

4-1BB Ligand-mediated Imbalance of Helper 17 T Cells and Regulatory T Cells in Patients with Allergic Asthma

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OBJECTIVES: To investigate the presence of 4-1BB ligand (4-1BBL) in the peripheral blood of patients with allergic asthma and evaluate its role in controlling the balance between helper 17 T (T_H17) and regulatory T (T_{reg}) cells. **METHODS:** Soluble 4-1BBL (s4-1BBL) was quantified by enzyme-linked immunosorbent assay in plasma from patients with asthma ($n = 45$) and from healthy control subjects ($n = 35$). The proportion of monocytes positive for membrane-bound 4-1BBL (m4-1BBL) was determined by flow cytometry. Peripheral blood mononuclear cells from patients with asthma were incubated with anti-4-1BB monoclonal antibody *in vitro*. Concentrations of interleukin (IL)-17 and

transforming growth factor (TGF)- β_1 in the culture supernatant were analysed. **RESULTS:** Plasma s4-1BBL concentrations and the proportion of m4-1BBL-positive monocytes were significantly lower in patients with asthma than in control subjects. The culture supernatant concentration of TGF- β_1 was increased and that of IL-17 was decreased by incubation with anti-4-1BB monoclonal antibody. **CONCLUSIONS:** Both soluble and membrane-bound 4-1BBL were reduced in patients with allergic asthma compared with control subjects. 4-1BBL/4-1BB signalling may play an important role in allergic asthma by regulating the T_H17/T_{reg} balance.

KEY WORDS: 4-1BB; 4-1BB LIGAND; ALLERGIC ASTHMA; HELPER T CELLS; INTERLEUKIN 17; REGULATORY T CELLS; TRANSFORMING GROWTH FACTOR- β

Introduction

Allergic asthma is a chronic inflammatory condition of the airways. The prevalence of asthma continues to increase, and the optimal treatment of this condition remains a challenge.¹ Asthma is believed to result from an inappropriate type 2 helper T (T_H2) cell-mediated immune response to common

aeroallergens in genetically susceptible individuals.² In allergic asthma, activated T_H2 cells and their secreted cytokines promote the development of eosinophilia and airway hyper-responsiveness.³ Experimental and clinical data indicate that T_H2 cells and an imbalance in the type 1 helper T cell (T_H1)/ T_H2 ratio are central to the pathogenesis of asthma.^{4,5}

It is recognized that the T_H1/T_H2 imbalance paradigm does not fully explain

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the aetiology of asthma, and other $CD4^+$ T cell subsets such as helper 17 T (T_h17) and regulatory T (T_{reg}) cells may play a role in asthma.^{6,7} Both T_h17/T_{reg} and T_h1/T_h2 imbalances have been found in patients with moderate-to-severe asthma.^{8–11}

The type 2 transmembrane glycoprotein 4-1BB (also known as CD137 or tumour necrosis factor [TNF] receptor superfamily member 9), is mainly found on the surface of activated T cells.¹² The 4-1BB ligand (4-1BBL) is present on the cell surface of activated antigen presenting cells (APCs), particularly at sites of inflammation *in vivo*.¹³ 4-1BBL/4-1BB has been shown to costimulate both primary and secondary T cell immune responses¹⁴ and to have diverse and opposing effects on the regulation of the immune system.¹⁵ Evidence suggests that 4-1BBL/4-1BB is more important for effective cytotoxic T cell responses than for T_h cell responses.¹⁶

Anti-4-1BB antibody treatment was found significantly to increase intracellular forkhead box P3 (*FOXP3*) expression, *in vitro* suppressive activity and T_{reg} numbers in a murine model of autoimmune type 1 diabetes.¹⁷ Anti-4-1BB treatment dramatically reduced ovalbumin-specific serum immunoglobulin (Ig)E and IgG₁ production in a murine asthma model,¹⁸ suggesting that 4-1BB might be a possible target for asthma immunotherapy.¹⁹ The effect of anti-4-1BB treatment in humans, particularly in relation to T_h17/T_{reg} balance, has not been fully elucidated.

The present study determined 4-1BBL protein levels in patients with allergic asthma and their relationship to the T_h17/T_{reg} imbalance paradigm in order to gain a better understanding of the role of 4-1BBL/4-1BB in asthma.

Patients and methods

STUDY POPULATION

Patients with chronic persistent asthma,

attending the Department of Pulmonary Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, were sequentially enrolled in this case-control study between October 2009 and January 2011.

Pulmonary function and skin prick tests were performed, and a self-administered asthma control questionnaire (ACQ) was reviewed for all participants as described previously.^{20–23} Forced expiratory volume in 1 s (FEV_1) and forced vital capacity were measured, and $FEV_1\%$ (actual FEV_1 as a percentage of predicted FEV_1) was calculated. The skin prick test was performed for 12 common allergens on the inner forearm. The severity of asthma was classified into four categories based on the level of symptoms, airflow limitation and lung function variability: intermittent (wheezing < once per week; $FEV_1\% > 80$; lung function variability < 20%); mild persistent (wheezing once per week – once per day; $FEV_1\% > 80$; lung function variability 20 – 30%); moderate persistent (wheezing at night > once per week; $FEV_1\% 60–80$; lung function variability > 30%); or severe persistent (frequent wheezing at night; $FEV_1\% < 60$; lung function variability > 30%), according to Global Initiative for Asthma guidelines.^{1,24} None of the patients had been treated with systemic glucocorticoids or other immunosuppressive agents within 1 month prior to study entry. Patients with acute infections within 1 week of recruitment and those with other chronic diseases were excluded.

Sex- and age-matched healthy control subjects were enrolled from the physical examination centre of Ruijin Hospital, Shanghai, China. All control subjects were nonsmokers with normal pulmonary function and negative allergy tests, according to the lung function and skin prick tests described above.

The study was approved by the Research Ethics Board of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from all study participants prior to enrolment.

PBMC ISOLATION AND CULTURE

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized (0.9 mmol/l) peripheral blood by Ficoll-Hypaque density gradient centrifugation at 3000 *g* for 20 min at room temperature. PBMCs were suspended at a density of 2×10^6 cells/ml in RPMI medium 1640 with GlutaMAXTM, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (complete medium; all cell culture reagents from Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA). Cells were cultured at 37 °C with 5% carbon dioxide in air.

STIMULATION WITH ANTI-4-1BB

The PBMCs isolated from patients with asthma were cultured in 96-well plates at 1×10^5 cells/well in complete medium with 5 µg/ml anti-4-1BB monoclonal antibody (kindly provided by Dr Lieping Chen, Professor of Immunobiology, Yale Cancer Center, New Haven, CT, USA) or 5 µg/ml IgG_{2a} (isotype control) for 72 h. Culture supernatants were then collected by centrifugation at 1500 *g* for 5 min and stored at -80 °C until analysis of cytokine production. PBMCs were retained for flow cytometric analysis.

FLOW CYTOMETRY

For analysis of T_{reg} (CD4⁺CD25⁺FOXP3⁺) and T_H17 (CD4⁺IL-17⁺) cell numbers, PBMCs were resuspended in complete medium containing 20 ng/ml phorbol 12-myristate 13-acetate, 1 µg/ml ionomycin and 2 nmol/ml monensin (all Sigma-Aldrich, St

Louis, MO, USA) in 24-well plates (1×10^6 cells/well in 1 ml). After 4 h the cells were transferred to tubes, washed once with 10 mM phosphate buffered saline (PBS; pH 7.4), and incubated with phycoerythrin-cy5-conjugated antihuman CD4 (0.125 µg in 5 µl, each sample) at 4 °C for 30 min. Cells were then fixed and permeabilized according to the manufacturer's instructions (eBioscience, San Diego, CA, USA), and stained with fluorescein isothiocyanate (FITC)-conjugated antihuman FOXP3 (0.5 µg in 5 µl, each sample) or phycoerythrin (PE)-conjugated antihuman interleukin (IL)-17A (0.25 µg in 20 µl, each sample) according to the manufacturer's instructions (eBioscience).

For the analysis of membrane-bound 4-1BBL (m4-1BBL) on PBMCs, cells were cultured for 4 h as described above (with the omission of ionomycin), transferred to tubes and washed once with 10 mM PBS (pH 7.4). Cells were then stained with FITC-conjugated antihuman 4-1BB (5 µg/ml) at 4 °C for 30 min, followed by incubation with PE-conjugated antihuman CD14 (1.0 µg in 5 µl, each sample) at 4 °C for 30 min according to the manufacturer's instructions (eBioscience). Isotype controls were used to correct for nonspecific binding.

Stained cells were analysed by flow cytometry using a BDTM LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with FlowJo software, version 7.6 (Tree Star, Ashland, OR, USA).

ELISA

Plasma was isolated from heparinized (0.9 mmol/l) peripheral blood by centrifugation at 1500 *g* for 10 min and stored at -80 °C until analysis of cytokine production. The concentrations of soluble 4-1BBL (s4-1BBL) in plasma, and IL-17 and transforming growth factor- β_1 (TGF- β_1) in culture supernatant were measured using enzyme-

linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The minimum detectable concentrations were 1 pg/ml for s4-1BBL, 8 pg/ml for IL-17 and 16 pg/ml for TGF- β_1 . Intra- and interassay coefficients of variation for all ELISAs were < 10% and < 15%, respectively. All samples were analysed in duplicate.

STATISTICAL ANALYSES

Statistical analyses were undertaken using the SPSS® statistical package, version 11.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Homogeneity of variance was assessed in all groups. In the case of homogeneity, data were analysed using one-way analysis of variance followed by the Student–Newman–Keuls test and expressed as mean $\pm$ SD. In the case of heteroscedasticity, data were analysed using the Mann–Whitney *U*-test and expressed as median (interquartile range). Pearson's correlation coefficient was used to analyse the relationship between FEV₁% and the proportion of 4-1BBL-positive monocytes. A *P*-value < 0.05 was considered to be statistically significant.

Results

The study enrolled 45 patients with chronic persistent asthma, who were divided into two groups according to the severity of asthma: intermittent-to-mild (*n* = 25); and moderate-to-severe (*n* = 20). The control group comprised 35 sex- and age-matched healthy subjects (Table 1). FEV₁% was significantly higher in controls than in patients with asthma, and in patients with intermittent-to-mild asthma compared with moderate-to-severe asthma (*P* < 0.01 for both comparisons).

Plasma concentrations of s4-1BBL were significantly higher in healthy control subjects compared with patients with asthma (298.29 $\pm$ 40.32 μ g/l versus 202.47 $\pm$ 60.68 μ g/l; *P* < 0.05). There was no significant difference in plasma s4-1BBL concentration between the intermittent-to-mild and moderate-to-severe asthma groups.

The proportion of CD14⁺m4-1BBL⁺ monocytes was significantly lower in patients with asthma compared with control subjects (2.69 $\pm$ 1.85% versus 9.06 $\pm$ 6.79%; *P* < 0.05) (Fig. 1). The proportion of CD14⁺m4-1BBL⁺ monocytes in patients with moderate-to-severe asthma was significantly lower than in patients with intermittent-to-

TABLE 1:

Baseline demographic and clinical characteristics of the healthy control subjects and patients with allergic asthma (stratified according to disease severity) included in this study to evaluate the role of 4-1BB ligand in allergic asthma

	Control subjects <i>n</i> = 35	Patients with asthma	
		Intermittent-to-mild <i>n</i> = 25	Moderate-to-severe <i>n</i> = 20
Age, years	32.10 $\pm$ 6.51	31.76 $\pm$ 8.32	34.69 $\pm$ 10.42
Gender, males/females	19/16	12/13	11/9
FEV ₁ %	96.29 $\pm$ 5.42	87.53 $\pm$ 7.40 ^a	62.37 $\pm$ 10.49 ^{a,b}
ACQ score	–	1.33 $\pm$ 0.45	2.27 $\pm$ 0.89 ^b

Data expressed as mean $\pm$ SD or number (*n*) of patients.

FEV₁%, actual forced expiratory volume in 1 s (FEV₁) as a percentage of predicted FEV₁; ACQ, asthma control questionnaire.^{22,23}

^a*P* < 0.01 versus control subjects, ^b*P* < 0.01 versus intermittent-to-mild asthma; one-way analysis of variance followed by the Student–Newman–Keuls test.

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mild asthma ($1.90 \pm 0.32\%$ versus $3.28 \pm 0.93\%$; $P < 0.05$). The proportion of $CD14^+m4-1BBL^+$ monocytes was significantly positively associated with $FEV_1\%$ ($r = 0.571$, $P < 0.01$, Fig. 2). The concentration of $TGF-\beta_1$ in the culture

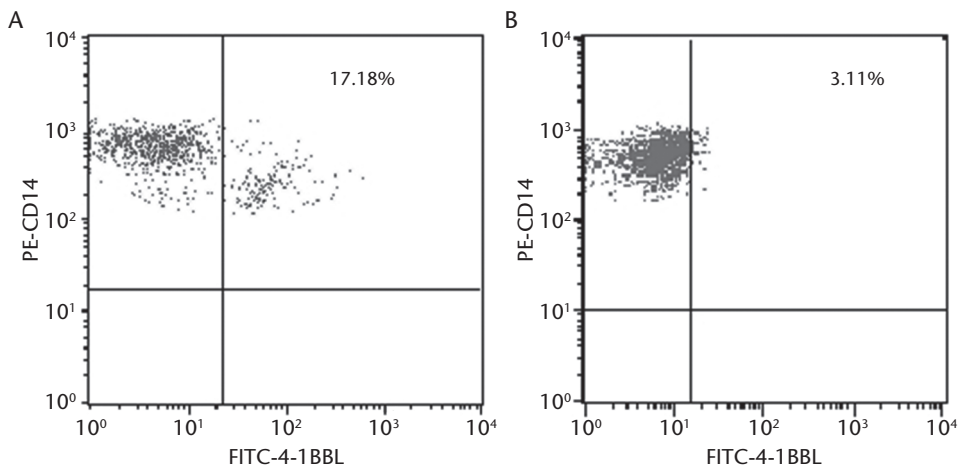


FIGURE 1: Representative results of flow cytometric analysis of CD14 and 4-1BB ligand (4-1BBL) on peripheral blood mononuclear cells from (A) a healthy control subject and (B) a patient with allergic asthma. Cells were stained with phycoerythrin (PE)-conjugated anti-CD14 monoclonal antibody and fluorescein isothiocyanate (FITC)-conjugated anti-4-1BBL monoclonal antibody

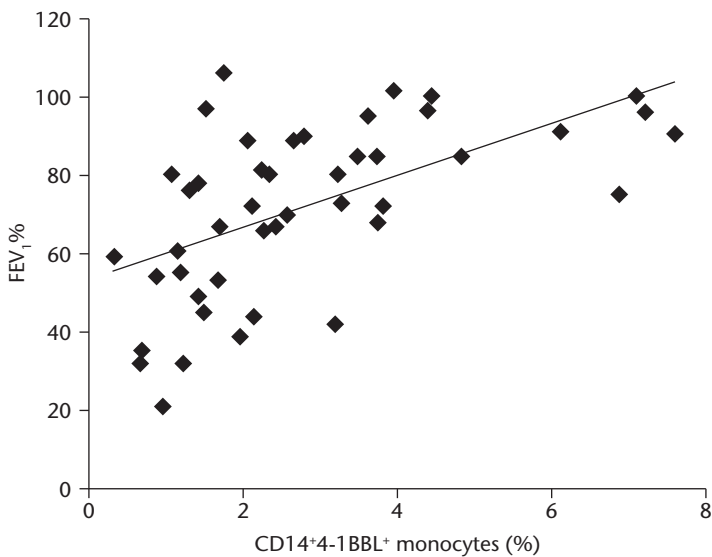


FIGURE 2: The relationship between severity of asthma (percentage predicted forced expiratory volume in 1 s [$FEV_1\%$]) and the percentage of $CD14^+4-1BBL^+$ monocytes in peripheral blood mononuclear cells from patients with allergic asthma ($P < 0.01$, $r = 0.571$; Pearson's correlation coefficient analysis)

supernatant from PBMCs treated with anti-4-1BB was significantly higher than from cells treated with isotype control antibody (655.81 ± 476.26 ng/ml versus 410.50 ± 368.03 ng/ml; $P < 0.05$), and the concentration of IL-17 was significantly lower (5202.00 ± 4143.74 ng/ml versus 8377.00 ± 3839.98 ng/ml; $P < 0.05$). There were no significant differences in the proportions of T_h17 or T_{reg} cells in PBMCs treated with anti-4-1BB or isotype control antibody (Table 2).

Discussion

The present study found that plasma s4-1BBL concentrations and the proportion of $CD14^+m4-1BBL^+$ monocytes were reduced in patients with allergic asthma compared with healthy control subjects. In addition, *in vitro* treatment of PMBCs from patients with allergic asthma with anti-4-1BB monoclonal antibody stimulated $TGF-\beta_1$ and inhibited IL-17 production compared with isotype control treatment.

It has been demonstrated that s4-1BBL binds to the 4-1BB receptor and stimulates IL-2 and interferon- γ release from peripheral T cells.²⁵ Transmembrane 4-1BBL is present at high levels on the surface of activated APCs, including monocytes, B cells and mature dendritic cells, and can be induced on T lymphocytes.²⁶ Monocytes are the major source of 4-1BBL in peripheral blood.²⁷

The paucity of 4-1BBL on resting APCs and the requirement for T cell activation to induce 4-1BB upregulation suggests that 4-1BBL/4-1BB is required for sustaining T cell responses following CD28 costimulation.²⁸ Accumulating evidence indicates that 4-1BBL-mediated signals regulate the activity of professional APCs.²⁷ Monocytes stimulated with 4-1BBL exhibit several phenotypic changes, including cell proliferation, proinflammatory cytokine and chemokine production and upregulation of cell adhesion molecules.²⁹ In addition, 4-1BBL/4-1BB interactions have been shown to participate in T_h cell-induced inflammation. Various inflammatory/autoimmune diseases including rheumatoid arthritis, autoimmune myocarditis, herpetic stromal keratitis, allograft rejection and graft-versus-host disease are less severe in the absence of 4-1BBL/4-1BB interactions.²⁹

The anti-4-1BB monoclonal antibody has been shown to alleviate airway inflammation and reverse established disease in a murine asthma model, via anti-4-1BB-mediated T_h cell anergy.⁴ This finding was supported by others who found that 4-1BB-mediated signalling anergized T_h cells in lupus prone mice.³⁰ In a murine model of multiple sclerosis, anti-4-1BB treatment inhibited disease relapse and initially promoted the activation and proliferation of

TABLE 2:

The proportions of helper 17 T (T_h17) cells and regulatory T (T_{reg}) cells in the total T cell population of peripheral blood mononuclear cells from patients with allergic asthma, treated *in vitro* with anti-4-1BB monoclonal antibody or isotype control

Treatment	Proportion of T cell population (%)	
	T_h17	T_{reg}
Anti-4-1BB	1.30 ± 0.53	3.45 ± 1.03
Isotype control	1.54 ± 1.01	2.76 ± 0.97

Data expressed as mean $\pm$ SD.

No statistically significant between-group differences ($P \geq 0.05$); one-way analysis of variance followed by Student–Newman–Keuls test.

antigen-specific T_h cells.³¹ Ultimately, however, the level of activation-induced T_h cell death was increased, inhibiting effector T cell responses.³¹

The important role of 4-1BBL/4-1BB signalling in asthma has focused research on s4-1BBL plasma concentrations and m4-1BBL on monocytes. Plasma s4-1BBL concentrations were found to be higher in patients with various haematological diseases compared with healthy subjects.²⁵ It was suggested that s4-1BBL release may regulate 4-1BB/4-1BBL interactions *in vivo* and this, in turn, might alter disease progression or outcome.²⁵ Compared with healthy control subjects, patients with multiple sclerosis had increased m4-1BBL on CD14⁺ monocytes and higher s4-1BBL plasma concentrations,²⁶ suggesting that elevated s4-1BBL might function as a self-regulatory mechanism of 4-1BB/4-1BBL interaction and costimulation. In contrast, the proportion of CD14⁺4-1BBL⁺ monocytes and s4-1BBL plasma concentrations were significantly lower in patients with asthma than in healthy control subjects in the present study. It is possible that a deficiency in 4-1BBL/4-1BB signalling (including s4-1BBL) may contribute to a shift from a T_h1 response to a T_h2 response in asthma.³² As such, the decrease in 4-1BBL might promote the development of allergic asthma. Studies have found that 4-1BBL/4-1BB signalling is involved in the proliferation and function of T_{reg} cells.³³ A novel chimeric form of 4-1BBL that was fused to streptavidin (SA-4-1BBL) demonstrated better costimulation of T_h and CD8⁺ T cells than an agonistic antibody, both *in vitro* and *in vivo*.³⁴ IL-2 treatment increased 4-1BB on T_{reg} cells, and stimulation with SA-4-1BBL expanded these cells up to 110-fold within 3 weeks.¹⁵ The expanded T_{reg} cells showed upregulation of CD25, 4-1BB and membrane-bound TGF- β , suppressed T_h

cell proliferation and prevented graft rejection in mice.¹⁵ The relationship between 4-1BBL/4-1BB and T_h17 cells remained unclear, however.^{15,35}

A T_h17/T_{reg} imbalance has been shown to be present in patients with asthma, particularly in moderate-to-severe disease.⁸ The present study, therefore, investigated the impact of 4-1BBL/4-1BB on the T_h17/T_{reg} balance in patients with allergic asthma. Treatment of PBMCs from patients with asthma with anti-4-1BB monoclonal antibody had no significant effect on the proportions of T_h17 and T_{reg} cells, but TGF- β_1 was significantly increased and IL-17 significantly inhibited. TGF- β_1 and IL-17 are critical for the function of T_{reg} cells and T_h17 cells, respectively.³⁶ It is possible that 4-1BBL/4-1BB signalling regulates the T_h17/T_{reg} balance in asthma by changing T_h17 and T_{reg} cell functions, independently of proliferation. It has been shown that 4-1BB triggering inhibits generation of those T_h17 cells responsible for induction and progression of experimental autoimmune encephalomyelitis and increases FOXP3⁺CD4⁺ T_{reg} cells.³⁵ Modulation of the T_h17/T_{reg} balance may be a mechanism by which 4-1BB affects immune responses in autoimmune and allergic diseases.

In conclusion, this preliminary study found lower proportions of m4-1BBL⁺ monocytes in patients with allergic asthma compared with healthy controls. This may lead to a T_h17/T_{reg} imbalance and more severe airway inflammation. Treatment of PMBCs with anti-4-1BB monoclonal antibody is associated with increased TGF- β_1 and decreased IL-17 concentrations *in vitro*. These results suggest that 4-1BBL/4-1BB signalling may play an important role in allergic asthma by regulating the T_h17/T_{reg} balance and that 4-1BBL/4-1BB may be a potential target for asthma immunotherapy. Future studies are

required to determine whether 4-1BBL levels in patients are correlated with asthma progression and outcome. In addition, it remains to be determined whether modulation of 4-1BBL release is beneficial in asthma treatment. The mechanism by which 4-1BBL/4-1BB regulates T_H17/T_{reg} balance also requires further investigation.

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Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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