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Bronchoalveolar lavage fluid cytokines and chemokines changes after bronchial thermoplasty in severe asthma

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Abstract

Background: Bronchial thermoplasty (BT) is a non-pharmacological intervention in severe asthma with a well-known mechanism of reducing airway smooth muscle. However, its effect on airway inflammation remains uncertain.

Objectives: To investigate the effect of BT on bronchoalveolar lavage fluid (BALF) cytokines and chemokines in severe asthma patients before BT, after the first BT, and 12 weeks after BT

Methods: Ten severe asthma patients were recruited, and BALF was obtained from right lower lobe before BT, after the first BT, and 12 weeks after BT. BALF analytes were measured and values were compared among the time points. Lung function, asthma control test (ACT), and asthma quality of life questionnaire (AQLQ) were also measured.

Results: Tumor necrosis factor (TNF)-α concentration was significantly decreased after the first BT and significantly increased at 12 weeks after BT. Interleukin-6 (IL-6) and TNF-related apoptosis inducing ligand (TRAIL) concentration were significantly increased at 12 weeks after BT. There were no significant changes in Regulated upon activation, normal T-cell expressed and secreted (RANTES) and transforming growth factor-beta1 (TGF-β1) concentration over time after BT. At 12 weeks after BT, there were significantly greater improvements in the scores on AQLQ (3.93 ± 0.88 to 5.3 ± 0.99, p = 0.002) and ACT (13.6 ± 3.27 to 19 ± 4.44, p = 0.002). The lung function did not differ significantly between pre- and post-BT.

Conclusions: BT has limited effect on TNF-α, IL-6, TRAIL, RANTES, and TGF- β1 in BALF suggesting that its clinical benefit is not primarily related to this local airway inflammation. The effect on long-term airway inflammation probably needs further studies.

Key words: Severe asthma, Bronchial thermoplasty, Airway inflammation, Airway smooth muscle cells, Airway remodeling

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Introduction

Asthma is one of the most common airway diseases affecting a population of over 200 million worldwide. The disability-adjusted life year of this disease is estimated to be 22-26 million, which significantly contributes to the global burden of asthma.[1] The mainstay of treatment for asthma in adults is an inhaled corticosteroids (ICS)-containing regimen. However, approximately 20% of asthma patients still have uncontrolled disease despite receiving an ICS-containing regimen. Bronchial thermoplasty (BT) is a novel intervention considered to be a non-biologic treatment for difficult-to-treat or severe asthma patients who have a non-type 2 phenotype or type 2 phenotype that failed biologic treatment.[2] The efficacy of this technique was first demonstrated in dogs, showing a reduction of airway smooth muscle (ASM) and airway hyperresponsiveness to methacholine in radiofrequency-treated airways.[3] Later, BT was proven effective in treating moderate to severe asthma patients based on several non-randomized trials and randomized trials including Asthma Intervention Research (AIR), Research in Severe Asthma (RISA) trial, and AIR2 trial.[4-6]

Despite its well-known effect on reducing ASM in airway remodeling, other potential mechanisms of action involved in extracellular matrix, airway epithelium, neural innervation could also underlie its effectiveness in controlling airway inflammation.[7] Given the complex interaction between ASM and inflammatory mediators, recent study has addressed the ability to release several inflammatory mediators, but little is known about the effects of BT on airway inflammation.[8] To date, only a few studies have investigated the effect of BT on inflammatory mediators and have provided conflicting results.[9,10] In a previous study, only the acute effect on local airway inflammation was examined, and the measured inflammatory mediators should be carefully interpreted because of the potential effect of corticosteroids in BT protocol. Therefore, our study aimed to investigate the early and sustained effects of BT on local airway inflammation in terms of the changes in the concentration of cytokines and chemokines in bronchoalveolar lavage fluid (BALF) over times after BT.

Materials and methods

Patient recruitment and sample collection

Ten subjects who were 18 years or older and had severe uncontrolled asthma, characterized by step 4 and 5 according to the Global Initiative for Asthma (GINA), were recruited for the study. Prior to enrollment, all medications were reviewed and optimized at the chest clinic for three months. Patients were evaluated to rule out any other conditions that can mimic asthma such as eosinophilic granulomatosis with polyangiitis (EGPA) and allergic bronchopulmonary aspergillosis (ABPA). Informed consent, approved by the Faculty of Medicine, Chulalongkorn University Institutional Review Board (IRB number 530/60), was obtained from all patients before enrollment. After enrollment, lung function test, fractional exhaled nitric oxide (FeNO), asthma control symptom using Asthma Control Test (ACT), and quality of life using Asthma Quality of Life Questionnaire (AQLQ) scores were evaluated.

The subjects were evaluated at 12 weeks after the last procedure (at the third session of BT). The following assessments were performed: lung function test, FeNO, ACT, and AQLQ scores, as well as an evaluation of asthma medications.

The lung function test was performed according to the American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines.[11] FeNO levels were measured using NIOX VERO® according to the manufacturer's instructions and the ATS/ERS guideline.[12] The AQLQ is a questionnaire consisting of 32 items that assess asthma-related symptoms and limitations during the 2 weeks preceding administration of the questionnaire. Participants respond on a scale of 1 to 7, with higher numbers indicating a better quality of life. The ACT is a tool used to identify asthma control and consists of 5 items. It assesses the frequency of shortness of breath and general asthma symptoms, use of rescue medications, the effect of asthma on daily functioning, and overall self-assessment of asthma control. The scale ranges from 5 to 25, with a higher ACT score indicates better asthma control.

Bronchial thermoplasty and BALF collection

BT was performed in accordance with current recommendations using Alair Bronchial Thermoplasty System (Boston Scientific, Natick, MA, USA).[13] The system consists of an Alair Bronchial Thermoplasty controller and catheter. Briefly, the three sessions of BT were given at 3-week intervals using flexible bronchoscopy under general anesthesia. The sequence of three sessions was performed at the right lower lobe, left lower lobe, and both upper lobes consecutively. All patients were treated as inpatients for close observation after each session of BT. A 50-milligram of prednisolone was given orally 3 days before, on the day of, and 1 day after each session of BT.

BALF samples were collected at three timepoints during the study. Bronchoalveolar lavage (BAL) was performed with a total volume of 100-150 ml of normal saline solution and the BALF was collected by suction specimen trap. Each BALF sample was collected from the same location, which was the lateral segment of the right lower lobe. The first BALF sample was collected 2-4 weeks prior to the first session of BT, as part of surveillance bronchoscopy to assess airway anatomy and detect any airway abnormalities. The second BALF sample was collected before performing the second session of BT. Twelve weeks after the third session of BT, the patients underwent bronchoscopy to collect the third BALF sample, which was performed as an outpatient. After the procedure, the subjects were routinely observed for adverse events, and if any adverse events were detected, the subjects would be admitted for close observation or treatment.

At the timepoints when the first and third BALF were collected, the investigators interviewed the subjects regarding their use of corticosteroids. If the subject had used corticosteroids within 2 weeks prior to the scheduled bronchoscopy, the procedure would be postponed for an additional 2 weeks after the last dose of corticosteroid.

BALF processing and cytokine/chemokine measurement

Fresh BALF was immediately processed after collection by centrifuging at 1,500 rpm for 10 minutes. The BALF supernatant was then stored at -80°C until use. For cytokine and chemokine detection, 10-12 ml of BALF were concentrated to 300-500 µl at 4°C for 1 hour using Amicon Ultra-15 Centrifugal Filter-3K (Milipore, Ireland). The Bio-Plex Pro Human Cytokine 13-plex Assay kit and TGF-β1 (BioRad) were used to determine cytokines and chemokines via Luminex assay. Fifty microliters of 4-fold diluted samples were used for the assay, according to the manufacturer's instructions. The samples were measured by Bio-Plex 200 Systems (BioRad).

Statistical analyses

Continuous variables were reported as mean (standard deviation [SD]) and median (interquartile range [IQR]). Categorical variables were reported as the number of patients and percentages. Paired *t*-test were used to compare the outcomes at baseline (pre-BT) with those after the first BT and 12 weeks after the third BT. For outcomes that were not normally distributed and measured at various time points, we used Generalized Estimating Equations (GEE), repeated measures, model to estimate the parameters of a generalized linear regression and determine the interaction between the two groups over three timepoints, while adjusting for possible effects of age and sex. Furthermore, we applied a natural log transformation to adjust the non-normally distributed data before conducting the GEE analysis. The GEE model was computed with mean values, standard error (SE), and Wald test for each independent variable. We established the correlation between the mean changes of ACT scores and AQLQ scores with the changes in levels of cytokines and chemokines by calculating Pearson's correlation coefficient. Variables with a *p*-value of less than 0.05 were considered statistically significant. The data were analyzed by using SPSS Statistics for Windows, Version 28.0.

Results

Ten patients with severe uncontrolled asthma were recruited for BT. Of these, five were male with a median age of 56 years (IQR, 45.75-63). The median blood eosinophil level at baseline was 230 cells/mL (IQR, 87.5-365). Blood IgE levels were obtained from seven patients, and their values ranged between 11-977 IU/mL with a median of 187 IU/mL (IQR, 64-282). **Table 1** shows the baseline demographic and clinical characteristics. None of the patients were lost to follow-up.

At baseline, the pre-bronchodilator Forced Expiratory Volume in one second (FEV_1) had a mean value of 74.6 ± 21.59% predicted. After administering 400 µg of salbutamol, there was an improvement in FEV_1 (post-bronchodilator FEV_1) with a mean value of 80.7 ± 21.93%. The mean ratio of residual volume (RV) to total lung capacity (TLC) was 40.78 ± 8.8. The mean FeNO was 30.78 ± 21.93 for the 9 patients for whom the value was available.

Table 1. Patient demographics.

Characteristics	Variables
Number of patients (n)	10
Age	56 (46.75-63)
Sex (Male/Female) (n)	5/5
Medication use, % (n)	
ICS /LABA	10 (100%)
LAMA	8 (80%)
OCS	4 (40%)
Leukotriene receptor antagonist	9 (90%)
Omalizumab	1 (10%)
Blood eosinophil count, cells/µL	230 (87.5-365)
Serum IgE level, IU/mL†	187 (64-282)

Definition of abbreviations: ICS = inhaled corticosteroids; LABA = long-acting b2-agonist; LAMA = long-acting muscarinic antagonist; OCS = oral corticosteroids. Data are presented as median (interquartile range) unless otherwise noted.
†data were available in 7 patients.

After 12 weeks following the third session of BT, there were no significant changes in the values of pre-bronchodilator FEV_1, post-bronchodilator FEV_1, RV/TLC, and FeNO. However, there was a significant improvement in the mean AQLQ (integrated score) from 3.93 ± 0.88 to 5.3 ± 0.99 (*p*-value = 0.002) and mean ACT increased from 13.6 ± 3.27 to 19 ± 4.44 (*p*-value = 0.004), indicating an improvement in asthma-related quality of life and asthma control, respectively. The mean dose of inhaled corticosteroids (budesonide propionate or equivalent) and long-acting beta 2 agonist (formoterol or equivalent) used before the procedure was 1180 ± 502.88 mg and 25.8 ± 8.96 mg, respectively, and these doses did not significantly change at 12 weeks after the third BT. **Table 2** summarizes the values before and after BT.

At the first and third timepoints, none of the patients had a recent history of oral corticosteroids use within 2 weeks prior to BALF collection. The BALF samples were analyzed for various regulatory cytokines and chemokines involved in CD4+ T helper (Th) cells, specifically Th1, Th2, and Th17 cells. The results for each cytokine and chemokine at each timepoint were summarized in **Figure 1**. Among the cytokines and chemokines measured, Interleukin (IL)-6, Regulated upon activation, normal T-cell expressed and secreted (RANTES), Tumor necrosis factor (TNF)-α, TNF-related apoptosis inducing ligand (TRAIL), and Transforming growth factor-beta 1 (TGF-β1) were consistently detectable in BALF over the three timepoints, and their mean levels are presented in **supplementary appendix**. However, the levels of IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, GM-CSF, and Interferon gamma (IFN-γ) in the collected BALF were below the lowest detection limit of the assay. The mean levels of the detectable cytokines and

Table 2. Asthma symptom scores, pulmonary function test, and medications before and 12 weeks after bronchial thermoplasty.

Values	Before BT	12 weeks after 3rd BT	*p*-value
ACT	13.6 (3.27)	19 (4.44)	0.004
AQLQ	3.93 (0.88)	5.3 (0.99)	0.002
Pre-BD FEV_1 (% pred)	74.6 (21.59)	78.8 (18.57)	0.385
Post-BD FEV_1 (% pred)	80.7 (21.93)	83.5 (16.91)	0.529
RV/TLC	40.78 (8.8)	41.88 (7.30)	0.503
FeNO, ppb†	30.78 (21.93)	44.44 (41.14)	0.358
ICS (BDP or equivalent), mcg	1180 (502.88)	1160 (556.17)	0.937
LABA (formoterol or equivalent), mcg	25.8 (8.96)	26.4 (7.58)	0.678

Definition of abbreviations: ACT = asthma control test; AQLQ = Asthma Quality of Life Questionnaire; pre-BD FEV_1 = pre-bronchodilator forced expiratory volume in 1 second; post-BD FEV_1 = postbronchodilator forced expiratory volume in 1 second; RV/TLC = the ratio of residual volume to total lung capacity; FeNO = fractional exhaled nitric oxide; ICS = inhaled corticosteroids; BDP = budesonide propionate; LABA = long-acting b2-agonist. Data are presented as mean ± SD unless otherwise noted.
†data were available in 9 patients.

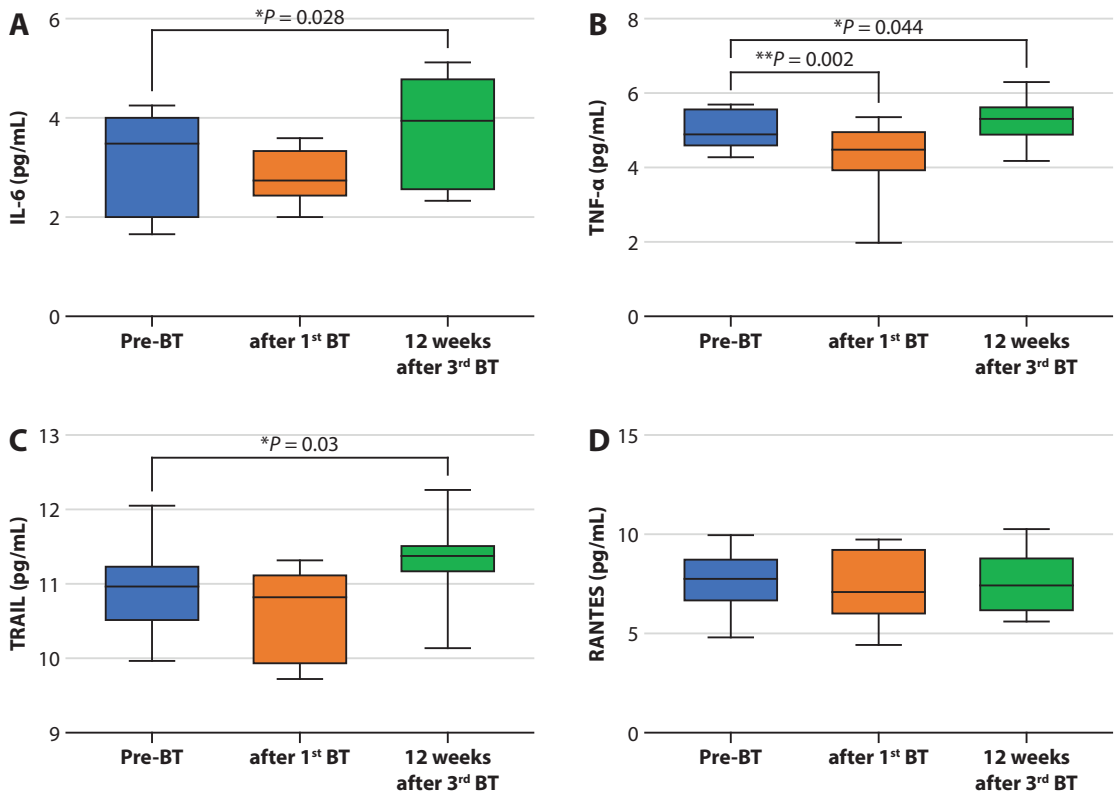

Figure 1. Changes in cytokines and chemokines in BALF. The box plots represent the levels of interleukin (IL)-6 (A), tumor necrosis factor (TNF)-α (B), tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) (C), regulated upon activation, normal T-cell expressed and secreted (RANTES)/CCL5 (D), and transforming growth factor (TGF)-β1 (E) after natural log transformation. $^*P < 0.05$ and $^{**}P < 0.01$ by GEE analysis, repeated measures, across three timepoints, including before BT, after 1st BT, and 12 weeks after BT.

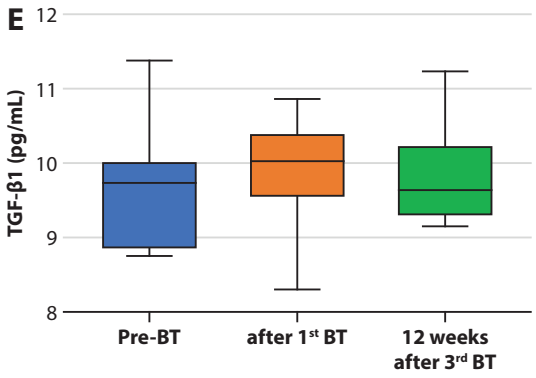

Figure 1. (Continued)

chemokines were compared after the first BT and 12 weeks after BT with those of pre-BT levels, which were reported as a mean difference (SD). A trend was observed towards a lower mean level of IL-6 after the first BT (-13.63 (20.06), p = 0.06), but its mean level significantly increased at 12 weeks after BT (34.58 (45.88), p-value = 0.041). TNF-α significantly reduced after the first BT (-68.01 (71.64), p-value = 0.015) but there was a trend towards elevation at 12 weeks after BT (61.36 (87.58), p-value = 0.054). The mean level of TRAIL did not significantly change after the first BT (-19216.40 (45747.45), p-value = 0.217), but it was significantly elevated at 12 weeks after BT (27260.91 (32081.52), p-value = 0.025). However, the mean level of TGF-β1 did not significantly change after the first BT (1891.15 (17159.60), p-value = 0.735) and 12 weeks after BT (725.08 (29391.40), p-value 0.940), when compared to pre-BT levels. Moreover, there was no significant change in the mean level of RANTES after the first BT (715.66 (7719.83), p-value = 0.776) and 12 weeks after BT (939.54 (9463.75), p-value = 0.761).

The results of the GEE analysis demonstrates that the levels of IL-6, TRAIL, and TNF-α in BALF were found to be statistically different among the three timepoints, whereas the level of RANTES and TGF-β1 in BALF remained unchanged over time (data shown in **supplementary appendix**). The level of IL-6 in BALF was significantly increased at 12 weeks after the third BT (3.71 ± 40.27) compared to pre-BT (3.10 ± 40.27, P-value = 0.028). However, there was no statistical difference observed between pre-BT and after the first BT. When compared to pre-BT (4.97 ± 28.70), the level of TNF-α in BALF significantly decreased after the first BT (4.27 ± 28.87, P-value = 0.002) but it significantly increased at 12 weeks after the third BT (5.24 ± 28.75, P-value = 0.044). Additionally, the level of TRAIL in BALF remained unchanged after the first BT but it significantly increased at 12 weeks after the third BT (11.32 ± 23.16) compared to pre-BT (10.93 ± 23.16, P-value = 0.03).

Table 3 presents the Pearson's correlation between the changes in ACT scores and AQLQ scores, and the changes in the levels of cytokines and chemokines, including IL-6, RANTES, TNF-α, TRAIL, and TGF-β1. It indicates that there is no significant correlation between these variables.

Table 3. Correlation between ACT scores and AQLQ scores with the levels of cytokines and chemokines in BALF using Pearson's correlation coefficient.

Values	ACT		AQLQ	
	R	*P*-value	R	*P*-value
IL-6	0.049	0.892	0.245	0.495
RANTES	-0.171	0.637	0.022	0.953
TNF-α	0.478	0.163	0.199	0.581
TRAIL	-0.281	0.432	0.060	0.870
TGF-β1	-0.585	0.076	-0.480	0.160

Discussion

BT is an endoscopic procedure aimed at reducing ASM in moderate to severe asthma. It has been clinically proven to improve asthma symptom and reduce severe exacerbation.[13,14] The clinical efficacy of BT has been established in several studies that correlate with the changes in ASM.[15,16] In our study, we investigated the early and sustained effects of BT on local airway inflammation in severe asthmatic patients. We found that BT improved asthma symptom control but had no significant effect on the long-term local airway inflammation based on the substantial changes in the measured cytokines and chemokines before and 12 weeks after procedure. This suggests that local airway inflammation did not account for the beneficial effect of BT.

The emerging roles of ASM beyond its contractile properties have been widely investigated in the pathophysiologic mechanism of asthma. ASM is now believed to have secretory and immunomodulatory functions, as there is increasing evidence that human ASM is capable of secreting a variety of cytokines and chemoattractants.[17-20] Furthermore, experimental studies have demonstrated mast cells infiltration in isolated ASM from asthmatic patients, a condition referred to as mast cell myositis.[14,21] This suggests that mast cells within ASM may be activated and release inflammatory mediators inducing airway hyperresponsiveness as well as airway remodeling. Although studies have addressed a significant reduction of ASM mass in severe asthma undergoing BT, the exact mechanisms how BT improve asthma control are still not fully understood.[9,22-25]

Moreover, a study investigating the clinical response of BT could not demonstrate an association between treatment response and ASM reduction.[25] We hypothesized that the complex interaction between ASM and inflammatory mediators may be affected by BT, potentially affecting local airway inflammation. However, previous studies have shown conflicting results regarding the effect of BT on airway inflammation.[9,10,26] In one study, Denner et al. were able to demonstrate clear changes in the inflammatory mediators including TGF-β1, RANTES/CCL5, TRAIL from the treated lobe in BALF, but the results should be interpreted with the possible effect of corticosteroids given in the treatment protocol.[9] However, our study was designed to collect the BALF after the first BT (second timepoint) to assess the effect of oral corticosteroids on the change of cytokines and chemokines in BALF. We postulated that the changes in BALF cytokines and chemokines in the second timepoint could be explained by two reasons. Firstly, the effect from pre-treatment with oral corticosteroids should be considered, as our result could demonstrate a trend toward reduction in IL-6 and TRAIL and a significant reduction of TNF-α in BALF, followed by an eventual increase at 12 weeks after BT. Secondly, the early effect of BT on local airway inflammation could also be possible.

A recent study also investigated the cytokines and chemokines in BALF without pre-treatment with corticosteroids, which revealed no significant differences between pre- and 6 months post-BT, as well as between untreated and BT-treated lobe.[26] However, to investigate the sustained effect of BT on airway inflammation, we measured the BALF cytokines and chemokines at 12 weeks after BT. Our results showed a significant increase in IL-6, TRAIL, and TNF-α in BALF, despite significant improvement in asthma control symptom scores. This suggests that the underlying mechanism of BT could not be explained solely by changes in inflammatory cytokines and chemokines. Our study supports the existing evidence that BT may not be correlated with local airway inflammation. However, controversy remains regarding the variable cytokines and chemokines that have been consistently detectable in BALF in previous studies.[9,26] Wijsman et al. reported no difference in BALF cytokines and chemokines between BT responders and non-responders, except for a significantly higher level of eosinophils in BT responders.[26] Taken together, the heterogeneity of study population may explain why some analytes were not detectable in BALF.

IL-6 has long been known as a marker of ongoing inflammation, but increasing evidence shows that it also plays a functional role in T cell survival, T cell proliferation, and differentiation of effector CD4 T cells.[27] Bronchial epithelial cells can produce IL-6 which has been found to be elevated in the BALF or sputum in asthma patients.[28-33] Additionally, TNF-α, another pro-inflammatory cytokine, has been found to increase in symptomatic asthma or status asthmaticus, and can also be secreted by ASM.[30,34] In our study, both IL-6 and TNF-α were found to be significantly elevated in BALF at 12 weeks after the third BT, indicating that local airway inflammation was not mediated by BT. However, the previous study showed that corticosteroids could also suppress IL-6 secretion from the bronchial epithelial cell.[31] The trend toward decreasing the level of IL-6 and a significant decrease in the level of TNF-α after the first BT could imply that pretreatment with corticosteroids could have an effect on this slight change.

RANTES (CC chemokine ligand 5), which belongs to the CC chemokine family, is another chemokine that plays an important role in allergic airway inflammation, as it has a potent chemoattractant activity for eosinophils.[35] Denner et al. reported a significant reduction in BALF RANTES levels at 3 weeks and 6 weeks after BT, which correlated with a decrease in eosinophils in BALF, representing a reduction in local airway inflammation. In contrast, our study, in the absence of potential effect from pre-treatment corticosteroid, demonstrated the unaffected level of RANTES in BALF from BT throughout the study period. Therefore, this was another finding suggesting that BT may not involve in decreasing local airway inflammation.

In our study, TRAIL was found to be significantly elevated at 12 weeks after the third BT, which is consistent with a previous study.[9] TRAIL is a cytokine that belongs to the TNF superfamily and is known for inducing apoptosis in various cells.[35] Its effects in human depend on the expression of the binding receptors, which are differentially expressed in acute or chronic group. In ragweed-allergic asthma following segmental antigen provocation, there was increased TRAIL expression and decreased expression of DR4 and DR5, the canonical human death receptors. The significant increase in TRAIL expression, as well as the elevation of TRAIL in BALF, which significantly correlated with the increase in eosinophils in BALF, indicates that TRAIL has the potential to mediate allergic inflammation by prolonging eosinophil survival.[36] However, in an animal study with chronic long-term allergen challenge, increased expression of TRAIL could induce airway leukocyte apoptosis and result in resolution of the allergic inflammation.[37] TRAIL has been shown to be involved in both resolution of airway inflammation and the ongoing active allergic inflammation. Therefore, the significant change in TRAIL in our study remains to be determined in terms of how BT affects TRAIL expression and which TRAIL signaling pathways are involved in the underlying mechanism of BT.

TGF-β1 is a profibrogenic factor primarily released from bronchial epithelial cells, myofibroblasts, and inflammatory cells that has been extensively investigated in airway remodeling. It has been found to be associated with the proliferation of ASM and the increased deposition of extracellular matrix.[38] TGF-β1 signaling in bronchial tissue from asthmatic patients has been reported to be correlated with the thickness of basement membrane.[39] Denner et al. found that BT significantly reduced the ASM mass and the level of TGF-β1 in BALF after 6 weeks.[9] However, the reduction in TGF-β1 in BALF could not clearly indicate the reduction of airway fibrosis but should concern the improvement of airway inflammation, as they also found a decrease in BALF eosinophils. On the other hand, our study could not demonstrate a significant effect of BT on TGF-β1 in BALF in both the short- and long-term period. Furthermore, Ichikawa et al. also examined the effect of BT on airway inflammation in which TGF-β1 expression on

tissue cells was not affected even with a significant reduction of ASM.[10] However, airway eosinophil have also been shown to be an important source of TGF-β1, of which Anti-IL5 therapy, mepolizumab, reduced the level in BALF.[40] Taken together, the airway epithelium and other inflammatory cells recruited to the airways are also capable of secrete TGF-β1. Therefore, our data support the hypothesis that the level of TGF-β1 in BALF might not correlate with the reduction in ASM and airway remodeling, and the source of TGF-β1 in BALF could be from other structures within the airways.

Previous studies have shown that BT does not provide long-term benefit in improving pulmonary function. However, robust evidence supports the clinical efficacy of BT in improving asthma control symptom scores.[4-6,41] Although our study did not quantify a reduction in ASM, the clinical improvement of BT was evident through significant improvements in ACT and AQLQ scores. It is worth noting that pre-BD FEV_1, post-BD FEV_1, and FeNO remained unchanged at 12 weeks after the third BT compared to baseline.

There are some limitations to this study. Firstly, although we planned to measure the inflammatory mediators involving Th-1, Th-2, and Th-17, only five analytes were consistently detectable over the study period, which may have limited our ability to interpret the effect from BT. However, this is consistent with previous studies that attempted to investigate BALF cytokine and chemokine in patients undergoing BT and found limitations in analyzing the BALF analytes.[10,26] Secondly, the high variability of cytokines and chemokines levels in BALF observed in our study could be due to the heterogeneity of asthma phenotype in our patient population. Additionally, the collection technique used to obtain BALF samples at different timepoint and in different patients could potentially affect the amount of returning fluid and thus impact the level of measured analytes. Thirdly, the cytokines and chemokines in BALF may not truly represent the local airway inflammation. Therefore, addressing the gene expression that regulates cytokines and chemokines production may provide additional information for better understanding the mechanism of BT.

In conclusion, the study found no significant effect of BT on local airway inflammation, despite demonstrating significant clinical improvement. However, due to the trend toward IL-6, and RANTES reduction in BALF and significantly lower level of TNF-α in BALF at 3 weeks after the first BT compared to baseline, the potential effect of pretreatment corticosteroids should be considered. TGF-β1 in BALF could be associated with the presence of local airway inflammation, not only ASM remodeling. In summary, while the clinical benefit of BT was significant, it may not be clearly explained by the effect on local airway inflammation, and other mechanisms should be further investigated.

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Conflict of interest

Authors declare no conflict of interest.

Author contribution

- NL, PS, and TS contributed to the conception and design of the study, as well as data acquisition, analysis, and interpretation.
- KJ contributed to data analysis and interpretation.
- VT and VW contributed to data acquisition and interpretation.
- All authors read and approved the final manuscript.

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Supplementary appendix

Table 1. Cytokines and chemokines concentrations in bronchoalveolar lavage fluid before BT, after 1st BT, and 12 weeks after BT.

BAL analytes (pg/ml)	Pre-BT	After 1st BT		12 weeks after 3rd BT	
	Mean (SD)	Mean (SD)	*p*-value*	Mean (SD)	*p*-value†
IL-2	ND	ND	-	ND	-
IL-4	ND	ND	-	ND	-
IL-5	ND	ND	-	ND	-
IL-6	31.86 (23.35)	18.23 (9.59)	0.06	66.43 (59.52)	0.041
IL-10	ND	ND	-	ND	-
IL-12	ND	ND	-	ND	-
IL-13	ND	ND	-	ND	-
IL-17	ND	ND	-	ND	-
GM-CSF	ND	ND	-	ND	-
IFN-γ	ND	ND	-	ND	-
RANTES	4,546.33 (6,216.28)	5,261.98 (6,376.22)	0.776	5,485.87 (8,802.91)	0.761
TNF-α	162.71 (84.57)	94.7 (59.98)	0.015	224.06 (135.38)	0.054
TRAIL	66,450.37 (44,921.39)	47,233.97 (26,324.87)	0.217	93,711.29 (49,382.72)	0.025
TGF-β1	22,287.59 (24,205.75)	24,178.73 (13,594.48)	0.735	23,012.66 (20,290.91)	0.940

Definition of abbreviations: IL = Interleukin; GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN = Interferon-gamma; RANTES = regulated upon activation, normal T-cell expressed and secreted; TNF = tumor necrosis factor; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; TGF = transforming growth factor; ND = not detected. Data are presented as mean ± SD.
*compare values between baseline (pre-BT) with those after the first BT.
†compare values between baseline (pre-BT) with those at 12 weeks after the third BT.

Table 2. Results of generalized estimating equation (GEE) analysis, repeated measures, with cytokines or chemokines as dependent variables. The difference between three timepoints, between the first and second timepoint, and between the first and third timepoint were shown.

Variables	Timepoint	Mean values (SE)	Time		Time 1 × Time 2		Time 1 × Time 3	
			Wald	*p*	Mean difference (SE)	*p*	Mean difference (SE)	*p*
IL-6	1	3.10 (40.27)	13.49	0.001	0.32 (0.22)	0.13	-0.60 (0.27)	0.028
	2	2.77 (40.17)						
	3	3.71 (40.27)						
RANTES	1	7.64 (68.13)	0.14	0.93	0.24 (0.66)	0.71	0.05 (0.35)	0.869
	2	7.39 (68.13)						
	3	7.58 (68.10)						
TRAIL	1	10.93 (23.16)	19.22	0.00	0.34 (0.19)	0.08	-0.39 (0.18)	0.03
	2	10.59 (23.23)						
	3	11.32 (23.16)						
TGF-β1	1	9.67 (9.80)	1.22	0.54	-0.23 (0.21)	0.26	-0.13 (0.26)	0.61
	2	9.91 (9.75)						
	3	9.81 (9.79)						

Table 2. (Continued)

Variables	Timepoint	Mean values (SE)	Time		Time 1 × Time 2		Time 1 × Time 3	
			Wald	*p*	Mean difference (SE)	*p*	Mean difference (SE)	*p*
TNF-α	1	4.97 (28.70)	25.23	< 0.001	0.69 (0.22)	0.002	-0.27 (0.13)	0.044
	2	4.27 (28.87)						
	3	5.24 (28.75)						

The data were subjected to a natural log transformation to adjust for non-normal distribution. All results are shown as estimated marginal mean (SE) values obtained after GEE analysis, repeated measures. Shown are the effects of time, Time 1 × Time 2 (1st timepoint versus 2nd timepoint), and Time 1 × Time 3 (1st timepoint versus 3rd timepoint), while adjusting for the effects of age and sex.