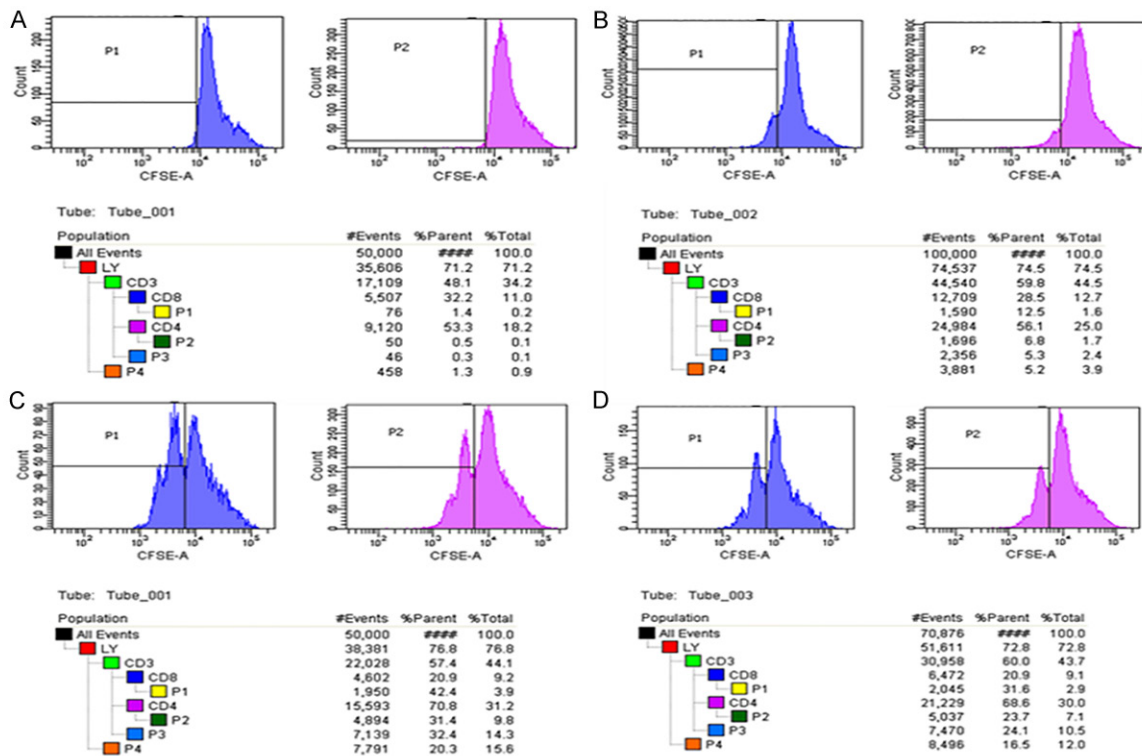

Figure 5. LTD4-stimulated proliferation of $CD4^+$ T and $CD8^+$ T in AdMC was inhibited by the ERK1/2 inhibitor PD98059. (A: Control group; B: PHA group; C: PHA+LTD4 group; D: PHA+LTD4+PD98059 group. Blue: $CD8^+$ T; Red: $CD4^+$ T). a***P<0.001, b***P<0.001, ***P<0.001 vs. control group. d**P<0.01 and c**P<0.01 vs. PHA group. b**P<0.01 and c***P<0.001 vs. PHA+LTD4 group. (n=5). The proliferation rates of $CD4^+$ T cells is 6.8%

and of $CD8^+$ T cells is 12.5% in PHA group. The proliferation rates of $CD4^+$ T cells is 31.4% and of $CD8^+$ T cells is 42.4% in PAH+LTD4 group. The proliferation rates of $CD4^+$ T cells is 23.7% and of $CD8^+$ T cells is 31.6% in PHA+LTD4+PD98059 group.

CysLT1 receptors *via* the ERK1/2 pathway. These findings indicate that CysLT1 receptors play a regulatory role in the pathogenesis of OSAS in children.

CysLTs are a family of inflammatory mediators that participate in a variety of inflammatory processes and allergic reactions. The biological effects of CysLTs are mediated through cysteinyl leukotriene receptors expressed on the target cell membrane. Kaditis and colleagues [11] demonstrated in their study that the expression of LT1-R is higher in tonsillar tissues of children with OSAS than in those with recurrent throat infections (RIs). Furthermore, LT1-R is expressed by $CD3^+$ lymphocytes located mainly in tonsillar extrafollicular areas. Previous evidence has suggested that the enhanced expression of cysteinyl leukotriene receptors is closely related to local inflammation and allergic responses in the upper airway. Another study demonstrated that LT pathways mediate intrinsic proliferative and inflammatory signaling pathways in adenotonsillar tissues from children with OSAS, but the precise cell types proliferating within these adenoids and tonsils were not specifically determined in that study [12]. In the present study, we confirmed that CysLT1 receptors are expressed by adenoid $CD3^+$ T cells. After treatment with PHA and LTD4 for 72 hours, both $CD4^+$ and $CD8^+$ T cells in AdMCs exhibited increased proliferation; which was dependent on the mechanism that involved CysLTs. This indicates that LTD4 regulated the activation of T cell proliferation.

The mechanism by which LTD4 regulates the proliferation of $CD4^+$ and $CD8^+$ T cells in adenoid tissues remains unclear. MAPKs are important signaling molecules that influence a broad range of cellular processes such as proliferation, differentiation, migration and apoptosis [14]. MAPKs belong to a group of serine/threonine kinases [15]. The MAPK family includes extracellular signal-regulated kinase (ERK), p38 kinase and c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs) [16, 17]. ERKs are activated by mitogenic and proliferative stimuli, and regulate proliferation, differentiation and migration; while JNKs and p38 kinase respond to environmental stress [18, 19]. The MAPK signal transduction pathway has been recognized as one of the signaling pathways downstream of CysLTs. LTC4 stimulates airway epithelial cells to produce TGF-β1, resulting in fibroblast proliferation *via* the activation of p38 MAP kinase [20]. Yuan [21] and colleagues reported that LTD4 induces a two-fold increase in ERK1/2 phosphorylation (but there was no change in p38 or JNK phosphorylation). Furthermore, the LTD4-stimulated migration of endothelial cells was mediated by CysLT1 receptors *via* the ERK1/2 pathway. Results from the present study demonstrates that LTD4 induces the increased phosphorylation of ERK1/2 and p38 (but not JNK), which was blocked by CysLT1 receptor antagonists. We also found that the ERK1/2 inhibitor, PD98059, inhibited LTD4-stimulated proliferation. Our findings suggest that ERK1/2 activation may play an important role in the CysLT1 receptor-mediated proliferation of $CD4^+$ and $CD8^+$ T cells in AdMCs. However, the precise signaling mechanisms by which CysLTs receptors lead to MAPK phosphorylation requires further investigation.

We did not observe a role for p38 in the proliferation of $CD4^+$ or $CD8^+$ T cells in AdMCs, although LTD4 induced an increase in p38 phosphorylation. The p38 pathways are primarily activated by cellular stress signals such as pro-inflammatory cytokines, heat shock or UV light; and have therefore been described as stress-activated protein kinases [22]. The p38 pathway is connected to diverse cellular processes such as protein degradation, cytoskeletal dynamics, apoptosis and migration [17]. The above evidence and our results provide greater insight into the role of p38 in the pathogenesis of OSA in children.

Conclusion

We have demonstrated that LTD4 stimulates the proliferation of $CD4^+$ and $CD8^+$ T cells in AdMCs *via* activation of the CysLT1 receptor. Furthermore, we found that the ERK1/2 MAPK pathway contributes to LTD4-induced proliferation. Our findings reveal a role for the CysLT1

receptor in the pathogenesis of OSAS in children.

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Disclosure of conflict of interest

None.

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