



PI3K and Notch signal pathways coordinately regulate the activation and proliferation of T lymphocytes in asthma

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ABSTRACT

Aims: In the present study, we determined whether Phosphoinositide 3-kinase (PI3K) and Notch signal pathways are involved in the expression of cyclinD1, cyclinA and p27kip1 which were key molecules in controlling cell cycling from CD4⁺ T lymphocyte in animal model of asthma.

Main methods: Ovalbumin (OVA) sensitized murine model of asthma was used to investigate the expression of cyclin D1, cyclin A, and p27kip1 by splenic CD4⁺ T lymphocytes. We further observed the effect of specific inhibitor of PI3K (LY294002) and specific inhibitor of Notch (DAPT) on the proliferation of such CD4⁺ T lymphocytes.

Key findings: We found that the expression of cyclinD1 and cyclinA was upregulated at both protein and mRNA levels in asthma group while p27kip1 was down-regulated. Both LY294002 and DAPT inhibit the proliferation of CD4⁺ T lymphocytes in a time- and dose-dependent manner. Furthermore, LY294002 and DAPT have additive effect in down-regulation of cyclinD1 and upregulation of p27kip1. An upregulation of cyclinA, although not statistically significant, was also observed.

Significance: These data suggested that PI3K signal pathway and Notch signal pathway may coordinately regulate the cell proliferation and differentiation processes through up-regulating cyclinD1 and down-regulating p27kip1 of CD4⁺ T lymphocytes.

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Introduction

Asthma is a chronic airway inflammatory disease increasing in prevalence. T lymphocytes play an important role in the pathogenesis of asthma such as airway inflammation and remodeling through the release of cytokines (Choy et al., 2011; Lloyd and Hessel, 2010; Levine and Wenzel, 2010). Phosphoinositide 3-kinase (PI3K) is one of the key enzymes in cell signaling and is widely distributed in the lung tissue. PI3K is crucial in T cell signaling (Alcazar et al., 2007; Okkenhaug et al., 2006; Deane et al., 2007). Notch signal pathway is also involved in T cell differentiation and participates in T cell activation (Laky and Fowlkes, 2008; Osborne and Minter, 2007; Dallman and Smith, 2005). However, those cell signal pathways are not completely independent of each other, i.e. cross talks take place between different signaling pathways. We hypothesize that signaling pathways between PI3K and Notch coordinate in regulating the proliferation and activation of T lymphocytes in asthma.

Cyclins, cyclin-dependent kinase (CDKs), and cyclin-dependent kinase inhibitor (CKIs) are the main players in the regulation of cell cycling to ensure the normal progress of cell cycling from G1 to G2 (CDKs) and from S phase to M phase (CKIs) (Sherr, 1996; Foijer and Riele, 2006). Cyclin D1 is a key regulator of cells from G1 to S phase, while cyclinA activity is essential for entry from G2 to M phase (Foijer and Riele, 2006; Blagosklonny and Pardee, 2002; Wang et al., 2009). In addition, the CDKs inhibitor p27kip1 also regulates the progression of the cells from G1 to S phase. Overexpression of p27kip1 results in inhibition of the progression of cells from G1 to S phase that halts the proliferation of cells (Boudová et al., 2003). In the present study, we determined whether PI3K and Notch signal pathways are involved in the expression of cyclinD1, cyclinA and p27kip1 of CD4⁺ T lymphocyte in an animal model of asthma.

Materials and methods

Mice

Male BALB/c mice, 4 to 6 weeks old, weighing 18–22 g at the beginning of the experiments, were purchased from Shanghai Slac Laboratory Animal Center (Shanghai, China). Throughout the entire study, they were kept in a pathogen-free environment in the animal center of Wenzhou Medical College. All animals were acclimated for at least 1 week prior to the experiments. This study was carried out

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in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Wenzhou Medical College (Permit Number: wydW 2009-0002). All surgery was performed under chloral hydrate anesthesia, and all efforts were made to minimize suffering.

Murine asthma model

Mouse asthma model was established as previously published, with some modifications (Park et al., 2011). Mice were sensitized by i.p. injection of 10 µg ovalbumin (OVA) (Sigma, USA)/20 mg Al (OH)₃ gel in 0.1 ml normal saline or PBS on days 1 and 13. From day 25, they were exposed to daily OVA (1 mg/ml) or PBS aerosol challenges for 30 min each for a total of 8 days. The aerosol treatment was performed in a plexiglas exposure chamber coupled Jet nebulizer (Pari IS-2 Jet nebulizer; PARI Respiratory Equipment) driven by compressed air at a flow rate of 6 L/min. The control group received normal saline on the same schedule. Mice were sacrificed 4 h after the last aerosol challenge.

Purification of CD4⁺ T cells

The spleen from each mouse was minced through a nylon screen into the medium. CD4⁺ cells were isolated by negative selection using magnetic labeled beads following the manufacturer's instruction (Dyna Bead, USA). The cells were routinely analyzed by flow cytometry using FITC conjugated anti CD4 antibody with CD4⁺ T cell purity >90%. Viability was over 95% by trypan blue exclusion. The cells were cultured in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% FCS (Hangzhou Sijiqing Biotech, China), 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 1:100 nonessential amino acids. Cells were maintained in an incubator at 37 °C, 5% CO₂.

Intracellular staining for cyclinA, cyclinD1 and p27kip1

CD4⁺ T cells from asthma and control groups were fixed with 4% paraformaldehyde and permeabilized in 0.25% polyethylene glycol tert-octylphenyl ether (TritonX-100) prior to staining with anti-cyclinA, anti-p27kip1, anti-cyclinD1 (rabbit IgG, Santa Cruz, USA), respectively. After washing, the cells were then incubated with PE labeled goat-anti-rabbit IgG (Santa Cruz, USA) on ice for 30 min and washed with PBS. Rabbit IgG was used as isotype control. Cells were then analyzed on FACSaria (BD Biosciences) and 10,000 events were collected from each sample. Data were analyzed using FlowJo software (Tree Star, San Carlos, CA, USA).

mRNA expressions of cyclinA, cyclinD1 and p27kip1

RT-PCR was used to assess the expression of cyclinA, cyclinD1 and p27kip1 at mRNA level. Total RNA was isolated from CD4⁺ T lymphocyte of asthma and control groups using Trizol according to the manufacturer's instruction. One microgram of RNA was reverse transcribed to cDNA by incubating with reverse transcriptase at 37 °C for 1 h. Primers specific for rat cyclinA, cyclinD1, p27kip1, β-actin1 and β-actin2 were designed to cross introns to avoid amplification of genomic DNA. For cyclinD1 the forward primer was 5-CCTGGATGCTGGAGGTCTG-3 and the reverse was 5-AGGGTGGGTGGAAATG AAC-3. For cyclinA the forward primer was 5-GGGCACCTCGAGGC ATTC-3 and the reverse was 5-CCTATTACCCGTCGAGTCTTGAG-3.

For p27kip1 the forward primer was 5-AGCCTACGCTCCGACT GTTG-3 and the reverse was 5-CCTCTGCCACTCGTATCTGC-3. For β-actin1 the forward primer was 5-AGAGGGAAATCGTGCCTGAC-3 and the reverse was 5-CCACAGGATTCCATACCAAG-3. For β-actin2 the forward primer was 5-CSGGACCTGACAGACTACCTCAT-3 and the reverse was 5-CCACAGGATTCCATACCAAGA-3. PCR conditions are as follows: preheating at 94 °C × 5 min, denaturation 94 °C × 30 s,

annealing × 30 s, extension 72 °C × 30 s. A total of 35 cycles was followed by a 10 min extension at 72 °C.

Cell proliferation assay

Cell proliferation was examined using protocols previously published in literature (Xiong et al., 2008; Liu et al., 2011). CD4⁺ cells (2 × 10⁵ cells in 100 µl) were stimulated in 96-well flat-bottomed microplates with PHA (20 µg/ml, Sigma) and IL-2 (1000 U/ml, Hua-xin Biotech, China) in the presence of inhibitors of Notch (DAPT, Sigma), PI3K (LY294002, Sigma) for up to 48 h at 37 °C. At 12, 24, and 48 h time points, cell proliferation was determined using cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cell viability was determined by scanning with a microplate reader at 450 nm. Data were expressed as the percentage of cell proliferation inhibition as follows: relative inhibition(%) = 1 - [A450 (treated) - A450 (blank)] / [A450 (control) - A450 (blank)] × 100%. According to the results of CCK-8, we selected the appropriate time and concentration to exclude the possibility of cytotoxicity caused by such reagents in this study.

The coordinated regulation between Notch and PI3K

CD4⁺ T cells (2 × 10⁶/2 ml in a 12 well plate) from asthma mice were activated with PHA (20 µg/ml, Sigma) and IL-2 (1000 U/ml; Hua-xin Biotech, China) for 4–6 h. Those cells were then divided into 4 groups: group a (control group), without further treatment; group b (intervention group b), treatment with 10 µM LY294002, a PI3K inhibitor (Sigma) for 48 h; group c (intervention group c), treatment with 10 µM DAPT, a Notch inhibitor (Sigma) for 48 h; and group d (double intervention group), treatment with both LY294002 and DAPT for 48 h. The protein and mRNA expressions of cyclinA, cyclinD1 and p27kip1 of CD4⁺ T cells were quantitated by PCR and flow cytometry.

Statistical analysis

All data were expressed as mean ± SEM. SPSS16.0 was used for statistical analysis. Comparison among multiple groups was performed using a one-way ANOVA followed by post hoc LSD tests. For samples with unequal variances, Dunnett's test was used instead. One-sample *t*-test (two-tailed) was used to compare treated samples from their own basal levels. *p* < 0.05 was considered to be statistically significant.

Results

Purity and viability of CD4⁺ T cells

After magnetic bead isolation, the purity of CD4⁺ T cells was determined by flow cytometry using FITC conjugated anti-CD4 as (90.04 ± 5.18) % (Fig. 1). Cell viability, as determined by trypan blue exclusion, was (94.82 ± 3.21) %.

The expression of cyclinA, cyclinD1 and p27kip1

Surface expression of cyclinA, cyclinD1 and p27kip1 from splenic CD4⁺ T cells was determined by flow cytometry. As shown in Fig. 2A and B, cyclinA and cyclinD1 levels were significantly higher in the asthma group [(28.0 ± 3.5)% and (14.9 ± 3.4)%] than that in the control group [(13.4 ± 3.5)% and (7.7 ± 1.8) %] (##*p* < 0.01 and #*p* < 0.05 respectively). However, CD4⁺ T cells from asthma group showed remarkable lower levels of p27kip1 [(23.3 ± 3.9)%] than that of the control group [(37.5 ± 5.8) %] (##*p* < 0.01).

We then determined the expression of cyclinA, cyclinD1 and p27kip1 at mRNA levels using RT-PCR. As demonstrated in Fig. 2C, cyclinA and cyclinD1 expression was upregulated in CD4⁺ T cells of

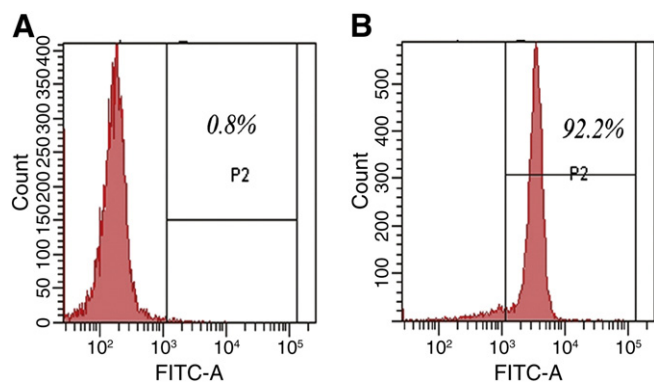


Fig. 1. Purity of CD4⁺ T lymphocytes. CD4⁺ T lymphocytes were isolated using magnetic beads. The cells were then incubated with FITC conjugated isotype IgG (A) or anti-CD4 mAb (B). After washing, the cells were suspended in PBS and subjected for flow cytometry. The figure shown is representative of 3 independent experiments.

asthma animals, while the expression of p27kip1 significantly declined. Quantitation data (Fig. 2D) revealed an increase for cyclinA and cyclinD1 at the mRNA level in the asthma group [(0.55 ± 0.16) and (1.38 ± 0.42)] when compared with the control group [(0.32 ± 0.10) and (0.92 ± 0.37)] (#p < 0.01 and #p < 0.05 respectively) and a decrease for p27kip1 at the mRNA level in the asthma group [(0.16 ± 0.02)] when compared with the control group [(0.32 ± 0.03)] (##p < 0.01).

LY294002 inhibits CD4⁺ T cell proliferation in vitro

To determine the role of PI3K in CD4⁺ T cell proliferation, LY294002, an inhibitor of PI3K was incubated with CD4⁺ T cells while being stimulated with PHA and IL-2. The result, as shown in Fig. 3A, demonstrated a time and dose dependent inhibition of such

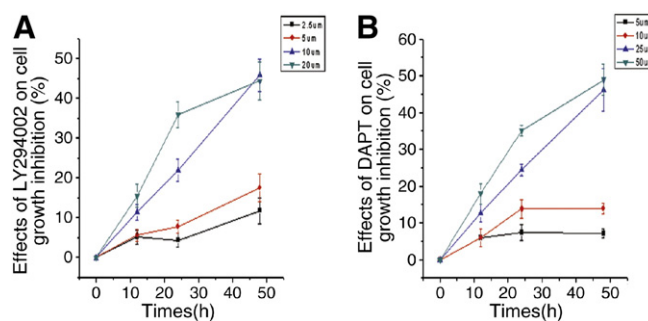


Fig. 3. LY294002 and DAPT inhibited the proliferation of CD4⁺ T lymphocytes. A. CD4⁺ T lymphocytes from asthma group were stimulated with PHA (20 μg/ml) and IL-2 (1000 U/ml). The cells were then incubated with different concentrations of LY294002 (0, 2.5, 5, 10, and 20 μM) in 4 wells each for 12, 24, and 48 h. CCK-8 (10 μl/well) was added for the last 6 h of incubation. OD at 450 nm and 650 nm was reader. Cell Proliferation Inhibition Ratio was calculated as (1 – medication group / the control group) × 100%. B. CD4⁺ T lymphocytes were stimulated as in A. DAPT at 0, 5, 10, 25, and 50 μM was added. CCK-8 was present during the last 6 h of incubation. The figures shown are the mean ± SEM of 3 experiments.

cells by LY294002. At 24 h, LY294002, at concentrations of 2.5, 5, 10, and 20 μM led to (4.39 ± 1.66)%, (7.78 ± 1.55)%, (21.86 ± 2.82)% and (35.79 ± 6.21)% inhibition respectively. On the other hand, CD4⁺ T cells treated with 10 μM of LY294002 for 12, 24, and 48 h, showed inhibition of proliferation at (11.41 ± 1.99)%, (21.86 ± 2.82)% and (45.79 ± 6.09)% respectively.

DAPT inhibits CD4⁺ T cell proliferation in vitro

Next, we test if Notch signaling is required for CD4⁺ T cell proliferation. Fig. 3B showed the result of PHA and IL-2 stimulated CD4⁺ T cells that were further treated with Notch inhibitor DAPT (at 5, 10, 25, and 50 μM) for 12, 24, and 48 h. At 24 h, DAPT at

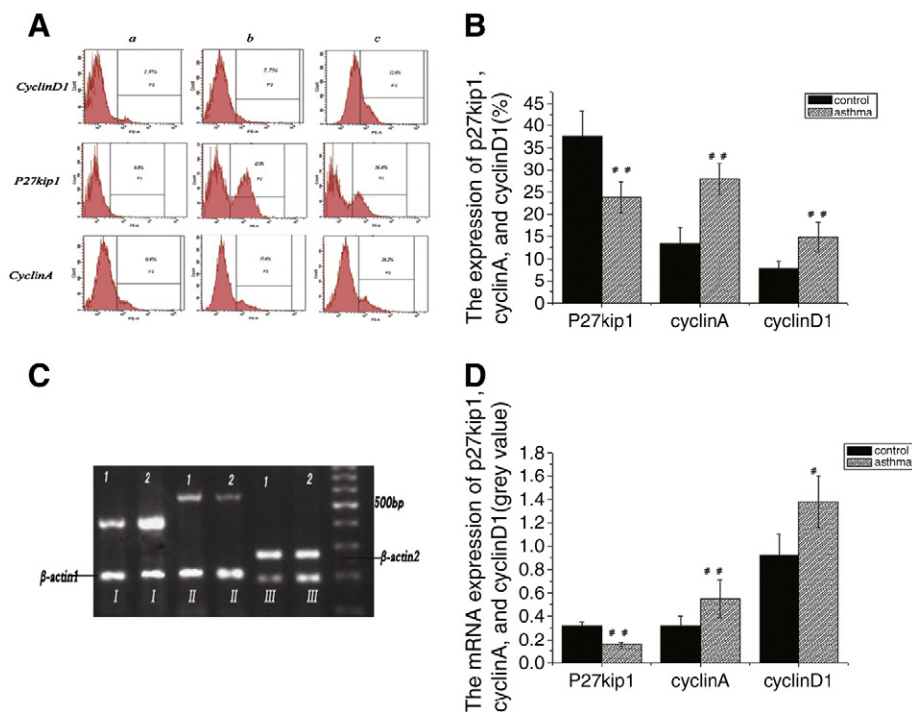


Fig. 2. Protein and mRNA expression of cyclinA, cyclinD1 and p27kip1. A. The expression of cyclinA, cyclinD1, and p27kip was determined by flow cytometry as stated in Materials and methods. Ten thousand events were collected and analyzed. a. Isotype control. b. Control group. c. Asthma group. B. Quantitation of the expression of cyclinA, cyclinD1 and p27kip1 from 3 independent experiments. #p < 0.05, ##p < 0.01 when compared to control. C. mRNA expression of cyclinA, cyclinD1 and p27kip1 using RT-PCR. Band I was cyclinD1, band II was p27kip1 and band III was cyclinA.1, Control group 2, Asthma group. Data shown are representative of 3 experiments with similar results. D. The quantitative data of the mRNA expression of cyclinA, cyclinD1 and p27kip1 from 3 independent experiments expressed as mean ± SEM. #p < 0.05, ##p < 0.01 compared to control.

concentrations of 5, 10, 25, and 50 μM caused $(7.39 \pm 2.05)\%$, $(13.80 \pm 2.57)\%$, $(24.43 \pm 1.51)\%$ and $(35.14 \pm 1.54)\%$ inhibition respectively. A time course study revealed that CD4^+ T cells treated with 25 μM of DAPT for 12, 24, and 48 h, showed inhibition of proliferation at $(12.80 \pm 2.42)\%$, $(24.43 \pm 1.51)\%$ and $(46.12 \pm 6.84)\%$ respectively. Therefore, DAPT significantly reduced CD4^+ T cell proliferation, again in a time and dose dependent manner.

PI3K and Notch signal pathways coordinately regulate the expression of cyclinA, cyclinD1 and p27kip1

To explore whether PI3K and Notch signal pathways can coordinately regulate the proliferation of CD4^+ T cells in an animal asthma model, we examined the expression of cyclinA, cyclinD1 and p27kip1 in CD4^+ T cells in the presence of PI3K inhibitor, Notch inhibitor, or both. The expression of cyclinD1 protein was significantly reduced by LY294002 alone, DAPT alone, and the combination of both when comparing with PBS $[(6.4 \pm 1.4)\%$, $(6.7 \pm 1.7)\%$, $(3.4 \pm 0.8)\%$, and $(11.2 \pm 2.1)\%$, $p < 0.01$, Fig. 4A, B]. There seems to be an additive effect of LY294002 and DAPT. The expression of p27kip1 is upregulated by LY294002 alone $(31.6 \pm 5.3)\%$, or with DAPT $[(4.6 \pm 2.8)\%$, control $(22.9 \pm 3.0)\%$, $p < 0.05$ and $p < 0.01$], respectively, but not changed by DAPT, indicating that it is mainly controlled by the PI3K pathway. The expression of cyclinA was not altered by either LY294002 or DAPT.

Consistent with protein expression, mRNA levels of cyclinA, cyclinD1 and p27kip1 showed a similar change in the presence of LY294002, DAPT, or both (Fig. 4C, D). These data suggest that the regulation is at transcription levels.

Discussion

The present study discovered previously unrecognized roles of Notch and PI3K signaling in the proliferation of CD4^+ T lymphocytes. First, we provided evidence that in an OVA-induced murine model of

asthma, the expression of cyclinA and cyclinD1 is increased in CD4^+ T lymphocytes, and p27kip1 expression is decreased. Second, we demonstrated that the proliferation of such CD4^+ T lymphocytes is regulated by both Notch and PI3K signaling pathways.

Based on the structure and substrate specificity, PI3K isoforms are classified into three groups, namely class I, class II and class III. Class I PI3K is further divided into class IA and class IB. In mammals, class IA PI3K is composed of 3 catalytic subunits (p110 α , p110 β and p110 δ) and 5 regulatory subunits (p85 α , p85 β , p55 γ , p55 α and p50 α). Class IB PI3K is composed of 1 catalytic subunit (p110 γ) and 3 regulatory subunits (p101, p84 and p87). Recent studies suggest that PI3K plays a crucial role in airway inflammatory response (Takeda et al., 2010; Thomas and Owen, 2008; Ito and Caramori, 2007). The finding of Alcazar et al. (2007) illustrates an essential role for p110 γ in T cell receptor (TCR)-induced T cell activation. p110 δ contributes significantly to Th cell expansion and differentiation in vitro and in vivo in the context of CD28 costimulation (Okkenhaug, 2006). Class IA PI3K regulatory subunits are essential for the function of a subset of T cells while challenging the notion that this signaling mechanism is only a critical mediator of costimulatory signals downstream of CD28 (Deane et al., 2007). PI3K inhibitor LY294002 suppresses the production of Th2 cytokine (IL-5 and IL-13) (Duan et al., 2005). The increased levels of Th2 cytokines (IL-4, IL-5 and IL-13) in both lung tissue and BAL fluids in a mouse asthma model were significantly reduced by IC87114, a selective p110 δ inhibitor, in a dose-dependent manner (Lee et al., 2006). Inhibition of the p110 δ signaling pathway suppresses IL-17 expression through regulation of NF- κB activity, indicating therapeutic potential in asthma (Park et al., 2010). One study demonstrates a novel functional role for p110 δ signaling in the induction of type 2 responses in vivo that may offer a new therapeutic target for Th2-mediated airway disease (Nashed et al., 2007). Our study herein shows that PI3K inhibitor LY294002 inhibits the in vitro proliferation of CD4^+ T lymphocyte in a time- and dose-dependent pattern. Therefore, it supports the notion that PI3K signal pathway participates in the immune response of asthma

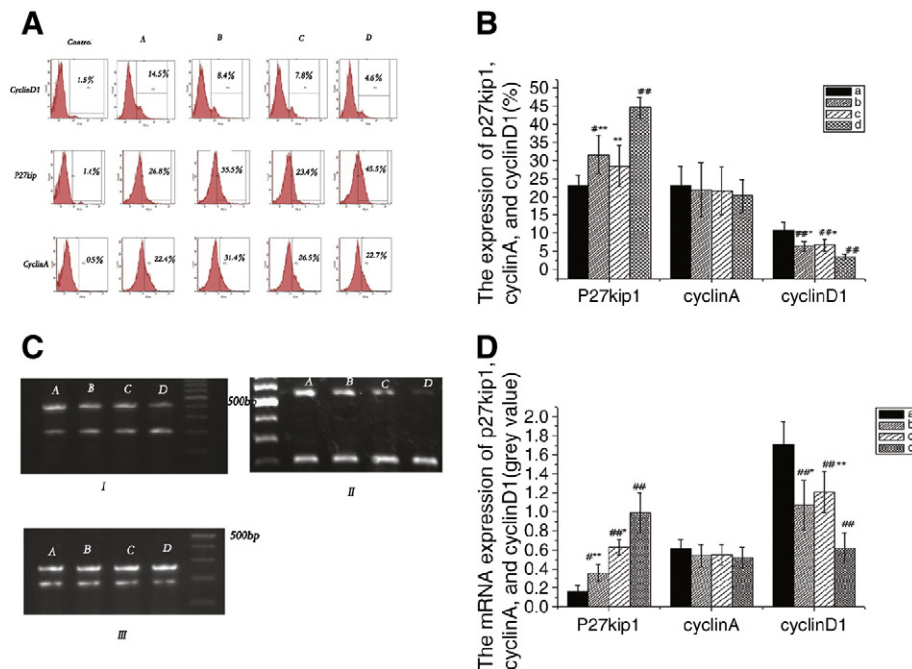


Fig. 4. The effect of LY294002 and DAPT on the expression of cyclinA, cyclinD1 and p27kip1 by CD4^+ T lymphocytes from asthma animals. CD4^+ T lymphocytes from the asthma group were incubated with PBS (a), LY294002 (b), DAPT (c), or LY294002 + DAPT (d) for 48 h. Surface expression of cyclinA, cyclinD1 and p27kip1 was determined by flow cytometry (A); data from 3 experiments were quantitated (B). mRNA expression was examined by RT-PCR (C). I, cyclinD1; II, p27kip1; III, cyclinA. mRNA levels from 3 experiments were quantitated (D). # $p < 0.05$, ## $p < 0.01$, compared to group a; * $p < 0.05$, ** $p < 0.01$, compared to group d.

through regulation of the production of Th cell expansion and differentiation.

In 1917, geneticist Thomas Hunt Morgan and his colleagues described fruit flies with notches at the margins of their wing blades. It turned out that this notched wing phenotype was the result of a partial loss of function of the *Drosophila* Notch gene. The Notch signaling pathway is involved in the control of cell identity, proliferation, differentiation and apoptosis in various animals (Gazave et al., 2009). Mammals possess four Notch receptors (Notch1–4) that are bound by five ligands (Delta-like1, 3, and 4 and Jagged 1 and 2) (Radtko et al., 2010). Inhibition of Notch activation dramatically decreases T cell proliferation in both CD4 and CD8 cells (Palaga et al., 2003). Jagged is an APC-derived signal for IL-4 independent Th2 differentiation, with Delta being an APC-derived Th1 differentiation signal (Amsen et al., 2004). After Notch1 is blocked by Notch1-specific small interfering RNA, IL-4 is decreased and IFN- γ is increased in activated lung T cells, suggesting that the Notch1 signal plays a role in Th1/Th2 differentiation of asthma (Guo et al., 2009). At the same time, our study shows that Notch inhibitor DAPT inhibits the proliferation of CD4⁺ T lymphocyte in a time- and dose-dependent manner. Therefore, it supports the idea that that Notch signal pathway plays a key role in Th1/Th2 balance of asthma. Whether the combination of LY294002 and DAPT may carry synergistic or additive effect is not clear. However, it is important to address this issue in the future.

Cell cycle is a process that is closely related to cell growth and proliferation through a series of intra-cellular reactions. Key molecules involved in such a process include cyclin D1, cyclin A, and p27kip1. CyclinD1 is a key regulator of a cell's progress from G1 to S phase, while cyclinA activity is essential for entry from G2 to M phase (Fojer and Riele, 2006; Blagosklonny and Pardee, 2002; Wang et al., 2009). P27kip1 is a CDK inhibitor that controls the progression of cells from G1 to S phase, therefore, inhibiting the proliferation of cells (Boudová et al., 2003). In the absence of p27Kip1, T cells proliferate faster than control cells with an increase of cell growth and division and an increased number of cells in the S phase (Zhang et al., 2000). P27kip1 has the opposing roles in the control of CD4⁺ T cell proliferation and effector function (Rowell et al., 2005). The result of our current study shows that the protein and mRNA levels of cyclinA and cyclinD1 of CD4⁺ T lymphocyte of the mice in the asthma group are significantly increased compared to those of the control group. Meanwhile, there is a significant reduction of p27kip1, both at protein and mRNA levels. We can speculate that asthma may cause the abnormal activation and proliferation of CD4⁺ T lymphocytes by promoting the expressions of cyclinA and cyclinD1 and inhibiting the expression of p27kip1.

Both PI3K and Notch signaling pathways participate in the activation and proliferation of T cell and cell cycling. Also, we discovered directly the up-regulation of PI3K and Notch signal pathways in a mouse asthma model in our previous studies. In the present study, we questioned whether PI3K signal pathway and Notch signal pathway can coordinately regulate the proliferation of T cells through the modulation of the expressions of cyclinA, cyclinD1 and p27kip1. By using immunomagnetic beads, primary CD4⁺ T lymphocytes were isolated from asthma animals and tested in vitro for their proliferation in response to PI3K and Notch inhibitors. We found that, although PI3K inhibitor LY294002 and Notch inhibitor DAPT inhibit cyclinD1 expression individually, the combination of the two reagents has an additive effect. The same is true for the upregulating effect on p27kip1. It should be pointed out that this part of the in vitro study was performed using cells from the asthma group. It is assumed that cells from control animals are also affected by such reagents in vitro. This will be addressed in our future studies.

Taken together, our findings suggest that CD4⁺ T lymphocyte proliferation is regulated by both PI3K and Notch pathways. The down regulation of cyclinD1 and the up-regulation of p27kip1 may be the

underlying mechanism for the progression of G1/S phase in cell proliferation and differentiation.

Author contributions

CCL and WXZ conceived and designed the experiments. YN, WXZ and LC performed the experiments. WXZ and YN analyzed the data. XHC, HZ, BBL and YFL participated in the statistical analyses. WXZ wrote the paper. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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