Enhanced proliferation and defective activation-induced cell death of CD4+ T cells in childhood asthma

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Summary

Background: **Hyperactivation of CD4+ T cells in peripheral blood and airway tissues has been suggested to play a key role in the development and maintenance of chronic inflammation in childhood asthma. However, the underlying mechanisms are not yet clear.**

Objective: **To investigate alterations in serum levels of T helper cell-related cytokines, mitogen-stimulated CD4+ T cell proliferation and activation-induced cell death (AICD) in childhood asthma.**

Methods: **21 children with untreated asthma and 21 healthy volunteers (age and gender matched) participated in this study. Th1/Th2/Th17 cytokines in serum were analyzed by flow cytometry. CD4+ T cells were isolated from participants by using immuno-magnetic beads and were stimulated by phytohemagglutinin (PHA). Cell proliferation was evaluated with a Cell Counting Kit-8 (CCK-8). Activation induced cell death (AICD) of CD4+ T cells was also induced by PHA and apoptosis was assayed by annexin V/PI staining. Quantitative RT-PCR was carried out to analyze Fas and FasL mRNA expression. $FLIP_L$, caspase-8 and Bcl-2 were detected by western blot analysis.**

Results: **In children with asthma, the proliferative capacity of CD4+ T cells was enhanced and AICD was inhibited significantly, while serum IL-4, IL-10 and TNF were markedly higher compared with the control group. Fas mRNA expression in the asthma group was obviously lower than that in the control group, while no change was detected in FasL mRNA expression. Western blot analysis showed that the levels of the anti-apoptotic proteins, $FLIP_L$ and Bcl-2 in CD4+ T cells of the asthma group were significantly higher than in the control group. Spearman's correlation tests showed that only IL-4 correlated positively with $FLIP_L$ and Bcl-2 expression, while IL-10 and TNF were unrelated to $FLIP_L$ and Bcl-2 expression.**

Conclusions: **These results indicate that enhanced proliferation and defective AICD of CD4+ T cells influence the T cell-mediated inflammatory reaction in childhood asthma and that increased IL-4, $FLIP_L$ and Bcl-2 expression and decreased Fas expression jointly participate in these changes in cell proliferation and apoptosis.** ***(Asian Pac J Allergy Immunol 2014;32:75-83)***

Key words: *childhood asthma, T cells, proliferation, apoptosis, $FLIP_L$, Bcl-2*

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Introduction

Childhood asthma is an immune-mediated and heterogeneous disease that is characterized by chronic inflammation of the airways and infiltration of lymphocytes into the airway submucosa.[1,2] Antigens in the external environment induce immune tolerance, especially T cell tolerance, through contact with the mucosal immune system in tissues such as the respiratory and intestinal tracts, in a process that effectively protects the organism and avoids the occurrence of asthma. Chu KH and Chiang BL demonstrated that regulatory T cells

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induced by mucosal B cells alleviate allergic airway hypersensitivity.[3] One of the characteristics of asthma is hyperactivation of CD4+T cells in peripheral blood and the airway tissues , which causes rapid proliferation of T cells and secretion of large amounts of cytokines that exacerbate the inflammatory reaction.[4] In allergic inflammation, lymphocytes are known to accumulate at the site of inflammation partly because of their prolonged survival.[5] The exact causes and underlying mechanisms are not yet clear, although in vivo, T cell activation-induced cell death (AICD) is an important mechanism for removal of hyperactivated T cells and maintenance of peripheral immune tolerance.[6] T cell-mediated immune injury occurs when the balance of immune tolerance is destroyed by external factors.[7,8]

The activation-induced cell death signal is transmitted through the binding of Fas ligand (FasL) to its receptor Fas.[9] Spinozzi et al. observed significantly decreased T cell surface Fas expression in the lungs of patients with asthma and inhibition of Fas expression following T cell co-culture with IL-4 in vitro.[10] The death inducing signaling complex (DISC) formed by the death receptor Fas, the adapter protein FADD and caspase-8 mediates the extrinsic apoptotic program.[11] When recruited to the DISC, pro-caspase-8 is activated through a series of proteolytic cleavage steps.[9] Fas-associated death domain-like interleukin-1-β-converting enzyme-inhibitory protein (FLIP) inhibits death receptor-mediated pro-apoptotic signals by blocking the signaling pathway prior to caspase-8 activation.[12-14] Two forms of FLIP, $FLIP_L$ (long form) and $FLIP_S$ (short form), have been characterized. $FLIP_L$ is homogenous with caspase-8 but does not exhibit caspase activity.[15,16] Our previous study showed defective AICD and elevated $FLIP_L$ expression in CD4+ T cells in an animal model of primary biliary cirrhosis (PBC), a type of autoimmune inflammatory disease, which indicated that AICD is of great importance in the maintenance of immune tolerance and inflammation.[17] However, the roles of CD4+ T cell AICD and the signaling pathway in inflammatory infiltration in children with asthma remain to be elucidated. The present study was performed to investigate AICD and proliferation of peripheral blood CD4+ T cells in childhood asthma.

Methods

Study population

The study group was comprised of 21 children (13 males and 8 females , mean age 9.6±2.3 years) with allergic asthma of whom 4 had intermittent, 8 mild, 5 moderate, and 4 had sever persistent asthma. The diagnosis of asthma and the assessment of severity were done according to the GINA 2002 criteria.[18] All children had a history of recurrent episodes of airway obstruction. Children presenting with wheezing due to non-asthmatic chronic airway diseases or patients with autoimmune inflammatory diseases were excluded from this study. All subjects in the study group had positive skin prick tests (SPTs) to one or more allergens. SPTs were performed with a panel of aeroallergens including pollens, house dust mites, animal dander and moulds (Allergopharma, Reinbek, Germany). A positive SPT was defined as a mean diameter of at least 3 mm in the presence of negative normal saline and positive histamine controls. The degree of allergic sensitization was measured by the wheal size of the SPTs. The normal control (NC) group consisted of 21 healthy children (12 males and 9 females, mean age 9.7±1.9 years) with no history of asthma, no record of food allergy, allergic rhinitis or eczema, and no family history of asthma. This study was approved by the Medical Ethics Committee of Changshu No.1 People's Hospital. Written informed consent was obtained from children's parents or guardians.

Cytokines

IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17A were assayed using a cytometric bead array (CBA) human Th1/Th2/Th17 cytokine kit (BD, USA). Serial dilutions of samples were prepared (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256). Capture Bead suspension was vortexed vigorously (3-5s) prior to addition (10 μl) to each sample. The mixture was vortexed thoroughly. Capture Beads were re-suspended in serum enhancement buffer and incubated for 30 min at room temperature with light protection. The cytometer was calibrated using setup beads. Capture Beads (50 μl) and secondary antibodies were added to control and sample tubes and incubated for 3 h at room temperature with light protection. Sample data were acquired by flow cytometry (FACSCalibur, BD) and analyzed using FCAP Array software.

CD4+ T cell isolation and purification

Heparinized peripheral blood (10 ml) was collected from each patient and control subject. Peripheral blood mononuclear cells (PBMCs) were isolated from participants by means of Ficoll

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density-gradient centrifugation and were collected and washed once with RPMI1640 medium (Gibco, USA). Cell viability assayed by trypan blue exclusion was greater than 95% in all cases. Immuno-magnetic beads (Miltenyi Biotec, Germany) were used to separate CD4+ T cells and cell purity exceeded 95%. The viability of all samples was greater than 95% as verified by Trypan blue exclusion.

Cell proliferation in vitro

CD4+ T cells were re-suspended at 1×10^6 cells/ml in complete medium (RPMI1640 supplemented with 10 fetal calf serum (FCS), 50 μg/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, 10 mM HEPES). Cells were cultivated in a 96-well plate and were activated with 10 μg/ml phytohemagglutinin (PHA) (Sigma, USA) for 3 days. Phosphate buffered saline (PBS) was used as a blank control in each group. 10 μl CCK-8 (Cell Counting Kit-8, Dojindo, Japan) was added to each well 4 h before the end of the stimulation when optical density (OD) values were measured at 450 nm. Data are presented as the mean±SD of triplicate wells.

Activation-induced cell death in vitro

CD4+ T cells were adjusted to 2×10^5 cells/ml in complete medium and plated (3 ml) in a 6-well plate. PHA was added at a final concentration of 10 μg/ml. Cells were cultured for 16 h before being collected, washed three times with PBS and re-suspended in complete medium. Cells were then cultured for 12 h in 96-well plates coated with 10 μg/ml anti-CD3 (Abcam, UK). PBS was used as blank control in each group. Apoptosis was analyzed by annexin V/PI staining (Bender MedSystems, Austria) using an FC500 flow cytometer (Beckman coulter, USA) as previously described.[17]

Quantitative RT-PCR analysis

Total RNA was extracted from CD4+ T cells using by the TRIzol (Invitrogen, USA) method. Total RNA (1 μg) was reverse transcribed using an RT kit (Invitrogen, USA). The resulting cDNA was used as a template and β-actin was used as an internal reference. Fas and FasL mRNA expression was detected using an ABI PRISM 7900 Sequence Detection System quantitative PCR instrument (Applied Biosystems, USA) with the following cycling conditions: 55°C for 2 min, 94°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle Threshold (Ct) values were read automatically and the value of $2^{-\Delta\Delta Ct}$ was calculated to quantify the expression of the target gene in each group. Primers were as follows:

Fas forward: CCGGAGCGGACCTTTGGCTTG
Fas reverse: CGTGGGGTGCGGACAGGAAT
FasL forward: GTGCCCAGAAGGCCTGGTCAA
FasL reverse: CAGGCCTGTGCTGTGGTTCCC
β-actin forward: GAAATCTGTCAAAGTTCA
β-actin reverse: AGGCAGCTCGTAGCTCTT

Western blot analysis

Protein concentration was determined following lysis using the Bradford method with BSA as a standard substance. Cell lysate samples (30 μg) were resolved by SDS-PAGE and electro-transferred to nitrocellulose membranes. The blots were probed with anti-$FLIP_L$ (Santa Cruz, USA), anti-caspase-8 (Cell Signaling Technology, USA) and anti-Bcl-2 (Cell Signaling Technology, USA) and detected with an anti-rabbit HRP-conjugated antibody. β-actin (Sigma, USA) was used as an internal reference. Levels of relative expression were assayed by densitometric scanning (Tanon, China). $FLIP_L$, caspase-8 and Bcl-2 expression was standardized against an arbitrary control value of 1.0.

Statistical analysis

Plasma cytokine concentrations were not in a Gaussian distribution. Therefore, the Mann-Whitney rank sum test was used to analyze differences in the concentration of cytokines in childhood asthma

Table 1. Serum cytokines characteristic of Th1, Th2 and Th17 cells in patients with childhood asthma (N = 21) and control subjects (N = 21)

Group	Cytokines (pg/ml)						
	IL-2	IL-4	IL-6	IL-10	TNF	IFN-γ	IL-17A
Childhood asthma	3.28	5.68	3.47	5.14	6.35	8.70	--
Median(min-max)	(2.64-3.92)	(3.50-8.38)	(1.11-12.70)	(4.61-7.15)	(4.75-25.84)	(8.3-12.56)	
Normal control	3.09	5.16	3.55	4.86	4.91	5.01	--
Median(min-max)	(2.72-3.64)	(4.97-7.80)	(2.34-6.74)	(4.55-5.14)	(4.62-7.23)	(4.82-9.96)	
***P* value**	0.172	0.018	0.429	0.006	0.009	0.966	--

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patients and normal controls. The Student's *t* test was used to assess differences in cell proliferation, apoptosis in AICD, relative mRNA expression and signaling protein levels. The correlation of two parameters was calculated with the Spearman correlation test. A probability of $P < 0.05$ was considered to be significantly different. All analyses were performed using the SPSS (version 11.0).

Results

Serum concentrations of T helper cytokines

Animal models and clinical studies in humans have indicated an important role for T helper (Th) cells producing multiple cytokines in the pathogenesis of allergic asthma.[16,17] In this study, we conducted a flow cytometric analysis of Th1/Th2/Th17-related cytokines to further elucidate the expression patterns of cytokines in the peripheral blood of children with asthma. Serum levels of IL-4, IL-10 and TNF in the childhood asthma group were significantly higher than those in the normal control group ($P = 0.018$, 0.006 and 0.009 respectively). In contrast, no differences were detected in the expression of IL-2, IL-6 and IFN-γ between the two groups ($P > 0.05$). IL-17A was not determined in each group.

Proliferation of CD4+ T cells

Patients with asthma show chronic respiratory inflammation and lymphocyte infiltration. Based on this information, it was hypothesized that T cell proliferation is altered by the inflammatory environment. CD4+ T cell proliferation was analyzed in response to PHA, with PBS as the negative control, and measured in terms OD values. The following results are presented in Figure 1: normal control group, PBS: 0.186±0.013, PHA: 0.274±0.032, $P < 0.001$; asthma group, PBS: 0.178±0.015, PHA: 0.498±0.052, $P < 0.001$. These data demonstrate that the proliferative capacity of CD4+ T cells in the asthma group was significantly higher than that in the normal control group ($P < 0.001$). No significant differences in proliferative capacity were observed between the two groups in response to PBS.

Activation-induced cell death of CD4+ T cells

In normal situations, the immune system removes hyper-activated T cells through AICD. However, activated CD4+T cells exist at high frequencies in the peripheral blood and tissues of asthma patients.[8,19] CD4+ T cells isolated from children with asthma were stimulated in vitro with

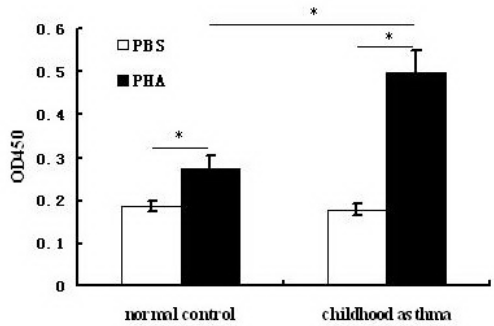

Figure 1. PHA-stimulated (10 μg/ml) proliferation of CD4+ T cells isolated from patients with childhood asthma and normal control subjects. PBS was used as blank control in each group. OD450 of CD4+ T cells in patients with childhood asthma was higher than that in control subjects and blank control (*$P < 0.001$).

PHA and anti-CD3 and apoptosis was analyzed by flow cytometry. The following results are presented in Figure 2: normal control group, PBS: 2.78±1.08, PHA: 65.28±3.85, $P < 0.001$; asthma group, PBS: 2.66±0.67, PHA: 35.62±0.05, $P < 0.001$. The rate of apoptosis in CD4+ T cells following stimulation with PHA and anti-CD3 was significantly lower in the asthma group compared with the normal control group ($P < 0.001$). No significant differences in the rate of apoptosis were observed between the two groups in response to PBS ($P > 0.05$).

Quantitative RT-PCR analysis

The induction of apoptosis mediated by the interaction of Fas and its ligand FasL is the main mechanism of AICD.[9] Relative quantitative PCR analysis of Fas and FasL mRNA expression in CD4+T cells showed that Fas mRNA expression in CD4+T cells was markedly lower in the asthma group than in the normal group ($P < 0.001$) (Figure 3). However, no significant difference in FasL expression was observed between the two groups ($P > 0.05$).

Western blot analysis

Western blot analysis was used to detect expression of caspase-8 and the anti-apoptotic proteins $FLIP_L$ and Bcl-2 in peripheral blood CD4+T cells in patients with childhood asthma and normal control subjects (Figure 4). Compared with the normal control group, no obvious difference was observed in caspase-8 expression in the asthma group, while $FLIP_L$ and Bcl-2 expression were markedly increased. Correlation analysis of the

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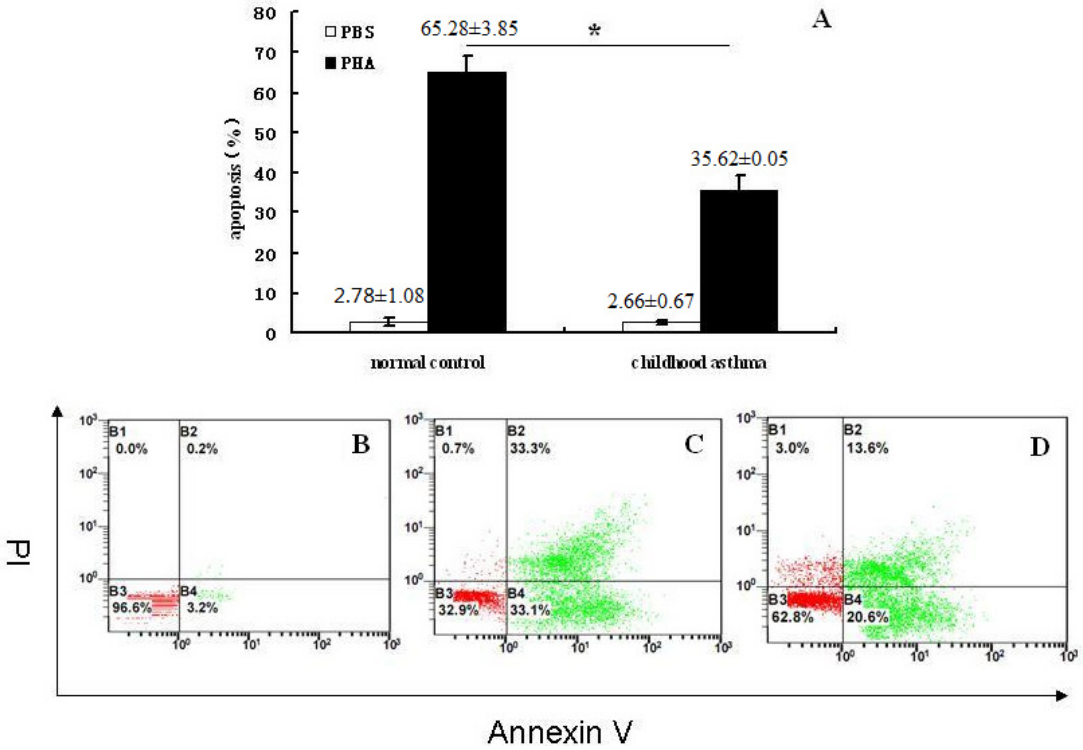

Figure 2. Apoptosis of CD4+ T cells activated by PHA (10 μg/ml) and anti-CD3 (10 μg/ml) (A) and representative dot-blot (FITC-Annexin V versus PI) in PBS (B), normal control (C) and childhood asthma (D) groups. Apoptosis was assayed by flow cytometry with annexin V-/PI+, annexin V+/PI- and annexin V+/PI+ representing dead cells, apoptotic cells and late stage apoptotic cells, respectively. Apoptosis was significantly inhibited in patients with childhood asthma compared to control subjects (*P<0.001).

relationship between IL-4, IL-10 and TNF and the relative expression of $FLIP_L$ and Bcl-2 showed that IL-4 correlated positively with $FLIP_L$ and Bcl-2 expression, although no correlation was detected between the expression of IL-10 and TNF and these two molecules (Figure 5).

Discussion

Childhood asthma is a complex allergic disease, mainly manifested as periodic respiratory injury, chronic respiratory inflammation and respiratory submucosal infiltration of large numbers of lymphocytes and eosinophils.[1,20] Studies have shown that Th cells play an important role during the onset of allergic asthma and allergens induce the immune system to generate large amounts of IgE, which activate eosinophils, basophils and mast cells to release a variety of cytokines. These cytokines promote Th cells to differentiate into Th2 cells and secrete Th2-related cytokines to mediate inflammatory infiltration of respiratory tissues in childhood asthma.[21] Investigation of the profile of serum Th1/Th2/Th17-related cytokines in children with asthma revealed significantly higher levels of IL-4, IL-10 and TNF compared with control individuals. Of these, IL-4 is the main Th2-related cytokine and TNF promotes aggregation of lymphocytes and secretion of Th2 cytokines to mediate inflammatory reactions.[22] Kaminuma observed that IL-4 participates in the mediation of local tissue inflammatory infiltration in patients with asthma and also activates eosinophils and aggravates asthma symptoms.[23] Furthermore, IL-4 participates in IgE generation through regulation of class switching.[24] These observations indicate that IL-4, IL-10 and TNF play important roles in respiratory inflammation in childhood asthma.

The site of inflammatory injury in patients with asthma contains large amounts of activated lymphocytes with significantly prolonged survival.[25] In normal situations, the immune system eliminates hyperactivated lymphocytes through the AICD mechanism, which effectively prevents the occurrence of immune inflammatory injury and

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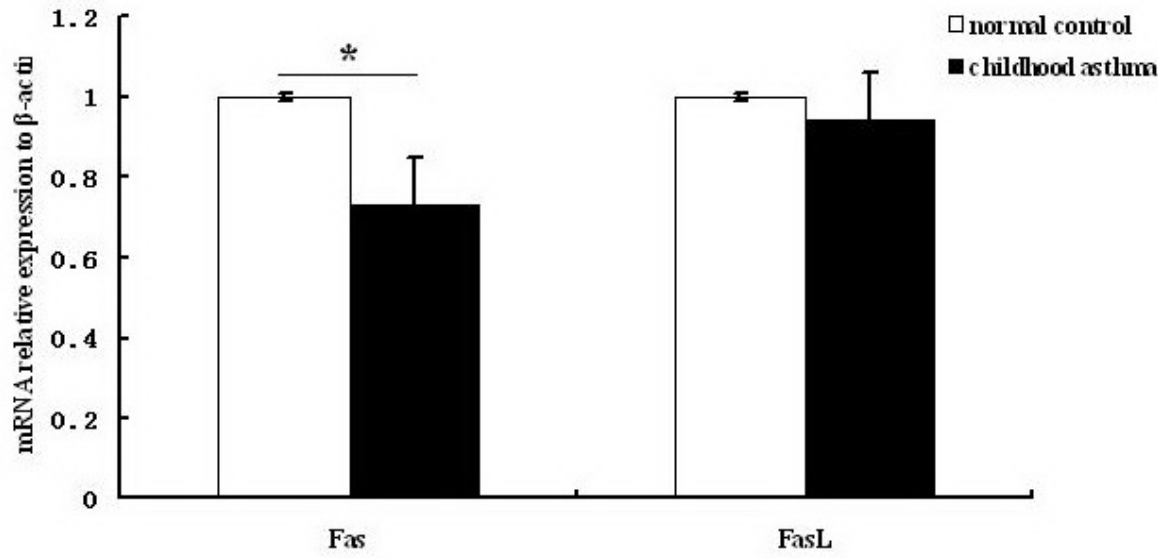

Figure 3. CD4+ T cell expression of Fas and FasL mRNA relative to β-actin in patients with childhood asthma and normal subjects. Fas mRNA expression in the childhood asthma group was significantly lower than that in control group. However, no obvious difference in FasL mRNA expression was observed between the two groups (*P<0.001).

autoimmune diseases.[26] Inflammatory infiltration, which is predominantly mediated by Th cells and the related cytokines, plays a dominant role in asthma. Therefore, proliferation and apoptosis of peripheral blood CD4+T cells stimulated with PHA were analyzed. Compared with the normal control group, PHA-stimulated proliferation of CD4+T cells in the asthma group was significantly enhanced, while apoptosis was significantly reduced. Thus, defective AICD and significantly enhanced proliferation may contribute jointly to inflammatory infiltration and prolonged survival of CD4+ T lymphocytes in patients with childhood asthma.

AICD is a death receptor-mediated form of apoptosis, mediated predominantly by the interaction of Fas and FasL resulting in recruitment of the adaptor protein FADD and activation of downstream apoptosis-related proteins.[27] Relative quantitative PCR analysis of CD4+T cell Fas and FasL expression in children with asthma demonstrated that Fas mRNA expression in the asthma group was significantly reduced compared with the control subjects, while no obvious difference was detected in FasL expression. These data indicate that reduced Fas mRNA expression may be one of the reasons for AICD inhibition. Studies have shown that Fas protein expression by lung T cells is significantly reduced in patients with asthma,[28] which is partially in accordance with our results. $FLIP_L$ inhibits Fas-FasL signaling pathway at the caspase-8 activation level to prevent the occurrence of apoptosis.[29] In spite of this apoptosis-inhibitory function, $FLIP_L$ also promotes cell proliferation and inflammatory reactions.[30] Western blot analysis of CD4+T cell caspase-8 and $FLIP_L$ expression in patients with childhood asthma and the control subjects showed that $FLIP_L$ expression in the childhood asthma group was significantly higher than that in control group, although no difference was detected in caspase-8 expression between the two groups. This implies that increased $FLIP_L$ expression and decreased Fas expression may be the main causes of CD4+T cell apoptosis-inhibition in childhood asthma and furthermore may be important in promoting cell proliferation and inflammatory infiltrations.

IL-4 downregulates Fas expression on T cells and induces Bcl-2 expression to inhibit apoptosis and promote proliferation.[31] Our study showed that both the serum IL-4 and Bcl-2 expression by CD4+ T cells were significantly increased in children with asthma. Accordingly, it can be speculated that apoptosis inhibition and enhanced proliferation of CD4+ T cells are related to this increase in IL-4

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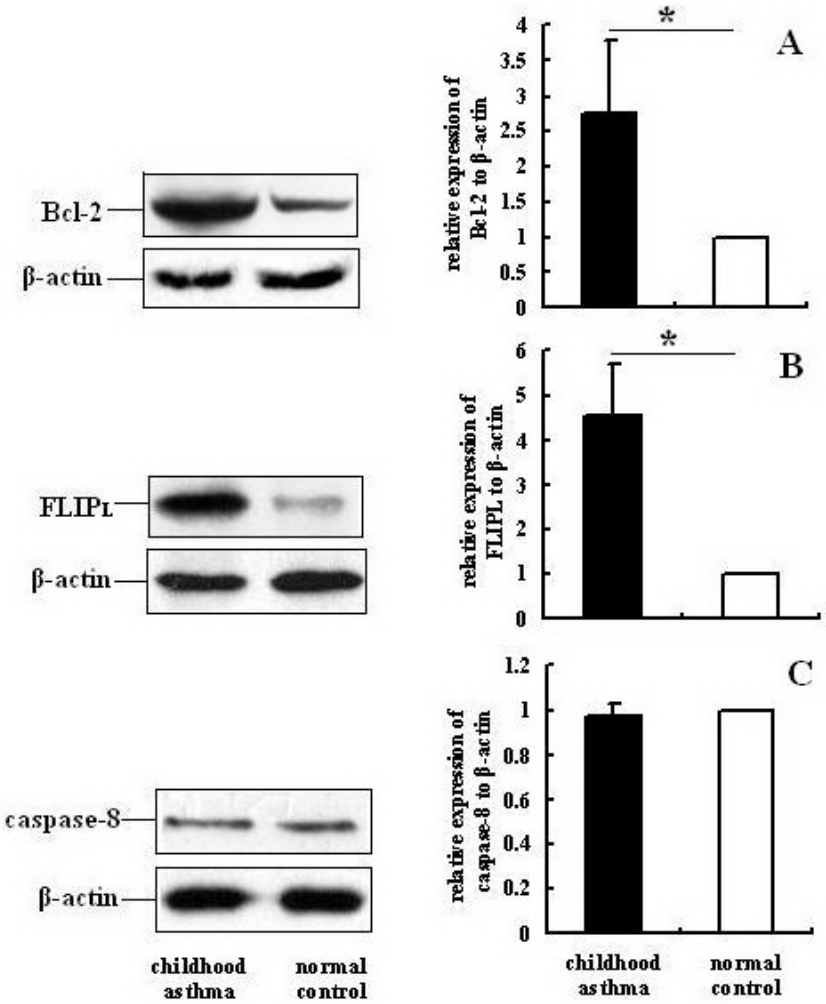

Figure 4. CD4+ T cell proliferation and apoptosis signaling in patients with childhood asthma and control subjects. Expression of Bcl-2 (A) and $FLIP_L$ (B) relative to β-actin expression was significantly higher in the childhood asthma group than in the control group although no obvious difference in caspase-8 (C) expression was detected between the two groups (*P<0.001).

expression. Correlation analysis demonstrated a positive correlation between serum IL-4 and $FLIP_L$ and Bcl-2 expression in the asthma group, which further confirms the hypothesis that IL-4 contributes to proliferation and AICD of CD4+ T cells.

In conclusion, raised IL-4, $FLIP_L$ and Bcl-2 expression and decreased Fas expression in childhood asthma may jointly determine defective AICD and enhanced proliferation of CD4+T cells and play important roles in promoting chronic inflammation. This study provides the basis of further studies of the pathogenesis and immune therapy of childhood asthma.

Acknowledgments

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Conflict of interest

We declare that we have no conflict of interest related to the publication of this manuscript.

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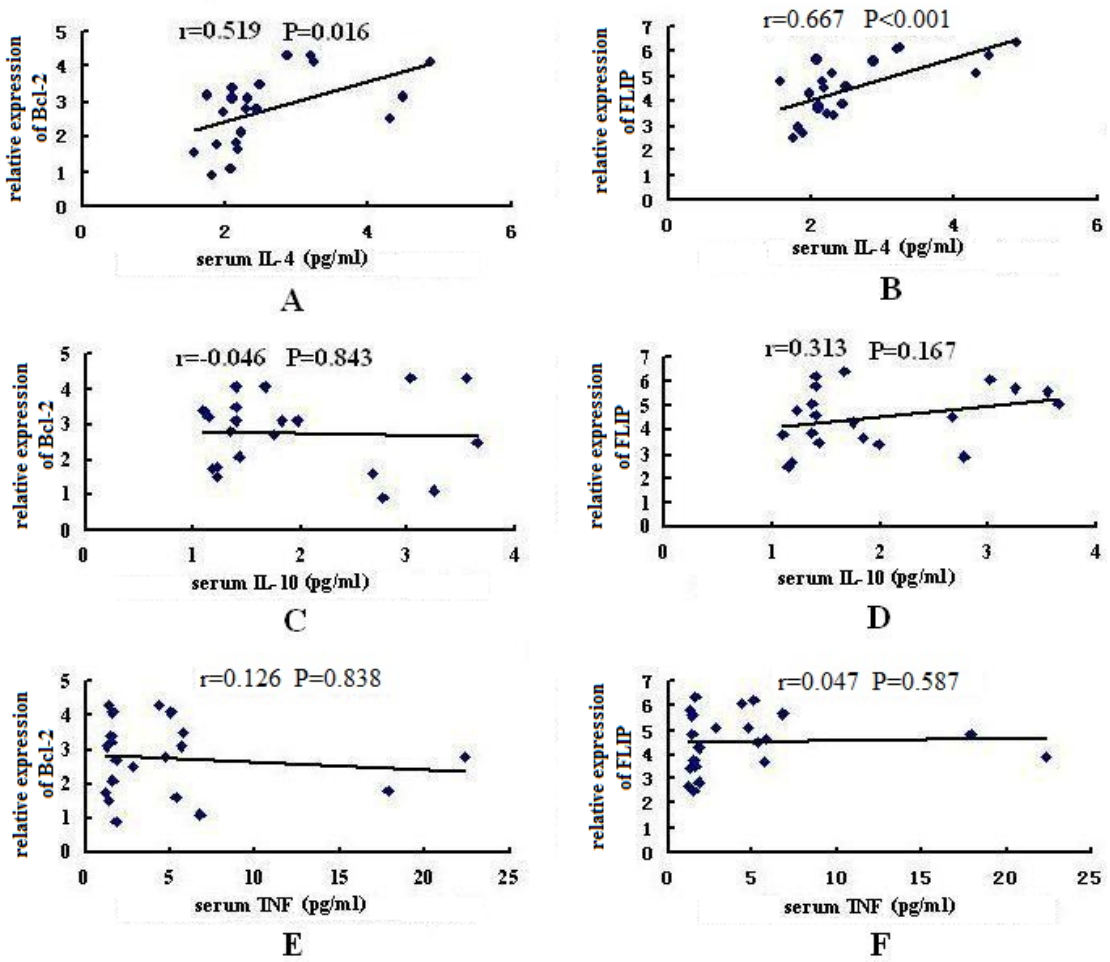

Figure 5. Results of Spearman's correlation test between CD4+ T cell serum cytokines and signaling proteins in the childhood asthma group. (A) Correlation between serum IL-4 and Bcl-2 (r=0.519, *P*=0.016); (B) Correlation between serum IL-4 and $FLIP_L$ (r=0.667, *P*<0.001); (C) Correlation between serum IL-10 and Bcl-2 (r=-0.046, *P*=0.843); (D) Correlation between serum IL-10 and $FLIP_L$ (r=0.313, *P*=0.167); (E) Correlation between serum TNF and Bcl-2 (r=0.126, *P*=0.838); (F) Correlation between serum TNF and $FLIP_L$ (r=-0.047, *P*=0.587).

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