



Inhibition of thymocyte autophagy-associated CD4⁺T thymopoiesis is involved in asthma susceptibility in mice exposed to caffeine prenatally

Han-xiao Liu¹ · Hui-yi Yan^{1,2} · Wen Qu¹ · Xiao Wen¹ · Li-fang Hou¹ · Wen-hao Zhao¹ · Jie Ping¹

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Abstract

Our previous studies demonstrated that prenatal caffeine exposure (PCE) caused thymopoiesis inhibition, immune disorders, and airway remodeling in offspring, which raises the question of whether PCE is a risk factor for postnatal asthma. Meanwhile, the mechanism of PCE-induced thymopoiesis inhibition is not clear yet. Considering caffeine's pro-autophagy effects (lacking evidence in thymus) and the important role of autophagy in maintaining thymopoiesis, this study aimed to investigate whether PCE contributes to asthma susceptibility, and further explore the molecular mechanisms of thymopoiesis inhibition from the perspective of pro-autophagy effects of caffeine both in vivo and in vitro. The PCE mouse model was established by 96 mg/kg/day caffeine administration from gestational day (GD) 9–GD 18, and an asthma model was established on the offspring by ovalbumin sensitization and challenge. The results confirmed our hypothesis that PCE could suppress pulmonary CD4⁺T development and aggravate allergen-induced asthma symptoms in the offspring. In fetuses, PCE significantly suppressed A2AR–PKA signaling, upregulated Beclin1–LC3II autophagy, promoted Bcl10 degradation, reduced A20 expression, and inhibited CD4⁺T thymopoiesis. Similar results were also observed in 4 μM caffeine-treated thymocytes in vitro. Moreover, inhibiting A2AR by antagonist (SCH 58261) performed the same downstream biological effects as caffeine treatment, and autophagy inhibitor (Bafilomycin A1) clearly abolished the caffeine-induced Bcl10 degradation and A20 suppression. In conclusion, our findings, for the first time, showed that PCE could attenuate CD4⁺T thymopoiesis and suppress pulmonary CD4⁺T development by directly enhancing autophagy in thymocytes, and provided a firm experimental evidence that PCE is a risk factor for postnatal asthma.

Keywords Prenatal caffeine exposure · Immunotoxicity · Asthma · CD4⁺T deviation · Thymocyte · Autophagy

Abbreviations

A2AR A2 adenosine receptor
 AHR Airway hyper resistance
 BafA1 Bafilomycin A1

BALF Bronchoalveolar lavage fluid
 Co-IP Co-immunoprecipitation
 GAPDH Glyceraldehyde phosphate dehydrogenase
 GD Gestational day
 HE Hematoxylin and eosin
 IL Interleukin
 IFN Interferon
 IUGR Intrauterine growth retardation
 Mch Methacholine
 OVA Ovalbumin
 PAS Periodic acid-Schiff
 PCE Prenatal caffeine exposure
 PD Postnatal day
 SP Single positive cells
 TEM Transmission electron microscope
 Th Helper T-cells
 Tregs Regulatory T-cells

Han-xiao Liu and Hui-yi Yan contributed equally to this work.

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✉ Jie Ping
 pingjie@whu.edu.cn

¹ Department of Pharmacology, School of Basic Medical Sciences, Wuhan University, 185, East Lake Road, Wuhan 430071, China

² Wuhan Mental Health Center, Wuhan Hospital for Psychotherapy, Wuhan 430012, China

TGF Transforming growth factor
TNF Tumor necrosis factor

Introduction

Asthma, one of the most common airway inflammatory diseases, affects approximately 300 million individuals worldwide as of 2018 (GINA 2018). Considering that asthma is currently incurable, it is vital to identify and avoid the triggers (GINA 2018). Epidemiological and experimental studies have linked asthma susceptibility to intrauterine growth retardation (IUGR), which refers to fetal development limitations due to adverse prenatal environments (Mu et al. 2014). Caffeine, widely found in beverages, food, and some analgesic drugs, has been recognized as one of the major risk causes of IUGR (Heckman et al. 2010; Sengpiel et al. 2013). Our and other labs have confirmed that the prevalence of various diseases was increased in the prenatal caffeine-exposed (PCE) offspring with IUGR (Ping et al. 2012, 2014; Steck et al. 2015; Wang et al. 2014b). Our recent researches further revealed that PCE could cause immune disorders and airway remodeling in the offspring (Liu et al. 2017a, b). Consequently, we hypothesized that PCE is a risk factor for asthma susceptibility after birth.

CD4⁺T deviation is a well-established paradigm for asthma. Generally, both type 2 helper T-cells (Th2)/Th1 and Th17/regulatory T-cells (Tregs) are balanced under physiological conditions, whereas Th2-bias and/or Th17-bias will polarize allergic responses (Liu et al. 2016). Despite our recent finding showed skewed splenic Th cytokine repertoires in PCE offspring, the effects of PCE on CD4⁺T deviation are still largely unknown and needed to be further investigated (Liu et al. 2017a). It is well known that all these peripheral CD4⁺T subsets (Th1, Th2, Th17, and Tregs) are derived from CD4⁺T thymopoiesis. During pregnancy, the peripheral CD4⁺T network is not yet present in fetuses, whereas CD4⁺T thymopoiesis is well-established but just in the vulnerable state (Holt and Jones 2000). It was reported that thymocytes express A2 adenosine receptor (A2AR), a common caffeine receptor, which makes thymocyte a target of caffeine toxicity (Kiss et al. 2006). Therefore, the effects of PCE on fetal CD4⁺T thymopoiesis and the underlying mechanisms are also needed to be systematically studied.

Macroautophagy (herein referred to as autophagy) has two main stages: first, the autophagy-inducible molecules (such as Beclin1) trigger the autophagosome initiation; second, the downstream components (such as LC3II) prolong and fuse the autophagosome. Recent reports have demonstrated that autophagy is an important regulatory mechanism in maintaining thymopoiesis (Oral et al. 2017). Both human and animal studies indicated that toxicant exposures in pregnancy can trigger autophagy and potentially

induce permanent changes in many physiological processes, resulting in increased disease susceptibility in the offspring (Huang et al. 2018; Mahemuti et al. 2018; Quan et al. 2017). Besides, firm evidence showed that caffeine can enhance autophagy signaling in multiple organs (Hughes et al. 2017; Mathew et al. 2014; Moon et al. 2014). Therefore, we hypothesized that caffeine may also upregulate autophagy activities and attenuate the thymopoiesis in PCE fetuses.

This study aimed to investigate PCE-caused pulmonary CD4⁺T abnormalities and asthma susceptibility in the offspring on the basis of our previous findings that PCE restrict fetal CD4⁺T thymopoiesis. The underlying mechanisms of PCE-induced CD4⁺T thymopoiesis inhibition were further explored by determining the autophagy signaling both in PCE fetal thymuses *in vivo* and in caffeine-treated primary thymocytes *in vitro*. This work will help, for the first time, provide evidence for the pro-autophagy effects of caffeine on thymocytes and the potential correlation between asthma susceptibility and PCE.

Materials and methods

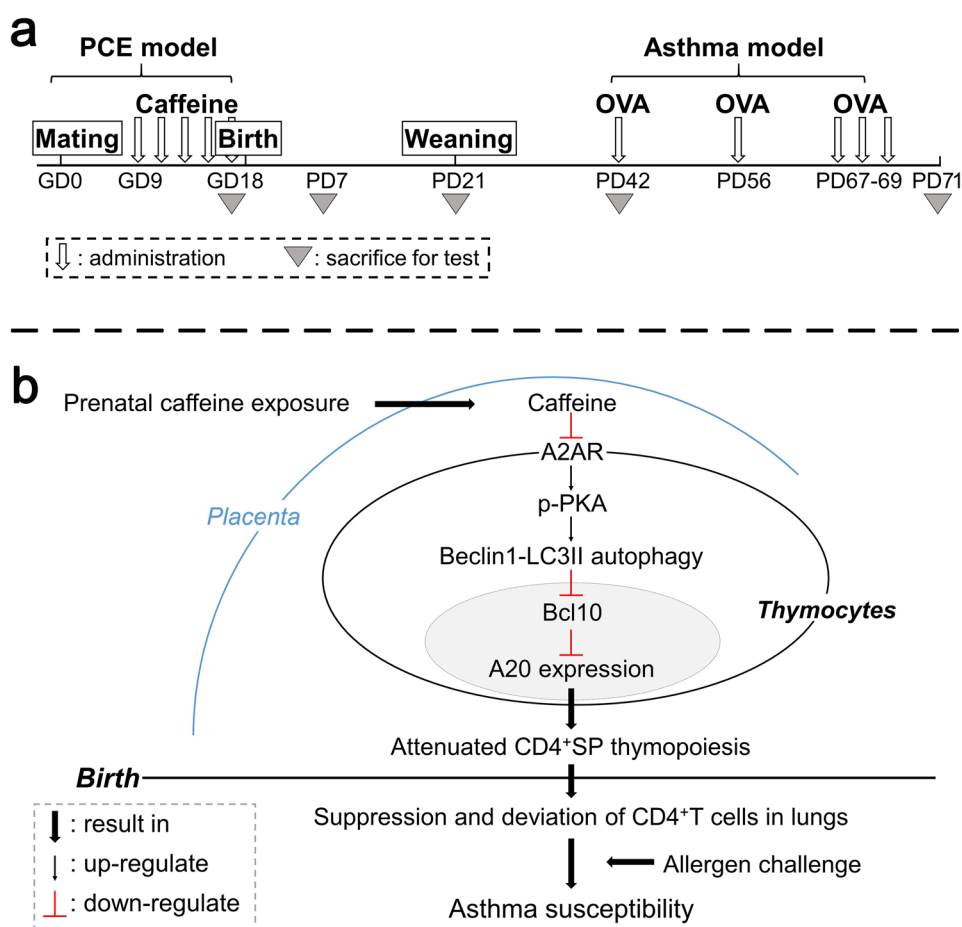
Animals and treatments

Balb/C mice were obtained from the Experimental Center of Hubei Medical Scientific Academy (no. 2015-0018) and maintained under specific pathogen-free conditions in the Center for Animal Experiment of Wuhan University (AAALAC International). Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Center for Animal Experiment of Wuhan University (AUP number: 2016009).

The PCE model was established as described before (Liu et al. 2017a) (Fig. 1a). Briefly, breeders at 8–10 weeks were mated on gestational day (GD) 0. From GD 9 to GD 18, pregnant mice were intragastrically administered 96 mg/kg of caffeine (Sigma-Aldrich) per day. The control group received the same volume of saline. On GD 18, a batch of pregnant mice were killed under isoflurane anesthesia and then fetuses from ten dams (each dam 6–8 fetuses) per group were obtained and weighed. Three fetal thymuses per group were randomly selected for fixing in a 2.5% glutaraldehyde solution for transmission electron microscope (TEM) analysis. The remaining fetal thymuses of the littermates were pooled together as one sample for subsequent analysis.

For the postnatal experiments, the pregnant mice were kept until normal delivery. When the offspring matured to adolescence, an asthma model was established. Briefly, offspring were divided into four groups (8–10 mice/group): prenatal control/saline challenge (C/S), PCE/saline challenge (Ca/S), prenatal control/ovalbumin (OVA) challenge (C/O), and PCE/OVA challenge (Ca/O). The C/O and Ca/O

Fig. 1 The experimental flow diagram and the mechanism hypothesis for the present study. **a** Caffeine or saline was administered to pregnant mice from gestational day (GD) 9 to GD 18. The postnatal asthma model was established by ovalbumin (OVA) sensitization and challenge, **b** caffeine inhibits A2AR on thymocytes and decreases PKA phosphorylation which in turn upregulates Beclin1–LC3II autophagy signaling followed by Bcl10 degradation and abnormal expression of A20 in thymocyte during CD4⁺T thymopoiesis. The impaired CD4⁺T thymopoiesis finally results in CD4⁺T abnormalities in lungs and susceptibility to asthma after birth



mice were sensitized intraperitoneally with 20 µg of OVA emulsified in 2 mg Imject Alum (Thermo) on postnatal day (PD) 42 and PD 56 and challenged with 100 µg OVA in 50 µL PBS by intranasal administration for 3 consecutive days (PD 67–69). The C/S and Ca/S mice were sensitized and challenged with PBS at each time-point. 48 h after the final OVA/PBS challenge, half animals were performed pulmonary function test. Others were killed to collect lungs for subsequent analysis.

Flow cytometry analysis of fetal thymocytes

Single thymocytes were obtained and surface stained (CD3-FITC, CD4-APC and CD8-PE-cy7) (eBioscience) as described before (Liu et al. 2017c). Data were analyzed and plotted using the FlowJo software. Three pooled thymus samples per group were used for detection as a three-time repeated experiment.

Western blotting

Proteins were prepared by lysis buffer and performed western blotting detection as described before (Liu et al. 2017c).

The primary antibodies (anti-Beclin1, anti-LC3I, anti-LC3II, anti-p62, anti-Bcl10, anti-PKA and anti-p-PKA) were obtained from Cell Signaling Technology. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the loading control.

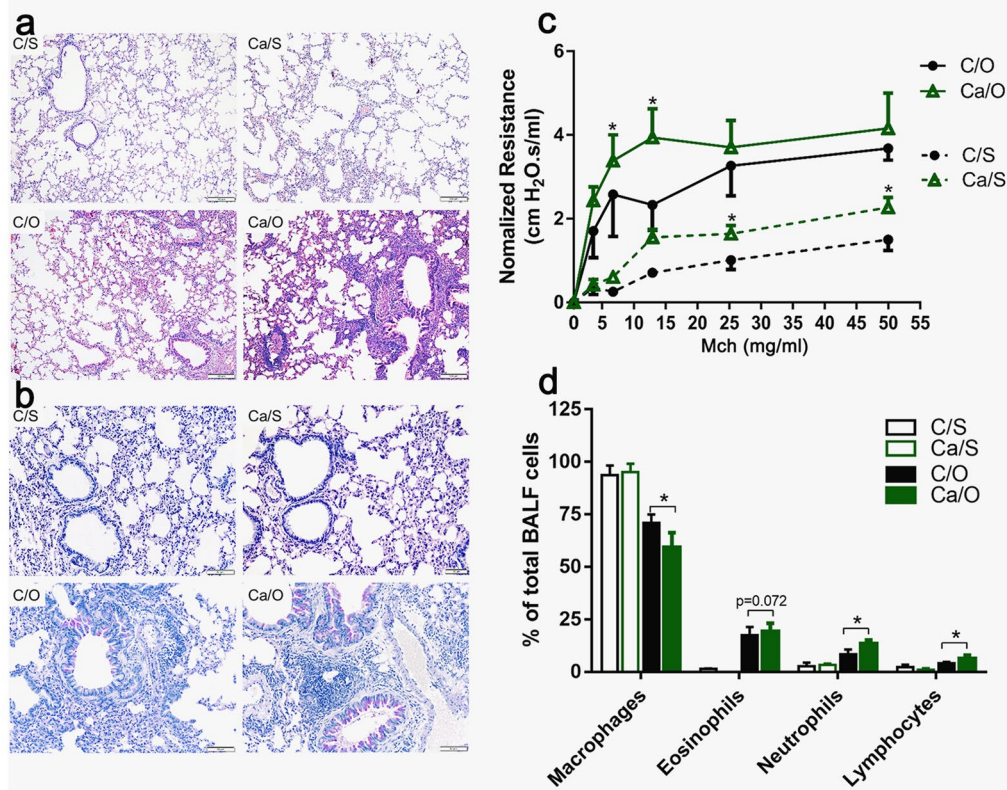
Co-immunoprecipitation (Co-IP)

Thymus was lysed with IP buffer and then mixed with the anti-p62-binding A/G and IgG binding beads, respectively. The beads with immunoprecipitation were pulled down by centrifugation at 3000×g for 5 min. Following four washes, the immunoprecipitation was eluted by boiling the beads in SDS-PAGE loading buffer. The Bcl10 and p62 levels in the thymus lysates (as input), the IgG immunoprecipitation, and the anti-p62 immunoprecipitation were analyzed by western blotting. The detection was repeated two times.

qPCR

Single-strand thymocyte cDNA was prepared and the qPCR was performed as described before (Liu et al. 2017a). A20 expression was calculated with the $\Delta\Delta C_t$ method and

Female



Male

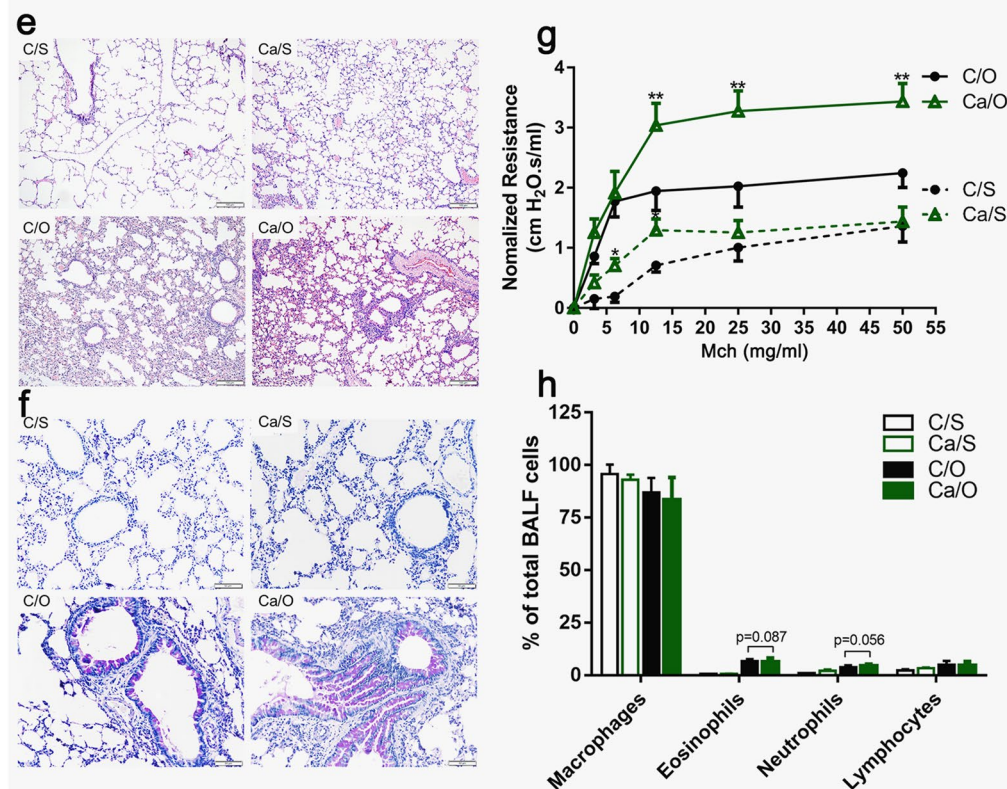


Fig. 2 The effects of prenatal caffeine exposure (PCE) on allergen-induced asthma symptoms in the offspring. **a, e** Hematoxylin and eosin staining of lungs, scale bar=100 μ m, **b, f** periodic acid-Schiff staining of lungs, scale bar=50 μ m, **c, g** airway hyper resistance (AHR), **d, h** cell counting in bronchoalveolar lavage fluid. Data were expressed as mean $\pm$ SD ($n=8-9$ /group). * $P<0.05$ vs control

normalized based on the GAPDH mRNA content. The primers of A20 (forward: GGCTGCTTCCTATGACTC; reverse: CACCTCACTCCATCCCTA) and GAPDH (forward: AAC TTTGGCATTGTGGAAGG; reverse: GGATGCAGGGAT GATGTTCT) were designed with Primer Premier 5.0 (PREMIER Biosoft) and queried with the NCBI BLAST database for homology comparison.

Pulmonary function test

Anesthetized mice were intubated and mechanically ventilated by a computer-controlled piston ventilator (Buxco FinePointe). Mice were then challenged with an aerosolized bronchoconstrictor, methacholine (Mch, Sigma-Aldrich), at increasing doses: 0, 3.125, 6.25, 12.5, 25, and 50 mg/mL. Airway hyper resistance (AHR) was calculated using the single compartment model. Individual AHR levels were normalized to their perspective baseline (0 mg/mL Mch).

Histological analysis

The left lungs were excised, fixed in 4% paraformaldehyde buffer, dehydrated, embedded, and sectioned. Paraffin-embedded 5 μ m sections were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS).

Cell counting and cytokine detection in bronchoalveolar lavage fluid (BALF)

BALF was collected by flushing lungs with 1.0 mL PBS containing 2% BSA. Cell smears were prepared by cyto-spin and stained with Wright–Giemsa to distinguish inflammatory cells (eosinophils, neutrophils, and lymphocytes). 200 cells were counted in each random location. In BALF supernatants, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-2, IL-4, IL-6, IL-10, and IL-17A were assayed by cytometric bead array kits (BD Bioscience), and transforming growth factor (TGF)- β was measured by enzyme-linked immunosorbent assay kits (Dakewei Biotech).

Flow cytometry analysis of pulmonary CD4⁺T-cells

The lungs of offspring (on PD 7, PD 21 and PD 42) were cut into pieces and incubated at 37 $^{\circ}$ C for 30 min in RPMI 1640 media [1 mg/mL collagenase I (Invitrogen)]. After

incubation, single cells were obtained through a 40 μ m cell strainer. Red blood cells were lysed, and the remaining cells were stimulated for 7 h with a PMA/ionomycin cocktail (eBioscience). After surface staining with 100 μ L of antibody cocktail (CD3-FITC, CD4-APC) (eBioscience), the cells were further fixed, permeabilized, and divided into four tubes for Th1 (IFN- γ -PE), Th2 (IL-4-PE), Th17 (IL-17-PE-cy7) and Tregs (Foxp3-PE) (eBioscience) staining, respectively. The detection was repeated three times.

Primary thymocytes culturing and treatments

Primary thymocytes were obtained from 3-week-old mice and cultured as described before (Liu et al. 2017a). To test the pro-autophagy effects of caffeine, the cells were treated with different concentrations (0.4, 4, and 40 μ M) of caffeine for 48 h, and with 4 μ M caffeine for different durations (24, 48, and 72 h). To investigate whether autophagy is involved in the caffeine-induced thymopoiesis inhibition, the cells were pretreated with 5 nM Bafilomycin A1 (BafA1), which could block the final fusion of autophagosomes, for 4 h before caffeine treatment (4 μ M, 48 h). To explore the role of A2AR in caffeine-induced autophagy, antagonist (SCH 58261, 25 μ M) of A2AR was used to treat the cells. A20 expression was detected by qPCR. The protein expression of the autophagy marker proteins (Beclin1 and LC3II), Bcl10, p62, and p-PKA was measured by western blotting. Bcl10–p62 complex level was detected by Co-IP. Thymocyte phenotypes were analyzed by flow cytometry.

Statistical analyses

Data were presented as the mean $\pm$ SD. Differences between two groups were compared using *t* tests, and differences among multiple groups were compared using analysis of variance (ANOVA). $P<0.05$ was considered statistically significant. Data were analyzed using SPSS 17 (SPSS Science) and Prism 6 (GraphPad Software).

Results

Offspring mice

PCE aggravated allergen-induced asthma symptoms

As compared to those in controls, AHR ($P<0.05$, Fig. 2c, g) and the percentages of eosinophils and neutrophils in BALF ($P=0.087$, $P=0.072$, $P=0.056$, $P<0.05$, Fig. 2d, h) were significantly increased in both female and male PCE offspring following OVA challenge. With OVA challenge, in both genders, HE (Fig. 2a, e) and PAS (Fig. 2b, f) staining also demonstrated more inflammatory cells, widespread

mucus production in the bronchioles and more mucus-producing cells in the lungs of PCE offspring than the controls. No obvious inflammatory infiltration or mucus production was observed in either the PCE or the control offspring without OVA challenge.

PCE altered pulmonary CD4⁺T responses after induction of asthma

Given that PCE aggravated pulmonary inflammation in the offspring, the cytokines of CD4⁺T responses in BALF were then analyzed. Before OVA challenge, no significant difference was found in the levels of Th/Tregs cytokines between the PCE and control offspring in both genders (Table 1). After the induction of asthma, the elevated levels of IL-4 (Th2 representative factor) and IL-17A (Th17 representative factor), and the reduced levels of IFN- γ and TNF- α (Th1 representative factors) were observed in the PCE offspring compared to the controls ($P < 0.05$, Table 1). The levels of TGF- β , IL-2, IL-10, and IL-6 were comparable between the two groups.

PCE reduced pulmonary CD4⁺T number and skewed CD4⁺T network

Before OVA challenge, CD4⁺T populations were detected at three time-points (PD 7, PD 21 and PD 42). As compared to the controls, both female and male PCE offspring exhibited a consistently lower number of pulmonary CD4⁺T-cells from PD 7 to PD 42 (Fig. 3a, h). On PD 7, in both genders, the percentages of Th2 (Fig. 3b, i) and Tregs (Fig. 3f, m) in PCE offspring were lower than those in the controls, whereas the percentages of Th17 (Fig. 3e, l) and Th1 (Fig. 3c, j) in PCE offspring were comparable with the control offspring. As the offspring matured, in both genders, the proportions of Th2 and Th17 and the ratios of Th2/Th1 and Th17/Tregs were increased in the PCE group (Fig. 3b–g, i–n), finally causing higher Th2/Th1 ratio and much higher Th17/Tregs ratio on PD 42 in PCE offspring compared to the controls. The flow cytometry paradigms were shown in Supplementary Figs. 1–3.

Fetal mice

PCE induced thymic dysplasia and impaired CD4⁺T thymopoiesis

Consistent with our previous data (Liu et al. 2017a, b), PCE significantly increased IUGR rates and decreased fetal thymus weight ($P < 0.05$, Fig. 4a–c). In our previous study, although the thymocyte phenotypes were not obviously altered, PCE significantly reduced CD4⁺ single positive cell (SP) absolute numbers and impaired CD4⁺T thymopoiesis

Table 1 Th1/Th2/Th17/Treg cytokines in BALF (pg/mL)

	C/S	Ca/S	C/O	Ca/O
Female mice				
IL-2 (pg/mL)	8.6 ± 0.9	8.0 ± 1.3	622.4 ± 51.9	630.1 ± 37.8
IFN- γ (pg/mL)	9.3 ± 1.3	8.1 ± 1.7	526.5 ± 29.7	439.1 ± 26.2*
TNF- α (pg/mL)	23.3 ± 7.9	23.6 ± 5.5	738.0 ± 59.2	657.9 ± 38.4*
IL-4 (pg/mL)	7.0 ± 1.3	6.7 ± 1.0	249.6 ± 30.7	307.1 ± 23.3*
IL-6 (pg/mL)	11.2 ± 3.8	10.2 ± 3.0	507.4 ± 34.6	503.7 ± 51.9
IL-10 (pg/mL)	42.0 ± 8.1	45.6 ± 10.3	531.3 ± 85.2	547.1 ± 43.4
IL-17A (pg/mL)	2.5 ± 0.7	2.6 ± 0.3	286.7 ± 34.9	389.0 ± 40.8*
TGF- β (pg/mL)	43.9 ± 10.2	38.7 ± 9.5	212.3 ± 23.4	213.6 ± 52.1
Male mice				
IL-2 (pg/mL)	7.1 ± 0.6	7.0 ± 0.9	627.1 ± 44.6	598.7 ± 62.9
IFN- γ (pg/mL)	10.2 ± 1.7	9.7 ± 2.1	506.9 ± 41.3	400.3 ± 47.9*
TNF- α (pg/mL)	24.5 ± 8.8	19.6 ± 4.6	713.0 ± 47.8	630.3 ± 59.1
IL-4 (pg/mL)	7.2 ± 0.9	7.2 ± 1.8	248.8 ± 25.3	314.4 ± 20.2*
IL-6 (pg/mL)	10.4 ± 2.0	10.4 ± 3.9	496.7 ± 48.7	508.5 ± 44.3
IL-10 (pg/mL)	37.6 ± 14.9	46.9 ± 11.1	528.0 ± 41.6	551.2 ± 63.4
IL-17A (pg/mL)	2.7 ± 0.5	2.5 ± 0.8	274.9 ± 20.4	353.7 ± 35.1*
TGF- β (pg/mL)	64.6 ± 10.5	55.8 ± 20.8	218.4 ± 64.9	174.6 ± 51.3

Cytokines in bronchoalveolar lavage fluid (BALF) were examined using cytometric bead array and enzyme-linked immunosorbent assay kits. Data were expressed as mean ± SD; $n = 8$ /group

* $P < 0.05$ Ca/O vs C/O

in fetuses. Similar results were observed in the present study ($P < 0.05$, Fig. 4d–f).

PCE reduced A20 expression, enhanced autophagy, and inhibited PKA phosphorylation during thymopoiesis

A20 is one of the key genes for maintaining CD4⁺ single positive cells (SP) survival (Matsuzawa et al. 2015). Consistent with our flow data, PCE significantly reduced A20 expression in thymus ($P < 0.05$, Fig. 4h). Enhanced autophagy can attach Bcl10, which is responsible for maintaining A20 expression (Xu et al. 2013), with the autophagy adaptor p62, followed by Bcl10–p62 degradation (Paul et al. 2012). Accordingly, as shown in Fig. 4g–j, more autophagic vacuoles under the

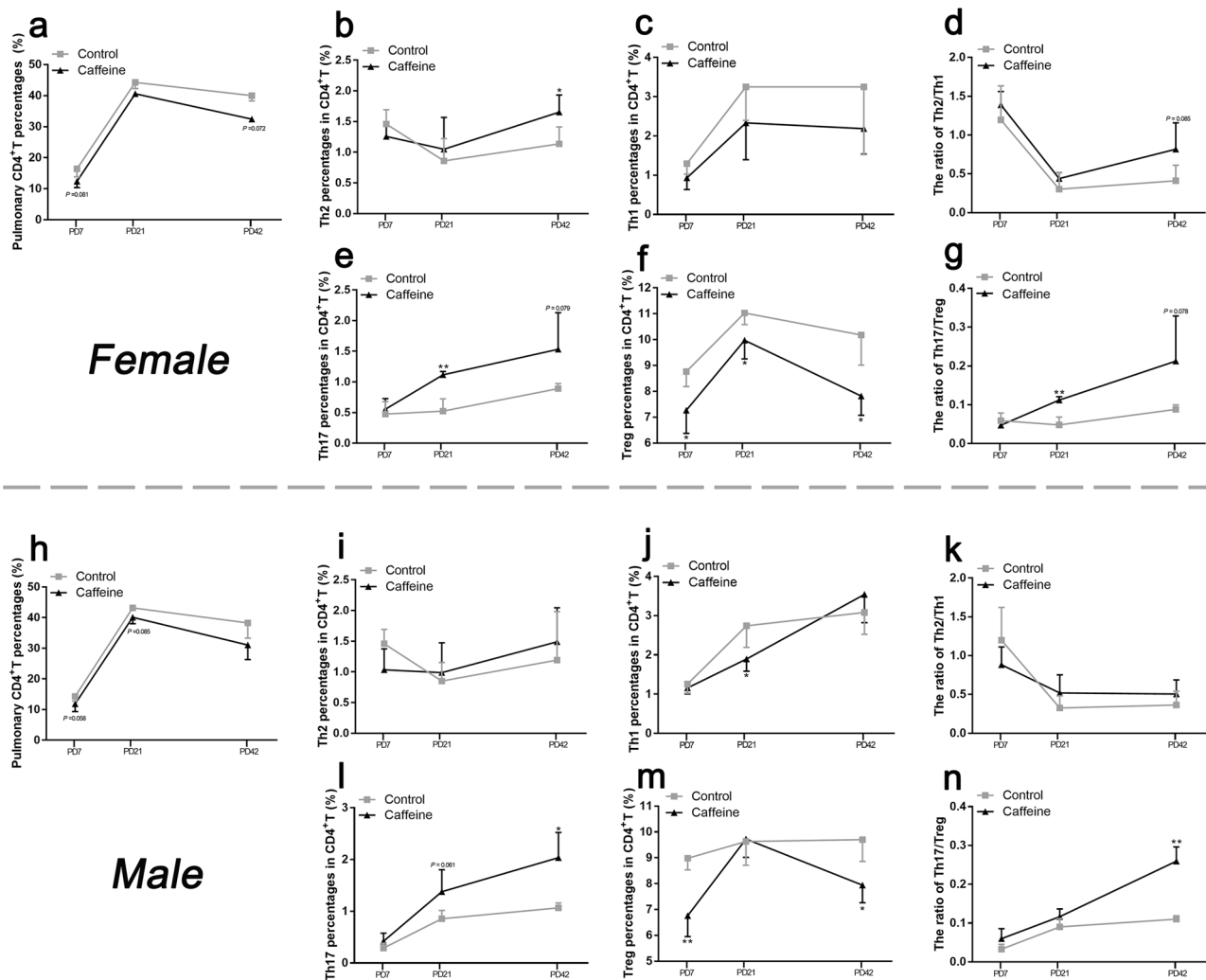


Fig. 3 The effects of prenatal caffeine exposure (PCE) on the pulmonary CD4⁺T populations in postnatal life. **a, h** Frequency of CD4⁺T-cell in lungs, **b, i** frequency of Th2 in lungs, **c, j** frequency of Th1 in lungs, **d, k** ratio of Th2/Th1 in lungs, **e, l** frequency of Th17

in lungs, **f, m** frequency of Tregs in lungs, **g, n** ratio of Th17/Tregs in lungs. Data were expressed as mean $\pm$ SD ($n = 3/\text{group}$). * $P < 0.05$, ** $P < 0.01$ vs control

TEM, clearly increased autophagy marker protein (Beclin1 and LC3II) ($P < 0.05$), enhanced Bcl10–p62 degradation, and reduced Bcl10 and p62 levels ($P < 0.05$) were observed in PCE fetuses. It is known that caffeine exerts biological function through antagonistic binding to A2AR followed by p-PKA dephosphorylation (Wang et al. 2014a). This phenomenon that p-PKA dephosphorylation was also observed in the PCE fetal thymuses ($P < 0.05$, Fig. 4g).

Primary thymocytes in vitro

Caffeine directly enhanced thymocyte autophagy in concentration- and time-dependent manners

After 48 h treatment, 4 and 40 μM caffeine significantly upregulated the expression of the autophagy marker proteins (Beclin1 and LC3II) in thymocytes ($P < 0.05$, Fig. 5a).

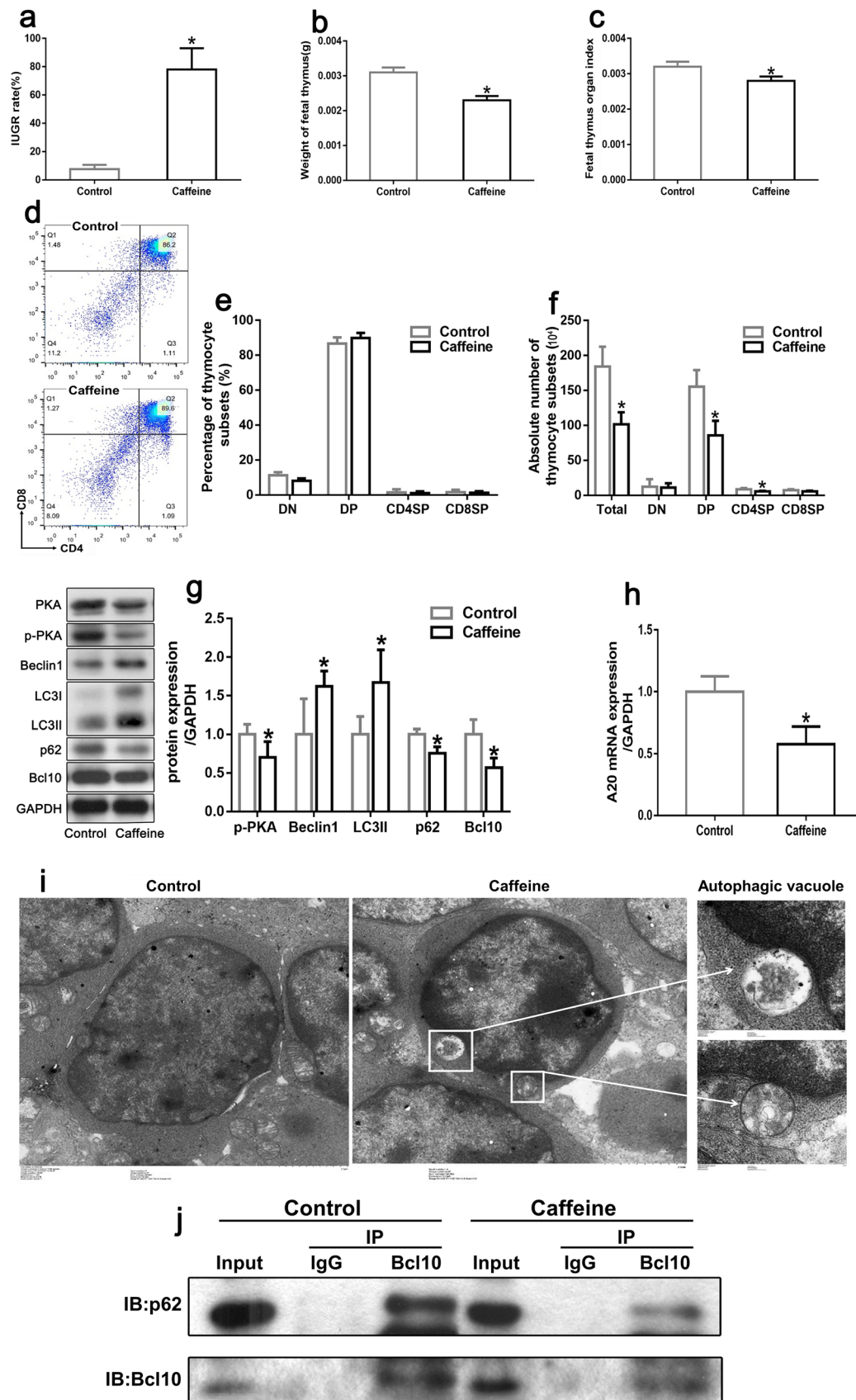


Fig. 4 The effects of prenatal caffeine exposure (PCE) on CD4⁺T thymopoiesis, A20 mRNA expression, the protein expression of p-PKA, p62, and Bcl10 and the autophagy levels during fetal thymopoiesis. **a** Intrauterine growth retardation (IUGR) rates, **b** fetal thymus weight, **c** fetal thymus organ index, **d, e** flow analysis of thymocytes, **f** the absolute number of fetal thymocyte subsets, **g** the expression of p-PKA, autophagy marker proteins (Beclin1 and LC3II), p62, and Bcl10, **h** A20 mRNA expression, **i** transmission electron microscope (TEM) detection ($\times 3000$ for thymocyte, $\times 20,000$ for autophagic vacuole), **j** Bcl10–p62 complex level. Data were expressed as mean $\pm$ SD [$n=8$ /group for **a, b, c** and **h, n=3/group for **e** and **f, n=5/group for **g**, the Co-IP detection (**j**) was repeated two times]. * $P<0.05$ vs control****

Besides, 4 μ M caffeine could enhance Beclin1 and LC3II expressions in thymocytes in a time-dependent manner (within the duration from 0 to 48 h) ($P<0.05$, $P<0.01$, Fig. 5b).

Autophagy-mediated caffeine-induced CD4⁺SP inhibition

Consistent with our data in vivo, caffeine clearly increased Bcl10–p62 degradation, followed by significantly decreased p62 and Bcl10 levels, A20 mRNA expression, CD4⁺SP ratio in the cells ($P<0.05$, Fig. 5c–f). Further, the BafA1 pretreatment totally abrogated the caffeine-induced Bcl10–p62 degradation, A20 expression inhibition, and CD4⁺SP inhibition ($P<0.05$).

A2AR mediated the effects of caffeine on enhancing thymocyte autophagy

To further investigate whether A2AR inhibition is involved in caffeine-induced autophagy in thymocytes, SCH 58261, an A2AR-specific antagonist, was used to treat thymocytes. As shown in Fig. 5g, the p-PKA level was reduced, and Beclin1 and LC3II expression were increased in SCH 58261 group, as same as the caffeine group ($P<0.05$, $P<0.01$).

Discussion

PCE mouse model

Based on our previous studies (Liu et al. 2017a, b; Ping et al. 2014), 96 mg/kg/day was the preferred caffeine dose to establish a typical IUGR mouse model and to avoid excessive numbers of absorbed or stillborn fetuses, and the exposure period from GD 9 to GD 18 not only covered the period of thymus development, but also protected the embryo implantation from the influence of caffeine. In fact, no miscarriage or stillborn fetus was observed in this study.

Increased asthma susceptibility in PCE offspring

OVA is typically used to achieve asthma model, and airway remodeling and allergic inflammation are two gold indicators for evaluating the severity of asthma (Aun et al. 2017). Researchers reported that female mice are sensitive to allergen, whereas healthy male mice fail to show obvious airway remodeling following OVA challenge (Antunes et al. 2010). However, some studies have found that adverse prenatal exposures could increase the vulnerability of offspring to allergens, leading to asthma susceptibility in two genders (Al-Sawalha et al. 2017; Sharkhuu et al. 2010; Wang et al. 2013). Hence, both female and male offspring were studied in the present study. Our data demonstrated that, in two genders, PCE offspring showed aggravated asthma symptoms following OVA challenge, confirming our hypothesis that PCE is a risk factor for asthma.

Th2- and Th17-bias in PCE offspring

CD4⁺T deviation plays pivotal roles during asthma development. Prenatal exposure to various exogenous could lead to different CD4⁺T deviation patterns (Th2-bias or Th17-bias or both) (Mandal et al. 2013; McFadden et al. 2015; Tarkowski et al. 2010; Xiao et al. 2013). We have reported that PCE could attenuate Th1 development and subsequently aggravate Th2 response following *Streptococcus pneumoniae* immunization in the offspring (Liu et al. 2017a). In the present study, after asthma induction, PCE offspring showed both enhanced eosinophilic infiltrations, which is mainly driven by Th2 response, and increased neutrophilic infiltration, which is mainly driven by Th17 response (Chang et al. 2017; Mitson-Salazar and Prussin 2017). This phenomenon indicated that PCE could aggravate both the Th2 and Th17 responses following OVA challenge. The data of cytokine repertoires in BALF further confirmed the collaborative participation of Th2- and Th17-bias during asthma development of PCE offspring.

PCE impaired CD4⁺T thymopoiesis and caused pulmonary CD4⁺T abnormalities in the offspring

Since PCE offspring showed both Th2- and Th17-bias during asthma development, we further focused on the pulmonary CD4⁺T development before OVA challenge in PCE offspring. Newborn, lactation, and adolescence were the three key checkpoints for the establishment of CD4⁺T immune homeostasis (Holt and Jones 2000). Hence, in this study, the pulmonary CD4⁺T populations at PD 7 (newborn), PD 21 (lactation), and PD 42 (adolescence) were detected. We found that, as compared to the controls, the Th2/Th1 ratio in PCE offspring increased faster with age and eventually became higher on PD 42, whereas

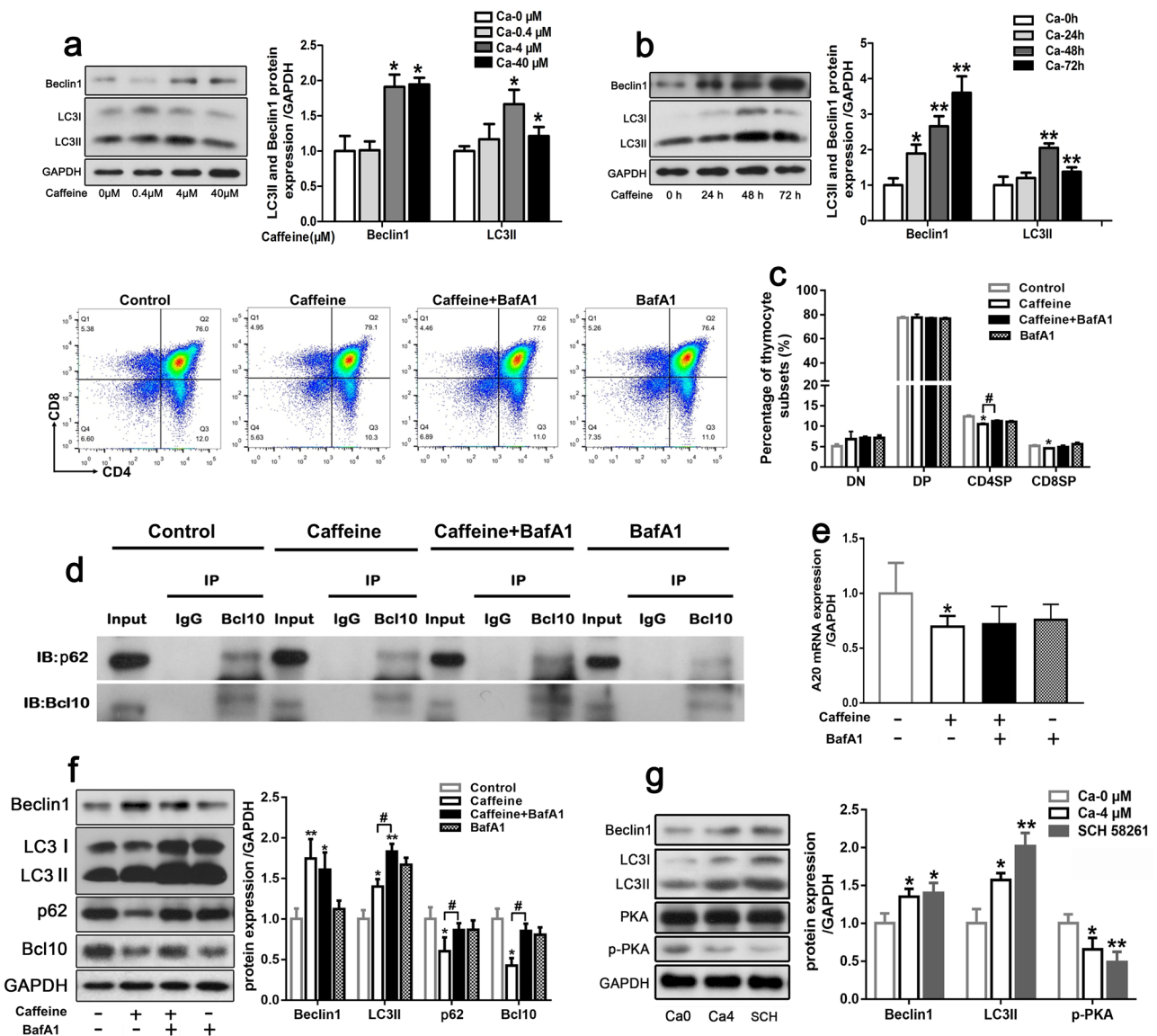


Fig. 5 The effects of caffeine treatment with or without Bafilomycin A1 (BafA1) pretreatment and SCH 58261 treatment on thymocyte autophagy in vitro. **a** The effects of caffeine (0–40 μ M) on the expression of autophagy marker proteins (Beclin1 and LC3II), **b** 4 μ M caffeine treatment for different time (24, 48, and 72 h) on the Beclin1 and LC3II expression. With or without BafA1 pretreatment, the effects of 4 μ M caffeine on **c** thymus phenotypes, **d** Bcl10–p62 com-

plex level, **e** A20 mRNA expression, **f** the protein expression of Beclin1, LC3II, p62, and Bcl10. **g** The effects of caffeine and SCH 58261 treatments on the protein expression of Beclin1, LC3II, and p-PKA. Data were expressed as mean $\pm$ SD [$n=3$ /group for **a–c**, **e–g**; the Co-IP detection (**d**) was repeated two times]. * $P<0.05$, ** $P<0.01$ vs control; # $P<0.05$, caffeine vs caffeine + BafA1

the Th17/Tregs ratio in PCE offspring was consistently higher from PD 7 to PD 42. These findings were consistent with the results that PCE aggravated both Th2 and Th17 responses after asthma induction. Besides, consistently stifled pulmonary CD4⁺T development was also observed in PCE offspring. It is well known that peripheral CD4⁺T development and homeostasis were based on well-developed CD4⁺T thymopoiesis (Tindemans et al. 2014). Firm evidence showed that exposure to various exogenous can

interfere fetal CD4⁺T thymopoiesis and lead to immune disorders after birth (Fenaux et al. 2004; Hanson et al. 2010; Holladay et al. 2011; Singh et al. 2012). Given that we have observed the permanently impair CD4⁺T thymopoiesis from GD 18 to PD 49 in PCE offspring (Liu et al. 2017a), the depressed CD4⁺T thymopoiesis might be one of the potential explanation for PCE-induced pulmonary CD4⁺T abnormalities.

Autophagy was involved in PCE-induced CD4⁺T thymopoiesis inhibition

Previously, we demonstrated that PCE could induce thymocyte apoptosis and impair CD4⁺T thymopoiesis in the fetuses. However, the increased apoptosis was turned out to be mediated by PCE-associated maternal glucocorticoid elevation in the fetuses, whereas caffeine showed few directly pro-apoptotic activity on thymocytes in vitro (Liu et al. 2017a). Interestingly, we noted that caffeine treatment enhanced the expression of Beclin1, which is a well-known autophagy trigger (Tanida 2011). Besides, Oral et al. (2017) pointed out that the upregulation of autophagy activity contributes to thymopoiesis inhibition. Therefore, we assumed that the direct pro-autophagy effects of caffeine were involved in PCE-induced CD4⁺T thymopoiesis inhibition. Our results showed PCE could increase autophagy activity in vivo, and caffeine in vitro treatment also directly enhances autophagy in thymocytes followed by CD4⁺SP inhibition. Combined these and our previous findings, PCE might impair CD4⁺T thymopoiesis by the synergistic action of the direct pro-autophagy effects of caffeine and the indirect pro-apoptotic effects of PCE-associated glucocorticoid elevation. Besides, this synergistic action might be responsible for the differences in the flow cytometry results of caffeine treatment in vitro (the pro-autophagy effects of caffeine directly induced CD4⁺SP suppression) and PCE in vivo (glucocorticoid elevation caused apoptosis in each thymocyte subpopulation, leading to no significant change in thymus phenotypes but significant suppression of CD4⁺SP absolute number).

Caffeine-triggered autophagy impaired CD4⁺T thymopoiesis by inhibiting A20 expression

In T-cells, TCR activation-mediated autophagy could lead p62 binding to Bcl10, causing gradual degradation of Bcl10 (Paul et al. 2012). Bcl10 is crucial for the thymopoiesis, whereas the Bcl10-deficient mice exhibited thymic dysplasia and reduced SP numbers (Xue et al. 2003). Similar results (thymic dysplasia, reduced SP numbers) were also observed in our studies. We assumed that autophagy-triggered Bcl10 degradation might be responsible for CD4⁺T thymopoiesis inhibition. Xu et al. (2013) showed that Bcl10 is a key regulator for maintaining A20 expression, which is essential for CD4⁺SP survival (Matsuzawa et al. 2015). The A20 knockout could drastically reduce CD4⁺T thymopoiesis. In the present study, data from western blotting, qPCR, and Co-IP showed decreased Bcl10–p62 complex levels, decreased Bcl10 expression, reduced A20 expression, and reduced CD4⁺SP number both in PCE thymus in vivo and in caffeine-treated thymocytes in vitro, which indicated that caffeine-induced autophagy could impair A20 expression and

its modulated CD4⁺T thymopoiesis. Besides, the autophagy inhibitor BafA1 not only abrogated caffeine-induced Bcl10 degradation but also reversed the caffeine-mediated A20 suppression and associated CD4⁺SP inhibition, further confirming our hypothesis that autophagy-triggered Bcl10 degradation may be responsible for caffeine-induced CD4⁺T thymopoiesis inhibition.

Caffeine enhanced autophagy by antagonizing A2AR–PKA signaling

In thymocytes, caffeine exerts its actions mainly by antagonistic binding to A2AR and depressing downstream PKA phosphorylation. Armstrong et al. (2001) have found that normal A2AR expression was needed to maintain thymopoiesis, whereas the A2AR knockdown could effectively decrease CD4⁺SP numbers and restrain thymopoiesis in mice. In this study, decreased p-PKA level and CD4⁺SP numbers were also observed in caffeine-exposed fetal thymocytes, suggesting an A2AR suppression following caffeine treatment. Although little report is available on whether A2AR suppression is associated with autophagy currently, PKA dephosphorylating is considered to be a common enhancer in promoting autophagy (Wu et al. 2018; Torres-Quiroz et al. 2015). In the present study, inhibiting A2AR signaling by SCH 58261 clearly reduced p-PKA level and performed the same downstream biological effects, which manifested as enhanced expression of autophagy marker proteins (Beclin1 and LC3II), as caffeine. These findings suggested that caffeine-induced autophagy and the subsequent A20 inhibition were mediated by A2AR–PKA suppression.

Conclusion

Our study demonstrated that PCE attenuated CD4⁺T thymopoiesis by increasing thymus autophagy directly, which results in suppression and deviation of pulmonary CD4⁺T-cells and asthma susceptibility following allergen challenge in offspring. The underlying mechanism (Fig. 1b) for the pro-autophagy effects of caffeine might be that caffeine suppressed A2AR–PKA signaling in thymocytes and subsequently upregulated the Beclin1–LC3II autophagy pathway, which could induce p62–Bcl10 degradation followed by decreased A20 expression and impeded CD4⁺SP survival. Our findings showed, for the first time, that caffeine could induce autophagy in thymocytes, and provided a firm experimental evidence that PCE is a risk factor for asthma.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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SUPPLEMENTARY DATA

Title: Inhibition of thymocyte autophagy-associated CD4⁺T thymopoiesis is involved in asthma susceptibility in mice exposed to caffeine prenatally

Han-xiao Liu^{1,+}, Hui-yi Yan^{1,2,+}, Wen Qu¹, Xiao Wen¹, Li-fang Hou¹, Wen-hao Zhao¹, Jie Ping^{1,*}

¹Department of Pharmacology, Wuhan University School of Basic Medical Sciences, Wuhan 430071, China.

²Wuhan Mental Health Center, Wuhan Hospital for Psychotherapy, Wuhan 430012, China.

⁺These authors contributed equally to this work.

***Corresponding author:** Jie Ping

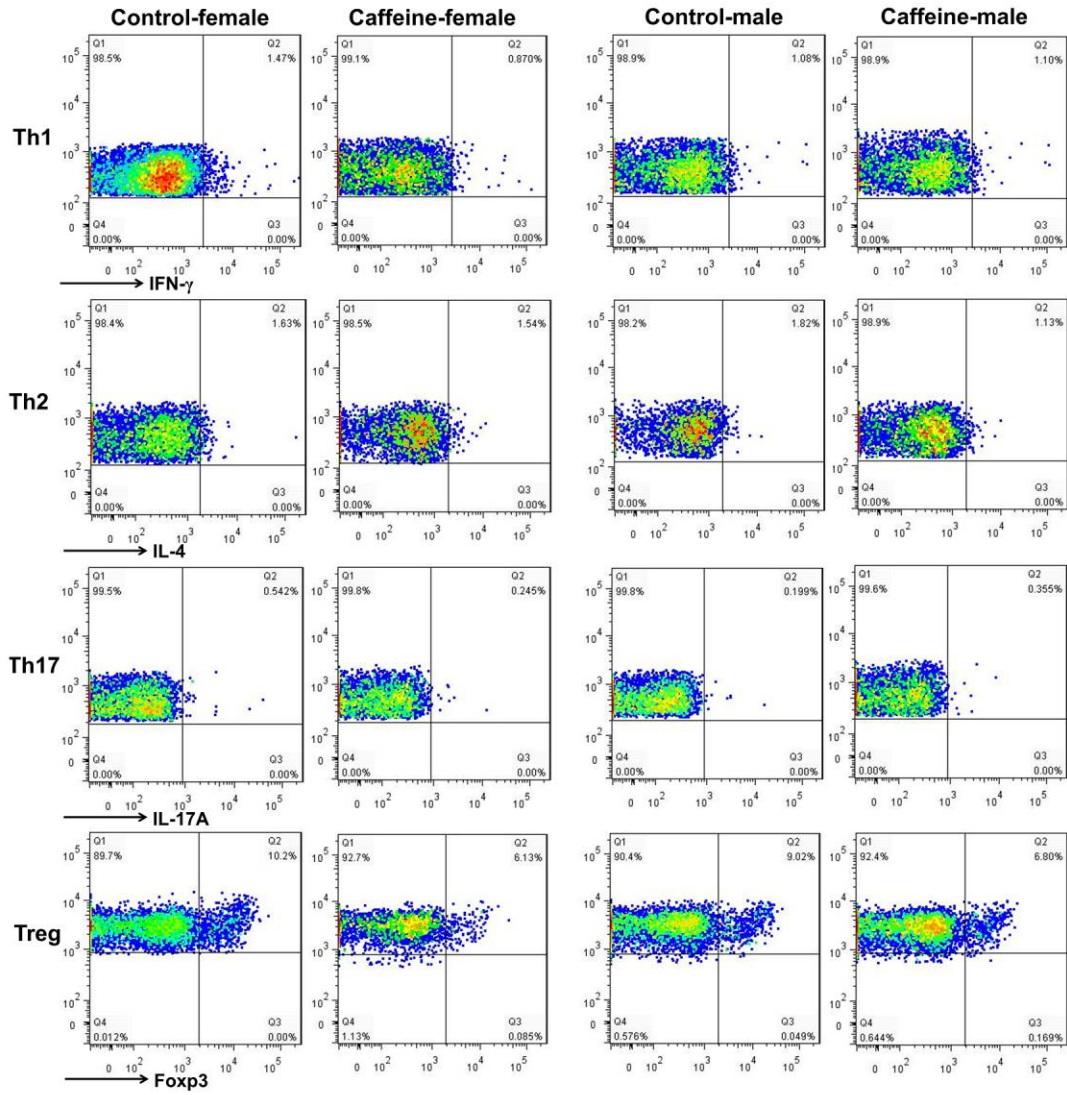
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Tel.: +86 27 6875 9310.

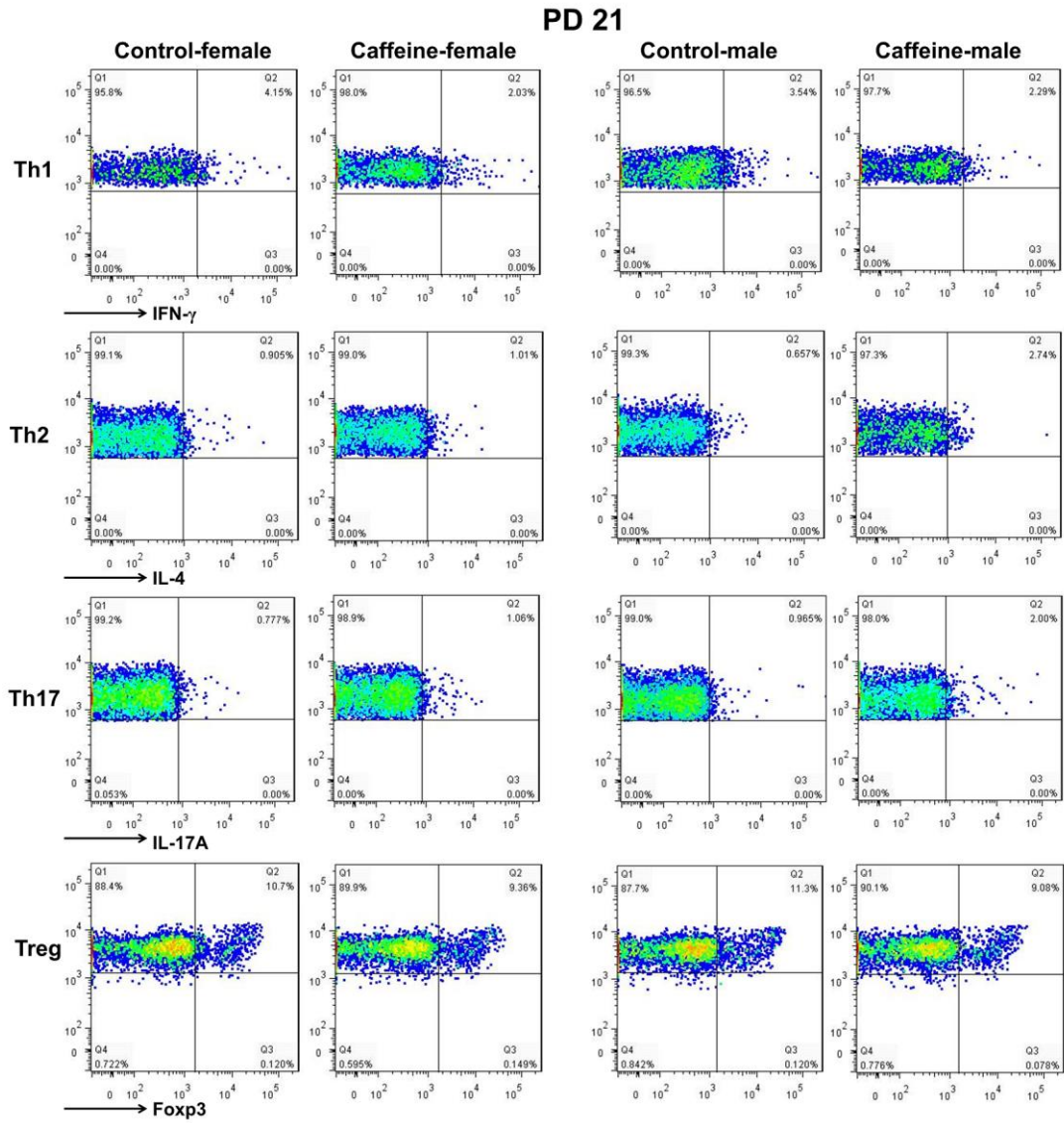
Fax: +86 27 8733 1670.

E-mail: pingjie@whu.edu.cn

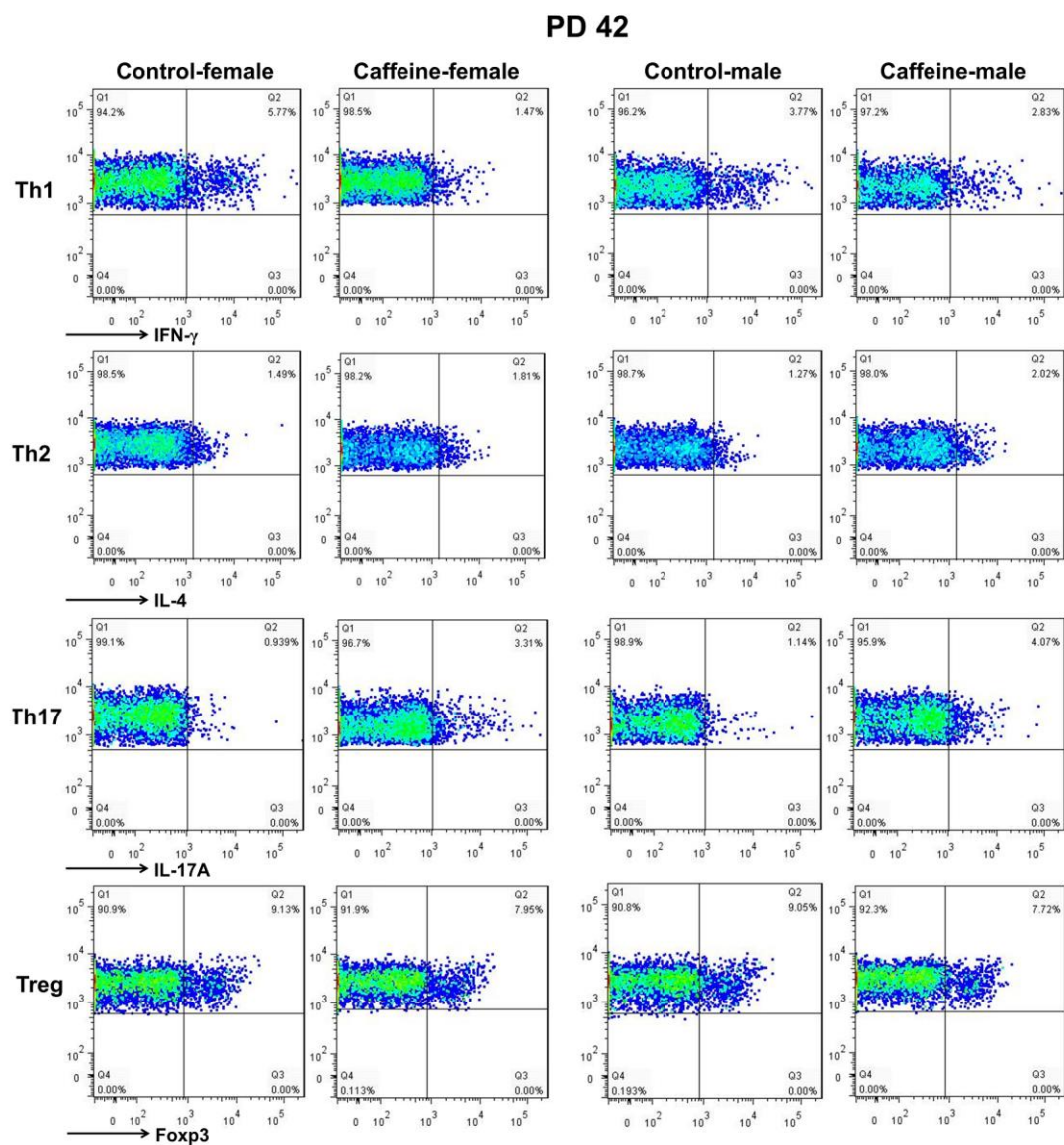
PD 7



Supplementary Fig. 1 The typical flow diagrams of pulmonary CD4⁺T phenotypes in offspring mice on postnatal day (PD) 7



Supplementary Fig. 2 The typical flow diagrams of pulmonary CD4⁺T phenotypes in offspring mice on postnatal day (PD) 21



Supplementary Fig. 3 The typical flow diagrams of pulmonary CD4⁺T phenotypes in offspring mice on postnatal day (PD) 42