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Original Article

The characterization and clinical significance of PI3K/Akt signaling pathway activation in the peripheral T cells of pediatric patients with atopic dermatitis

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Abstract: Objective: To investigate the activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway in the peripheral T cells of patients with atopic dermatitis (AD) and to explore its clinical significance. Methods: Peripheral T lymphocytes were isolated with a T cell isolation kit; PI3K expression was detected by immunoprecipitation and with an enzyme-linked immunosorbent assay (ELISA); Akt and its phosphorylation were analyzed with western blotting; T cell proliferation was analyzed with the MTT method; and interleukin (IL)-6 and IL-10 levels were detected with ELISA. Results: PI3K and p-Akt/Akt expression in freshly prepared peripheral T cells from AD patients were significantly higher than those in healthy controls ($P < 0.05$). When T cells from healthy controls were incubated with sera from AD patients for 24 hours, their PI3K and p-Akt/Akt expression significantly increased ($P < 0.05$). Treatment with the PI3K inhibitor LY294002 combined with a CD3/CD28 monoclonal antibody (mAb) significantly reduced the CD3/CD28 mAb-induced proliferation of T cells (195% ± 28% vs. 125% ± 22%, $P < 0.05$), IL-6 secretion (823 ± 128 ng/L vs. 431 ± 64 ng/L, $P < 0.05$), and IL-10 secretion (213 ± 35 ng/L vs. 120 ± 21 ng/L, $P < 0.05$) in AD patients. In addition, the PI3K and p-Akt/Akt protein levels in freshly prepared peripheral T cells from AD patients were unrelated to the patients' Eczema Area and Severity Index (EASI). Conclusion: The PI3K/Akt signaling pathway is abnormally activated in peripheral T cells from AD patients, and its activation corresponds to T cell proliferation and cytokine secretion, suggesting that serum cytokines that activate this pathway may be present in the peripheral blood of AD patients.

Keywords: Atopic dermatitis, T cell, PI3K/Akt signaling pathway

Introduction

Atopic dermatitis (AD) is a chronic, pruritic, inflammatory skin disease most common in early childhood, although up to 40% of the patients remain symptomatic throughout adulthood. Its cause is associated with many factors [1]. The pathogenesis of AD has been attributed to a complex interaction including environmental factors, host susceptibility genes, altered skin barrier function, and a deregulated immune system. T cells may play an important role in the development of AD [2-5]. Phosphoinositide 3-kinase (PI3K) is a key signaling protein that regulates T cell proliferation, activation and migration. PI3K protein is low in normal quiescent T cells and is significantly increased only when T cells are activated. Thus, we analyzed the expression of the PI3K/protein kinase B (Akt) signaling pathway in peripheral T cells from AD patients and explored the role and clinical significance of PI3K/Akt expression in the development of AD.

Patients and methods

Patients

A total of 30 pediatric AD patients (**Table 1**) who were treated at our hospital from October 2011 to October 2013 were included in this study. Their condition met the diagnostic criteria of Williams AD [6]. There were 8 male patients and 22 female patients who were aged 2 to 14 years (mean, 10 years). The severity of their AD was assessed according to the Eczema Area

Table 1. The characteristics of the patients

Characteristic	Normal individuals	All patients N = (30)
Female, No. (%)	22 (73)	22 (73)
Age, y (range)	10.5 (3-14)	10 (2-14)
Eczema Area and Severity Index (range)	-	6.23 (3.5-14.1)

and Severity Index (EASI) [7], and their EASI scores ranged from 3.5 to 14.1 (mean: 6.23 ± 2.71). None of the patients had a family history of allergies or a medical history of other marked skin lesions, autoimmune disease, respiratory disease, liver disease or tumors. Before this study, antihistamines had been discontinued for ≥ 2 weeks, and systemic and local glucosteroids and other immunosuppressants had been discontinued for ≥ 1 month. Thirty individuals were included in the healthy control group, of which 8 were male and 22 were female and who were aged 3 to 14 years (mean: 10.5 years). No significant difference was observed in gender or age between the 2 groups. This study was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

Isolation and culture of peripheral T cells

A peripheral blood sample (6 mL) was collected from each patient or healthy individual, and T cells were isolated according to the instructions for the RosettSep T cell isolation kit (StemCell Technologies, Canada). The purity of the isolated T cells was analyzed with flow cytometry and with an anti-CD3 monoclonal antibody (mAb) (BD Pharmingen); this analysis showed that the purity of the T cells was ≥ 95%. The isolated T cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Gibco), and the cells were treated with CD3 mAb (5 mg/L, BD Biosciences) and CD28 mAb (5 mg/L) or with the PI3K inhibitor LY294002 (10 μmol/L, Chemicon) as appropriate. A trypan blue exclusion assay was performed to analyze cell viability and showed that the cell viability was ≥ 95%.

Serum preparation and processing

Serum was routinely collected and stored at -80°C for subsequent use. To investigate the effect of AD patient serum on T cells from healthy controls, sera from AD patients were inactivated at 56°C for 30 minutes, centrifuged at 3,000 rpm for 10 minutes to remove the precipitate, and stored for later use.

Extraction of total cellular protein

T cells were washed once with cold 1 × PBS. Next, 50 μL of SDS sample lysis buffer A was added, and the T cells were then homogenized, placed on ice, sonicated for 10 to 15 seconds, boiled at 95°C for 5 minutes, placed on ice, and centrifuged at 850 g for 5 minutes. The resulting supernatant was collected, aliquoted, and stored at -20°C.

PI3K expression assay

A total of 2 × 10^6 T cells were washed with serum-free medium and placed on ice. Next, 2 mL of ice-cold buffer A (137 mmol/L NaCl, 20 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 0.1 mmol/L n-vanadate solution) was added and used to wash the cells 3 times. Lysis buffer (0.5 mL) was added, and the cells were incubated on ice for 20 minutes. The lysed cells were transferred to a 1-mL centrifuge tube and centrifuged for 10 minutes to precipitate any undissolved substances. After centrifugation, the supernatant was transferred to another centrifuge tube, 5 μL of anti-PI3K antibody was added, and the tube was incubated at 4°C for 1 hour. Next, 60 μL of 50% Protein A beads (dissolved in PBS) was added and mixed, and the mixture was incubated at 4°C for 1 hour. After centrifugation, the pellet was washed 3 times with 50 mmol/L Tris-HCl (pH 7.4), and the PI3K expression was analyzed by ELISA according to the manufacturer's instructions (Echolon).

Western blotting

Protein samples were boiled at 95-100°C for 5 minutes, and then equal volumes of the samples were electrophoretically separated by 15% SDS-PAGE. After electrophoresis, the proteins were transferred to a cellulose acetate membrane with transfer buffer, and the membrane was incubated with different concentrations of rabbit anti-human phosphorylated Akt (p-Akt) mAb (Cell Signaling) at 4°C overnight, followed by incubation with a secondary antibody at room temperature for 1 hour. After a TBST wash, an ECL solution was placed onto the

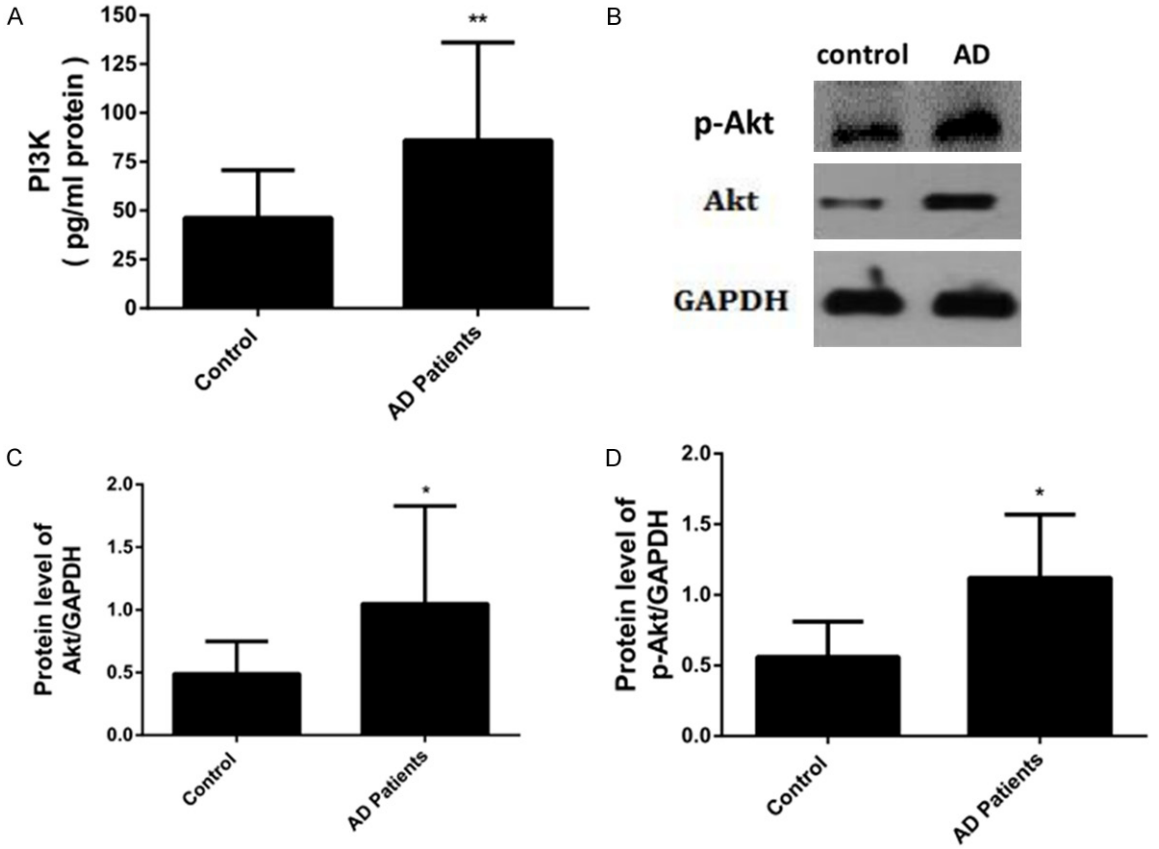

Figure 1. Expression of PI3K (A), p-Akt/Akt (B-D) in T cells from AD patients and healthy controls. The protein levels of PI3K in T cells were assessed by ELISA (A). The protein levels of p-Akt/Akt in T cells were assessed by western blot (B). The relative densities of p-Akt (C) and Akt (D) were corrected using GAPDH. Values represent means + SD at thirty experiments. *P < 0.05, **P < 0.001. Compared with healthy controls, *P < 0.05 **P < 0.001.

membrane for color development, and the membrane was analyzed with an imaging system. The optical density (A) was calculated, and the Akt expression was measured as the ratio of the A value of phosphorylated Akt to the A value of GAPDH.

MTT assay or Trypan blue assay of T cell proliferation

To analyze T cell proliferation, T cells were divided into 3 groups, namely those cultured in serum alone, those stimulated by a CD3/CD28 mAb, and those stimulated by CD3/CD28 mAb in the additional presence of LY294002. Each group was performed in a set of 3 duplicate samples. T cells (1×10^5) were seeded in 96-well plates and cultured in 100 μL of RPMI 1640 medium for 48 hours. The MTT assay was performed according to procedures described in the literature [8].

Also, a trypan blue exclusion assay was performed to analyze cell viability; the different group cells were collected and stained with equal volume of 0.4% trypan blue dye for 60 second. The number of viable cells (N, unstained cells) was quantified using a hemocytometer and an inverted microscope (Nikon). The percentage of viable cells was then determined from the equation as follows: Cell viability (%) = (N Experiment/N Blank control) × 100. The percentage of viable cells in each time was also considered as 100% for blank control group.

Measurements of cytokines in cell supernatants

Cell supernatants were collected and stored at -70°C. Levels of IL-6 and IL-10 were determined by using ELISA kits from R&D (Minneapolis, MN, USA) according to the manufacturer's instructions and specifications.

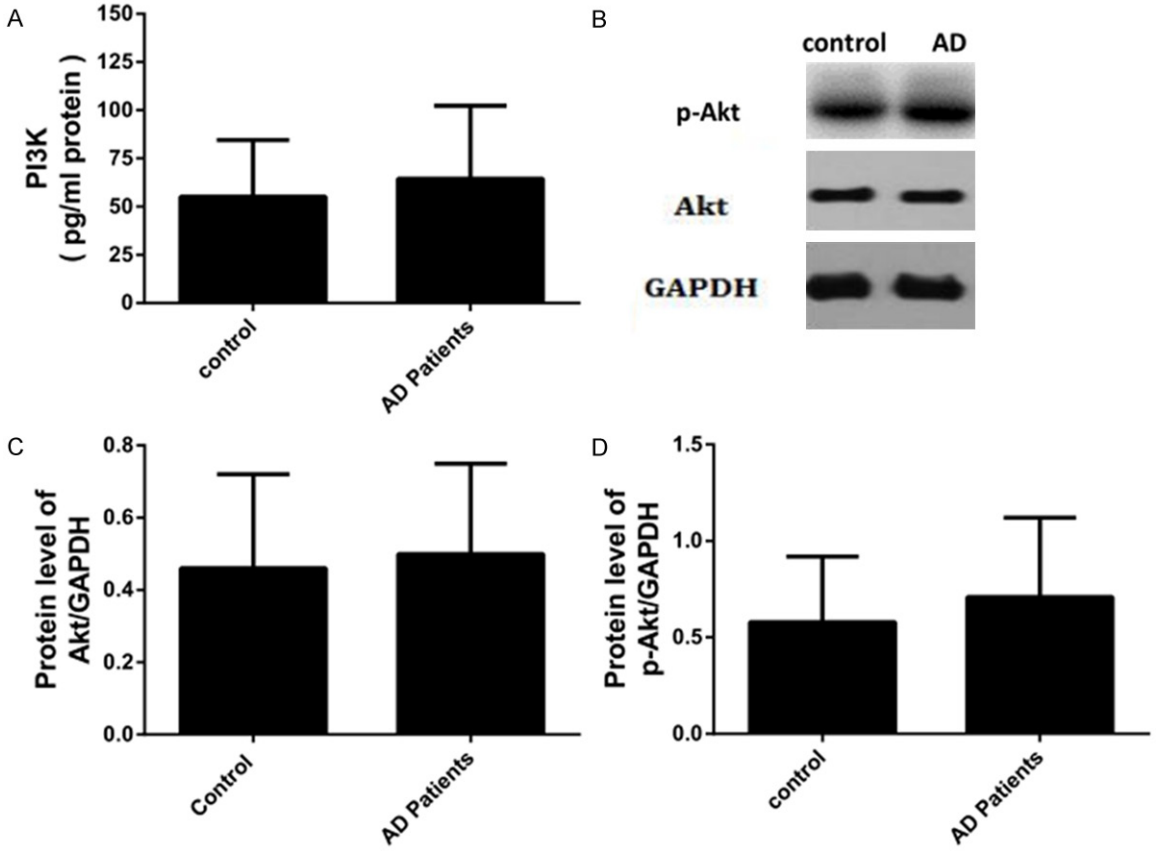

Figure 2. PI3K/Akt pathway expression after 24 h-culture of T cells from AD patients After T cells from AD patients were cultured for 24 hours, the protein levels of PI3K in T cells were assessed by ELISA (A) or the protein levels of p-Akt/Akt in T cells were assessed by western blot (B). The relative densities of p-Akt (C) and Akt (D) were corrected using GAPDH. Values represent means + SD at thirty experiments.

Statistical analysis

All data are expressed as x ± s and were analyzed with SPSS 19.0 software; α = 0.05 was considered statistically significant. Two independent samples with normal distributions were analyzed with a t or corrected t test, and those without normal distributions were analyzed with the Wilcoxon rank sum test. Pearson analysis was used to analyze correlations.

Results

Expression of the PI3K/Akt signaling pathway in T cells

PI3K expression was significantly higher in peripheral T cells from AD patients (85.96 ± 50.06 pg/ml) than in those from healthy controls (**Figure 1A**) (46.29 ± 24.38 pg/ml; t = 3.902, $P < 0.001$). p-Akt and Akt expression were also significantly higher in AD patients (1.12% ± 0.45% and 1.05% ± 0.78%) than in the healthy controls (**Figure 1B-D**) (0.56% ± 0.25%, t = 5.958 and 0.49% ± 0.26%, t = 3.373; both $P < 0.05$).

PI3K pathway expression after culture of T cells from AD patients in medium containing 10% fetal bovine serum for 24 hours

After T cells from AD patients were cultured for 24 hours, their PI3K expression was 64.45 ± 37.88 pg/ml (**Figure 2A**) and their p-Akt or Akt expression (**Figure 2B-D**) was 0.71% ± 0.41% or 0.50% ± 0.25%, which did not significantly differ from those in healthy controls (55.24 ± 29.33 pg/ml t = 1.053; 0.58% ± 0.34%, t = 1.028 and 0.46% ± 0.26%, t = 0.6074, respectively, both $P > 0.05$).

The effect of serum from AD patients on the PI3K signaling pathway in T cells from healthy controls

After AD patient-derived T cells were cultured in sera from AD patients, their PI3K and p-Akt/Akt

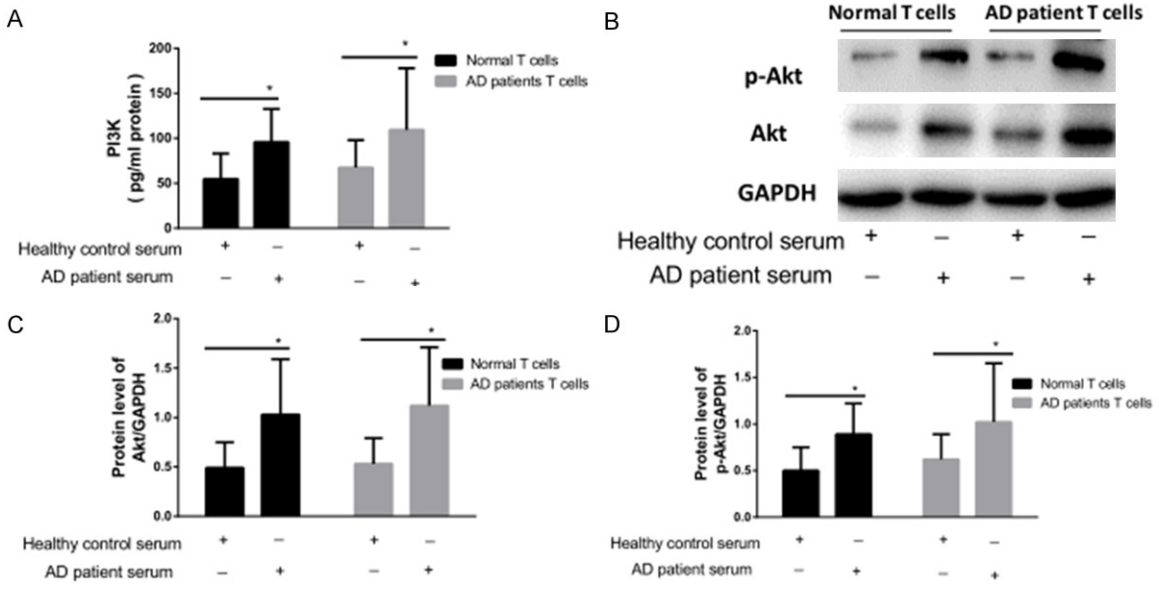

Figure 3. The effect of sera from AD patients and from healthy controls on the expression of the PI3K signaling pathway in T cells the protein levels of PI3K in T cells were assessed by ELISA (A). The protein levels of p-Akt/Akt in T cells were assessed by western blot (B). The relative densities of p-Akt (C) and Akt (D) were corrected using GAPDH. Values represent means + SD at thirty experiments. *P < 0.05.

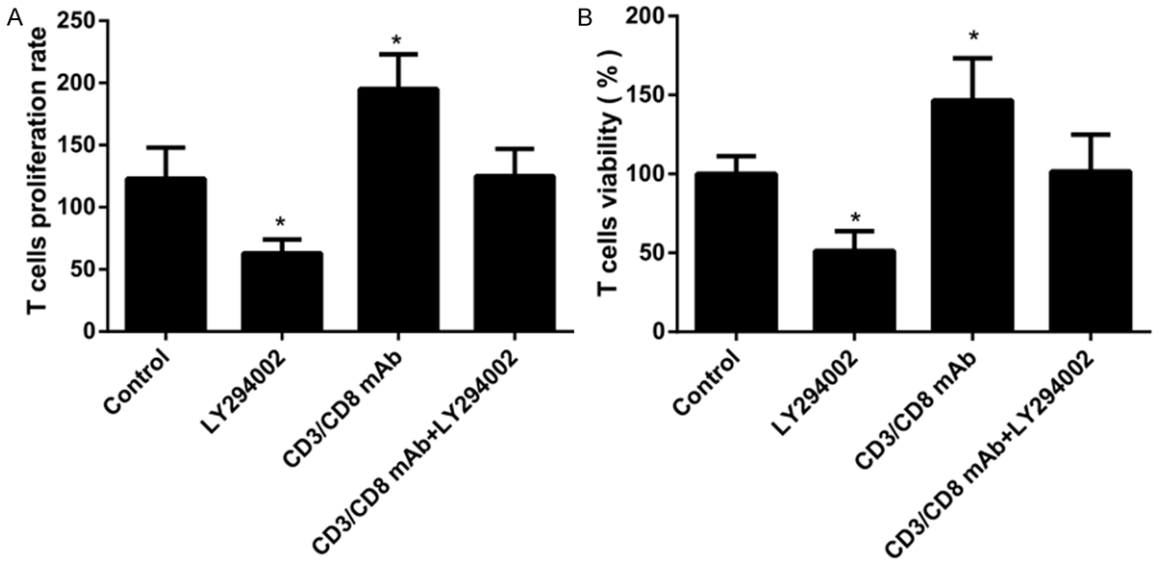

Figure 4. The effect of PI3K pathway inhibition on AD patient T cell proliferation. T cells were obtained from AD patients, and treated with serum alone, LY294002, CD3/CD28 mAb and CD3/CD28 mAb + LY294002. And then T cell proliferation by MTT assay (A) and typan blue assay (B). Compared to T cells cultured in serum, *P < 0.05.

expression were higher than those observed for the healthy controls (both P < 0.05) (**Figure 3**). After healthy control-derived T cells were cultured in sera from AD patients, their PI3K and p-Akt/Akt expression were significantly higher than those observed when the same T cells were cultured in sera from the healthy controls (P < 0.05) (**Figure 3**). This result suggests that the serum of AD patients may contain cytokines that adversely affect the activation of the PI3K pathway in T cells.

The effect of PI3K pathway inhibition on AD patient T cell proliferation

Compared to T cells cultured in serum alone as a control (**Figure 4**), treatment with the PI3K

Table 2. The effect of PI3K pathway inhibition and CD3/CD28 mAb stimulation on cytokine secretion by AD patient-derived T cells (x ± s)

	Control	LY294002	CD3/CD28 mAb	LY294002 +CD3/CD28 mAb
IL-6 (ng/L)	265 ± 46	168 ± 33	823 ± 128	431 ± 64[c]
t		2.242[a]		2.013[b]
p		< 0.05		< 0.05
IL-10 (ng/L)	98 ± 25	56 ± 14	213 ± 35	120 ± 21d
t		2.429[a]		2.143[b]
p		< 0.05		< 0.05

a: compared with no inhibitor; b: compared with CD3/CD28 mAb; c: compared with CD3/CD28 mAb.

inhibitor LY294002 significantly inhibited the proliferation of T cells from AD patients (123% ± 25% vs. 63% ± 11%; t = 2.687, P < 0.05). After CD3/CD28 mAb stimulation, AD patient-derived T cell proliferation reached 195% ± 28%; after CD3/CD28 mAb stimulation in the presence of the PI3K inhibitor LY294002, AD patient T cell proliferation was reduced to 125% ± 22% (**Figure 4**) (P < 0.05). These results suggest that PI3K inhibition significantly inhibits the proliferation of AD patient-derived T cells when cultured in serum alone or when stimulated with CD3/CD28 mAb.

The effect of PI3K pathway inhibition on cytokine secretion by AD patient T cells

Compared to T cells cultured in serum alone as a control, the PI3K inhibitor LY294002 significantly reduced IL-6 and IL-10 secretion by AD patient T cells (P < 0.05) (**Table 2**). IL-6 and IL-10 secretion were lower in T cells treated with both CD3/CD28 mAb and the PI3K inhibitor LY294002 than in T cells treated with CD3/CD28 mAb alone (P < 0.05) (**Table 2**). These results suggest that PI3K inhibition significantly inhibits IL-6 and IL-10 secretion in AD patient-derived T cells with or without CD3/CD28 mAb stimulation.

Correlation between PI3K pathway expression in AD patient-derived T cells and the disease severity of AD patients

The PI3K and Akt expression in AD patient-derived T cells were unrelated to the EASI scores of the AD patients from which the T cells were isolated (r = 0.25, P = 0.08 and r = 0.29, P = 0.13 for PI3K and Akt, respectively).

Discussion

PI3K is a heterodimer that consists of a regulatory subunit and a catalytic subunit. In quiescent cells, it is present in the cytoplasm as an inactive P85-P110 complex; once activated, it catalyzes the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on the cell membrane. PIP3 is a second messenger that activates downstream proteins, including Akt, to induce changes in many intracellular signaling proteins. PIP3 is involved in the synthesis of regulatory proteins, actin polymerization, cell survival and proliferation, and other biochemical processes [9]. Akt is the most important downstream signaling protein of PI3K. Recent studies have shown that the PI3K signaling pathway is involved in T cell migration and that it plays an important regulatory role in CD3- and/or CD28-mediated T cell activation, proliferation, and survival [10, 11].

Our results suggest that PI3K and Akt expression are significantly higher in freshly prepared peripheral T cells from AD patients than in healthy control T cells. When AD patient T cells were cultured in medium containing fetal bovine serum for 24 hours, the initially higher expression of the PI3K pathway returned to normal. Similarly, when AD patient T cells were cultured in sera from healthy controls, the expression of the PI3K pathway significantly declined. However, when healthy control T cells were cultured in sera from AD patients, the expression of the PI3K pathway significantly increased. These results suggest that the unusually high expression of the PI3K pathway in peripheral T cells from AD patients may be a secondary effect that is caused by certain pathogenic cytokine(s) in AD patient serum. Previous work has shown that in AD patients with acute skin lesions, Th17 cells may enhance the function of Th2 memory cells, promote the immune expression of keratinocytes, and induce the expression of inflammatory cytokines, such as transforming growth factor (TGF)-1, interleukin-11 (IL-11), and IL-6 [12]. The authors of that study inferred from these results that PI3K, following activation by inflammatory cytokines, converts phosphatidylinositol (4,5)-bisphosphate (PIP2) into the second messenger PIP3, which binds to the PH domain of Akt and leads to Akt phosphorylation and

hence to the ensuing changes in target genes in the nucleus. PI3K is thus involved in the physiological and pathological processes of AD, such as inflammatory responses and tissue remodeling.

To learn more about the clinical significance of PI3K pathway activation in peripheral T cells from AD patients, we investigated the relationships between PI3K pathway expression and T cell proliferation, cytokine secretion, and the disease severity of AD. The results showed that specific inhibition of unusually high PI3K expression significantly inhibited T cell proliferation and the secretion of cytokines such as IL-6 and IL-10 in AD patients, suggesting that the unusually strong activation of the PI3K pathway in peripheral T cells from AD patients may be associated with the intrinsic activation of T cells in these patients. Thus, we conclude that the pathological activation of T cells may be an important link in the development of AD and that the PI3K pathway may be an important target for regulating T cell expression in AD patients; inhibiting the expression of this pathway may be beneficial for AD treatment. This study showed no correlation between the PI3K expression and the EASI of AD patients, which may be related to the small sample size or to other factors; additional research is required for further in-depth investigation.

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Disclosure of conflict of interest

None.

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