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Original Article

$CD4^+$ T cell proliferation and inhibition of activation-induced cell death (AICD) in childhood asthma

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Abstract: *Objective:* The mechanism underlying immune inflammation and over-activation of T helper (Th) cells in childhood asthma was investigated through cell proliferation and activation-induced cell death (AICD) experiments. *Methods:* There were 30 children in the asthma group with an average age of 10.2 ± 3.1 years and 30 children in the normal control group with an average age of 10.5 ± 2.9 years. A cytometric bead array (CBA) was performed to detect Th1, Th2, and Th17 cytokines. After $CD4^+$ T cells were separated using immunomagnetic beads and stimulated by phytohemagglutinin (PHA) combined with anti-CD3 antibodies *in vitro*, cell proliferation and AICD were analyzed. Finally, mRNA expression of the apoptosis- and proliferation-related proteins Fas, FasL, and Bcl-2 was detected using quantitative polymerase chain reaction (PCR). *Results:* The serum cytokine levels of the children in the asthma group significantly increased compared to those of children in the normal control group (IL-4: 9.33 ± 2.25 ng/L *vs*. 4.59 ± 1.77 ng/L, P = 0.012; IL-10: 3.41 ± 0.80 ng/L *vs*. 1.36 ± 0.41 ng/L, P = 0.027; TNF: 7.63 ± 4.09 ng/L *vs*. 1.88 ± 0.52 ng/L, P = 0.023). The $CD4^+$ T cell proliferation ability in the asthma group was significantly higher than that in the normal control group (OD450: 0.66 ± 0.14 *vs*. 0.28 ± 0.07, P<0.001), whereas the AICD rate was significantly lower than that in the normal control group (29.46 ± 5.25% vs. 60.11 ± 4.93%, P<0.001). The Fas mRNA expression in the $CD4^+$ T cells from children in the asthma group was significantly decreased compared to that from the children in the normal control group, whereas the Bcl-2 expression was significantly higher than that from children in the normal control group. These differences were both statistically significant (P<0.001). Conversely, FasL expression did not differ (P > 0.05). *Conclusion:* Fas expression decreased and Bcl-2 expression increased in $CD4^+$ T cells from the children in the asthma group, inhibiting AICD, and promoting proliferation of Th cells to some extent. Apoptosis inhibition and cell proliferation might result in over-activation of Th cells and aggravation of inflammatory infiltration in children with asthma.

Keywords: Childhood asthma, proliferation, Th cells, activation-induced cell death

Introduction

Childhood asthma is an immune-associated disease caused by many factors, including genetics and the environment. An immune inflammatory reaction in the respiratory tract is a primary characteristic of asthma. Chronic inflammatory reactions mediated by T cells and related cytokines are significant in the initiation and progression of asthma [1]. Through contact with the mucosal immune system (such as the respiratory tract and intestinal tract), antigens in the external environment can induce the body to produce immune tolerance, particularly T cell tolerance. This process can effectively protect the body from developing asthma [2]. Over-activation of $CD4^+$ T cells in the peripheral blood and respiratory tract is a common feature of asthma patients and the resulting rapid proliferation of T cells and secretion of many cytokines aggravate the inflammatory reaction. The exact reasons and relevant mechanisms are currently unclear but activation-induced cell death (AICD) caused by T cell activation in the body is an important mechanism underlying the clearance of overly activated T cells and the maintenance of peripheral immune tolerance. When immune tolerance balance is destroyed by external factors, the body can suffer immune injury medi-

ated by T cells [3, 4]. This study observed proliferation of CD4+ T cells and conditions of AICD in the peripheral blood of children with asthma to investigate their mechanisms of action in asthmatic immune injury. These data provide new ideas for further development of peripheral immune tolerance therapy.

Material and methods

Study subjects

This study included 30 children with asthma (17 boys and 13 girls with an average age of 10.2 ± 3.1 years) who were admitted to the hospital. They all met the diagnostic criteria of asthma. There were 30 cases in the normal control group, which included 15 boys and 15 girls with an average age of 10.5 ± 2.9 years. An assessment of children with asthma according to the disease conditions showed that there were 6 mild cases, 15 moderate cases, and 9 severe cases. Blood was collected from all pediatric patients without receiving any drug treatment. In addition, patients with tumors, blood diseases, and other autoimmune diseases were excluded.

Experimental materials

The cytometric beads array (CBA) kit and T helper 1 (Th1), Th2 and Thl7 cytokine detection reagent kits were obtained from BD (USA). Lymphocyte separation solution was obtained from MP Biomedicals (China). CD4 antibody-labeled immunomagnetic beads were obtained from Miltenyi Biotec (Germany). RPMI1640 culture medium was obtained from Gibco (Australia). Phytohemagglutinin (PHA) was obtained from Sigma (USA). The CCK-8 cell proliferation reagent kit was obtained from Yeasen (USA). The Annexin V/PI apoptosis detection reagent kit was obtained from Shanghai Sangon Biotech (China).

Detection of cytokines

Serum samples from the children were separated, and interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF), interferon (IFN)-γ, and IL-17A were detected using CBA technology strictly according to the operational procedures in the reagent instructions. First, the standard samples were used for gradient dilution at 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256. In addition, fluorescent microspheres were mixed and re-suspended at room temperature in the dark for 30 min. Next, the relevant parameters of the flow cytometer (Beckman FC500) were adjusted and set up to enter the detection status. Finally, 50 μL of the diluted standard sample was added to a standard sample tube, and 50 μL of the specimen to be detected was added to a sample tube. Fifty microliters of PE-labeled human Th1/Th2/Th17 detection reagents was added to all experimental tubes, and the tubes were incubated in the dark for 3 h before being loaded onto the machine for detection.

CD4+ T cell separation and purification

A total of 5 ml of peripheral blood was collected from the children using EDTA-K2 as the anti-coagulant. Peripheral blood mononuclear cells (PBMCs) were separated using the lymphocyte separation solution and washed with pre-cooled phosphate-buffered saline (PBS) 3 times for future use. The above PBMCs and anti-CD4-labeled magnetic beads were vortexed at 4°C, incubated for 15 min, and passed through the column to separate the CD4+ T cells. The cells were washed with pre-cooled PBS 3 times. After being labeled with anti-CD4-FITC, the purity of the CD4+ T cells was analyzed using flow cytometry.

Cell proliferation experiments

CD4+ T cells with a purity > 95% were adjusted to 1×10^5/ml using RPMI1640 complete medium (10% fetal bovine serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, 2 mmol/L glutamine, and 10 mmol/L HEPES) and cultured in 96-well plates. PHA was added to the solution to reach a final concentration of 10 μg/ml. In addition, PBS was used as the experimental control in the asthma group and the normal control group. The cells were cultured at 37°C and 5% CO_2 for 3 d. Ten microliters of CCK8 was added to each well 4 h before harvesting the cells. Proliferation was observed by measuring the absorbance at 450 nm after 4 h.

AICD experiments

The cell concentration was adjusted to 2×10^5/mL using RPMI complete culture medium, and the cells were cultured in 6-well plates. The cells were combined with 10 μg/ml PHA and

Table 1. Expression of serum T cell-related cytokines of children in the asthma group and the normal control group (x ± s, ng/L)

Group	IL-2	IL-4	IL-6	IL-10	TNF	IFN-γ	IL-17A
Asthma	2.35 ± 0.41	9.33 ± 2.25	3.65 ± 1.42	3.41 ± 0.80	7.63 ± 4.09	2.05 ± 1.33	4.38 ± 1.32
Normal control	1.98 ± 0.66	4.59 ± 1.77	3.10 ± 1.78	1.36 ± 0.41	1.88 ± 0.52	1.99 ± 1.17	4.06 ± 1.50
P value	0.159	0.012	0.358	0.027	0.023	0.845	0.479

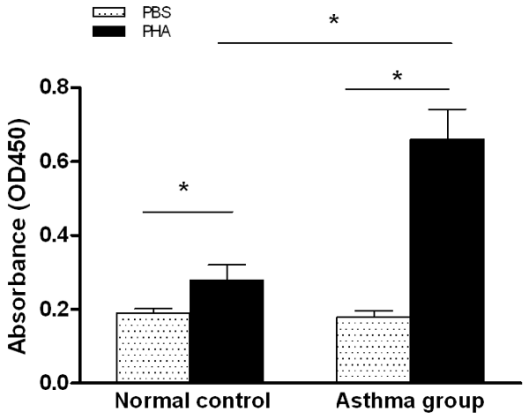

Figure 1. Proliferation of CD4+ T cells from asthmatic or normal children. CD4+ T cells were isolated from asthmatic or normal children and then activated by PHA or PBS. Cell proliferation was observed by measuring the absorbance at 450 nm. *P<0.001 *vs*. control.

cultured at 37°C and 5% CO_2 for 16 h. The cells were washed with sterile PBS 3 times and re-suspended in RPMI1640. The re-suspended cells were continuously cultured in 96-well plates coated with 10 μg/mL anti-CD3 for 12 h. PBS was added to each group of cells as the experimental control. The cells were collected, washed with PBS, and re-suspended in binding buffer (50 mmol/L Tris, 100 mmol/L NaCl, 1% BSA, 0.02% sodium azide, pH = 7.4). The cell concentration was adjusted to 5×10^5/mL, and the cells were thoroughly mixed with Annexin V and PI. Apoptosis was detected using flow cytometry [5].

Quantitative PCR

The total RNA of the CD4+ T cells was first extracted and then reverse transcribed into cDNA. The cDNA was used as the template for quantitative polymerase chain reaction (PCR) amplification using a PCR reaction reagent kit (Applied Biosystems), and β-actin was used as the internal control. $2^{-\Delta\Delta Ct}$ was calculated using the formula ΔCt = [Ct (target gene)]-[Ct (internal control gene)] and ΔΔCt = [ΔCt (asthma group)]-[ΔCt (control group)] to reflect the expression level of the target genes in the asthma group.

Statistical methods

SPSS 18.0 software was used. All data are measurement data and are expressed as mean ± standard deviation (SD). Cytokines were analyzed using analysis of variance, and other indicators were examined using the two-sample *t* test. P<0.05 indicated that the difference was statistically significant.

Results

Cytokine detection results

The serum IL-4, IL-10, and TNF levels of the children in the asthma group were all significantly higher than those of the children in the normal control group, and the differences were statistically significant (P<0.05). The expression levels of IL-2, IL-6, IFN-γ and IL-17A in the asthma group did not have significant changes compared to those in the normal control group, and the differences were not statistically significant (P > 0.05, **Table 1**).

Cell proliferation experiment results

The results of the absorbance of cell proliferation in each group are shown in **Figure 1**. In the normal control group, the proliferation of CD4+ T cells was 0.19 ± 0.02 when treated with PBS and 0.28 ± 0.07 when treated with PHA, (P<0.001) whereas in the asthma group, the proliferation of CD4+ T cells treated with PBS and PHA was 0.18 ± 0.03 and 0.66 ± 0.14 (P<0.001). The proliferation ability of the CD4+ T cells after PHA stimulation in the asthma group significantly increased compared to that in the normal control group (P<0.001). After treatment with PBS, the proliferation abilities between these two groups were not significantly different.

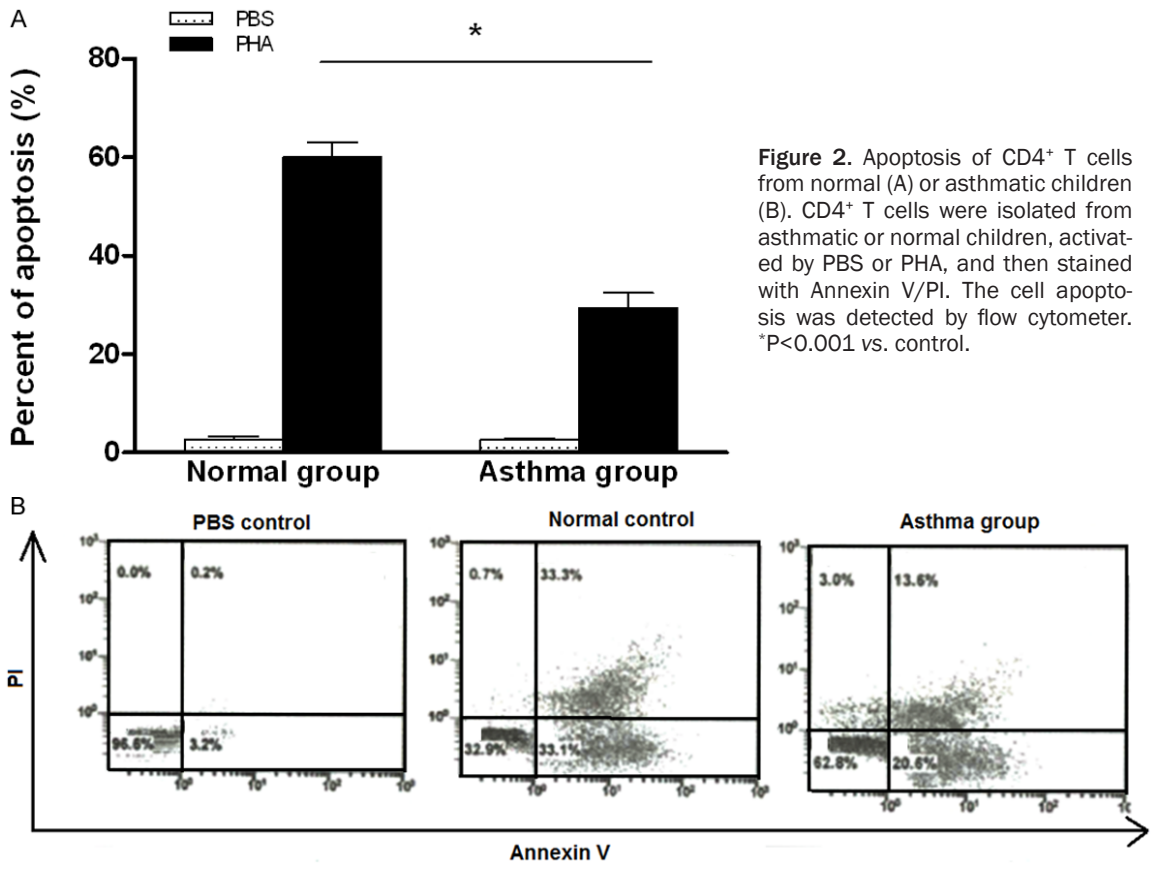

Figure 2. Apoptosis of CD4+ T cells from normal (A) or asthmatic children (B). CD4+ T cells were isolated from asthmatic or normal children, activated by PBS or PHA, and then stained with Annexin V/PI. The cell apoptosis was detected by flow cytometer. *P<0.001 *vs.* control.

Cell apoptosis detection results

In the normal control group, the results were 2.78 ± 1.08 in PBS group and 60.11 ± 4.93 in PHA group (P<0.001) and in the asthma group, the results were 2.66 ± 0.67 in PBS group and PHA 29.46 ± 5.25 in the PHA group (P<0.001). After PHA activation, the cell apoptosis rate in the asthma group was significantly lower than that in the normal control group (P<0.001). The difference after PBS treatment between these two groups was not statistically significant (P > 0.05, **Figure 2**).

The mRNA expression of Fas, FasL, and Bcl-2 in CD4+ T cells from children in the asthma group and children in the normal control group

The expression of Fas mRNA in the CD4+ T cells of children with asthma was significantly lower than that of children in the normal control group, and Bcl-2 expression in the asthma group was significantly higher than that in the normal control group. The differences were both statistically significant (P<0.001). The expression of FasL between these two groups did not display a significant change (P > 0.05, **Figure 3**).

Discussion

Childhood asthma is a complicated allergic disease. The main manifestations are periodic respiratory injury, chronic inflammation of the respiratory tract, and significant lymphocyte and eosinophil infiltration in the submucosal layer of the respiratory tract [2]. Studies have indicated that Th cells play important roles in the developmental process of allergic asthma. Allergens induce the body to produce a large amount of immunoglobulin E (IgE), which can activate eosinophils, basophils, and plasma cells to release various cytokines [3]. These cytokines can promote differentiation of Th cells into Th2 cells in the body to secret Th2-related cytokines and participate in mediation of the immune inflammatory reaction. The studies of Nakajima and Takatsu [6] and Kaminuma et al. [7] showed that IL-4 is involved in the mediation of local tissue inflammatory infiltration in asthma patients and can activate eosinophils to aggravate asthma symptoms.

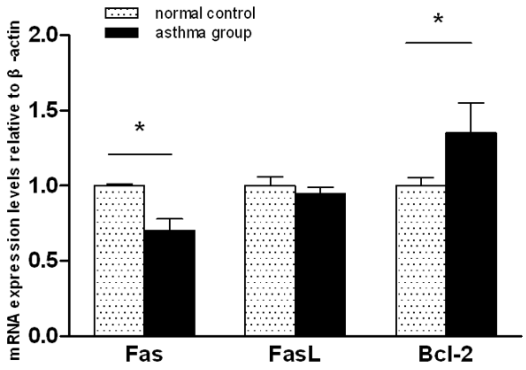

Figure 3. Expression of Fas, FasL, and Bcl-2 mRNA relative to β-actin mRNA in CD4+ T cells from asthmatic or normal children. Quantitative PCR was used to detect the mRNA expression of Fas, FasL, and Bcl-2.

In addition, IL-4 can participate in IgE production through the regulation of class switching. The study by Sahid El-Radhi et al. [8] showed that the IL-4 level in children with acute-phase asthma was significantly higher than that in a mild symptom group, and IL-4 was involved in the immune status changes in children with asthma. We detected serum Th1-, Th2- and Th17-related cytokines in children with asthma using CBA technology and show that IL-4, IL-10, and TNF are all significantly increased. IL-4 and IL-10 are the major Th2-related cytokines, whereas TNF can mediate immune inflammatory responses through promotion of lymphocyte aggregation and secretion of Th2-related cytokines [9].

The inflammatory injury sites of asthma patients contain many activated lymphocytes, and the survival time of these lymphocytes is significantly prolonged [10]. Under normal conditions, the immune system can clear overly activated lymphocytes through AICD to effectively prevent immune inflammatory injury and the development of autoimmune diseases. Because inflammation responses mediated by Th cells and its related cytokines play a leading role in asthmatic patients, we separated peripheral CD4+ T cells from children to perform *in vitro* stimulation to observe the ability of cell proliferation and the condition of AICD. The results show that CD4+ T cells in the asthma group are significantly enhanced after PHA stimulation compared to those in the normal control group, whereas AICD is inhibited. Furthermore, the apoptosis rate was significantly decreased. Therefore, lymphocyte infiltration and the prolongation of lymphocyte survival time in children with asthma might be induced by the joint effect of defects in the AICD clearance mechanism and the significant enhancement of cell proliferation ability.

AICD is cell apoptosis mediated by death receptors. Its main pathway is to recruit the adaptor protein FAS-associated death domain (FADD) through the interaction between Fas and FasL to activate downstream apoptosis-related proteins and induce cell apoptosis [11]. Under normal conditions, the body will clear overly activated T cells through AICD. However, children with asthma have many activated CD4+ T cells in the peripheral blood and tissues. Therefore, the AICD process in children with asthma might be suppressed to some extent. Detection of Fas and FasL expression in CD4+ T cells of children with asthma using quantitative PCR showed that Fas mRNA expression in the asthma group was significantly decreased compared to that in the normal control group, and FasL expression was not significantly different. These results indicate that reduction of Fas expression might be an important reason for the suppression of AICD. A study showed that the expression of the Fas protein on the surface of T cells derived from the lungs of asthma patients significantly decreased [12] and that result was consistent with our study results. Another study showed that co-culturing IL-4 with T cells could down-regulate Fas expression on the surface of T cells and that IL-4 could induce T cells to express Bcl-2 to inhibit cell apoptosis and promote cell proliferation [13]. We found that serum IL-4 levels in children with asthma significantly increased and that expression of Bcl-2 mRNA in CD4+ T cells also increased. Therefore, the inhibition of apoptosis and enhancement of proliferation ability in the CD4+ T cells of children with asthma might have an important relationship with the increase of IL-4 levels.

In summary, Th2-related cytokines significantly increased in children with asthma, the proliferation ability of CD4+ T cells significantly increased, and AICD was inhibited. The possible mechanism might be the reduction of the Fas expression level and the increase of Bcl-2 expression in CD4+ T cells caused by the increased IL-4 expression level.

Disclosure of conflict of interest

None.

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