

## Original Article

# Thiol peroxiredoxin, a novel allergen from *Bombyx mori*, modulates functions of macrophages and dendritic cells

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**Abstract:** *Bombyx mori* (*B.mori*, also known as silkworm) plays a role in the pathogenesis of allergic diseases. However, its allergens are to be characterized. The aim of this paper is to identify new silkworm allergens. Two-dimensional gel electrophoresis (2-DE) and mass spectrometry were employed to separate and identify potential allergens from silkworm pupa. Six potential allergens were identified in this study. Thiol peroxiredoxin (TP), one of the 6 allergens, reacted to serum IgE from patients sensitized to silkworm. By sensitizing with TP allergic asthma like symptoms were induced in mice, including elevation of the levels of serum IgE, IL-4 from bronchoalveolar lavage fluid and culture supernatant of spleen cells. In vitro experiments showed that TP significantly induced RAW264.7 cells (a macrophage cell line) apoptosis via modulating the BCL2 and Caspase9 pathways. The levels of CD80, CD40, CD83 and TNF- $\alpha$  in DC2.4 cells (a dendritic cell line) were increased in the culture after exposure to TP. In summary, TP is an allergic component of silkworm. It induces allergic asthma, and modulates the functions of macrophages and dendritic cells.

**Keywords:** *Bombyx mori*, allergy, thiol peroxiredoxin, macrophages, dendritic cells

## Introduction

*B.mori* is an economic insect; its pupa can be a delicious food [1]. *B.mori* is also an allergen in the pathogenesis of allergic disease [2, 3]. Published data show that a relative high incidence of people with respiratory allergy is sensitized to *B.mori* in the southern region of China [4]. The genome sequence of *B.mori* has been reported [5]. However, *B.mori* allergens are to be further characterized.

Macrophages and dendritic cells (DCs) are important cell fractions in the immunity [6]. They are the first line immune cells to encounter allergens, and play a central role in maintaining immunological homeostasis and host defense [7]. In the asthma mouse model, depletion of macrophages elevates airway hyper-reactivity, interleukin (IL)-13-dependent eosinophilic and Th2 inflammation; allergen-specific IgG1 and IgE are increased [8]. The

phagocytic ability of macrophages from pediatric asthmatics is impaired [9]. In children with severe asthma, macrophage apoptosis is increased [9].

DCs are classic antigen presenting cells (APC), and express a wide variety of pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are well known PRRs, and play a very important role in recognition of allergens [10]. Atopic asthma is mainly dependent on skewed helper type 2 CD4+ T cell responses (Th2) [11]. DCs capture allergens to present allergens to naive CD4+ T cells to differentiate into either Th1 or Th2 cells. MHC II-allergen peptide complex, cytokines, and co-stimulatory molecules from DCs are required in T cell differentiation [12]. CD80, CD40, MHC II, CD83 and TNF- $\alpha$  play a role in the activation and regulation of T cells [13]. However, the factors modulating the function of DCs are not fully understood. Therefore, while studying the mechanisms of allergen-

induced diseases, it is important to clarify the role of the macrophages and dendritic cells.

The aim of this study is to identify new allergens from silkworm. Recently, proteomics have been a useful tool to identify new allergens [14, 15]. In our paper, silkworm pupa extracts were separated by 2-DE, and six new potential allergens were identified by proteomics. The thiol peroxiredoxin (TP) protein was expressed and purified. The results showed that TP was an allergen as it responded to serum specific IgE from patients sensitized to *B.mori* and induced airway hyperresponsiveness and Th2 polarization in mice.

### Materials and methods

#### Chemicals

CCK8 kits were purchased from Transgen (FC-101-02). Antibody against GAPDH and BCL2 was purchased from Proteintech (10494-1-AP, 12789-1-AP); Caspase9 antibody was obtained from ABclonal (A0281); TLR4 signaling inhibitor was purchased from Invivogen (CLI-095); PE-CD80, FITC-CD40, PE-CD83 and FITC-MHC2 antibodies were obtained from Ebioscience (12-0801, 11-0402, 12-0831 and 11-5321).

#### 2-DE and immunoblotting

Silkworm pupa extracts were separated by 2-DE, as described previously [14]. Briefly, immobilised pH gradients (IPG) gels with linear gradients (pH 3-10) were rehydrated overnight. The extracts were focused to the isoelectric points by an Ettan IPGphor 3 apparatus for 40,000 volt-hours at 20°C. The IPG strips were equilibrated for 15 minutes in SDS equilibration buffer (50 mmol/L Tris-HCl pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS and 1% DTT) before the second dimension. SDS-PAGE was performed, and one of gels was dyed with Coomassie brilliant blue (CBB) solution, whereas the other gel was processed for further immunoblotting analysis. The proteins of 2-DE were transferred to a PVDF membrane; the membrane was blocked with 5% skim milk for 1 hr. Pooled allergic patients' sera were added to incubate at 4°C overnight. Biotinylated goat anti-human IgE antibody was used as the secondary antibody, and incubated with the streptavidin-conjugated-HRP at 37°C for 2 hrs. After each step, the membrane was washed with TBST for 3 times.

The results were developed by adding ECL substrate (Millipore, WBKLS0500), and the positive spots were excised and analyzed by Mass spectrometry.

#### Mass spectrometry

Protein spots from 2-DE gels were excised and washed with Milli-Q water; they were dissolved in 50% ACN/50 mM ammonium bicarbonate for 15 mins to remove the CBB dye, and then dehydrated twice in 100% ACN for 30 mins; Trypsin (10 ng/μL) digestion was performed at 37°C overnight; The tryptic peptides were extracted with 50% ACN/0.1% TFA and lyophilized for 4 hrs. MALDI-TOF MS and MALDI-TOF MS/MS were carried out by the Beijing Genomics Institute (BGI) company, Shenzhen.

#### Preparation of recombinant TP

The PCR products of TP were ligated into a pET-32a vector (Takara), and transformed into BL21 for expression. The bacteria were induced by isopropyl-D-thiogalactopyranoside (IPTG) at 25°C for 20 hrs, then harvested and resuspended in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5. After sonication, The TP proteins were purified by affinity chromatography.

#### Enzyme-linked immunosorbent assay (ELISA)

Serum specific-IgE antibodies for TP were measured by an in-house ELISA. The plates were coated with 100 ng TP/well in 100 μl buffer at 4°C overnight. After washed with PBST for 2 times, the plates were blocked with 200 μl 5% bovine serum albumin (BSA) in PBS at room temperature for 1 hr. The patients' sera (100 μl/well) was added to each well and incubated for 2 hrs, and then incubated with peroxidase-labeled goat anti-human IgE for 2 hrs. Each step was washed with PBST for 3 times. The results were developed by adding tetramethylbenzidine (TMB) and stopped by 2 M H<sub>2</sub>SO<sub>4</sub>. The plates were read by ELx808 microplate reader (BioTek, Shanghai, China) at 450 nm. In our study, an informed consent was obtained from each human subject, and approved by the Human Ethic Committee at Shenzhen University.

#### Development of an asthma mouse model

Female BALB/c mice (6-8 weeks) were purchased from Guangzhou Experimental Animal

Center and maintained in a pathogen-free facility, and the experimental procedures were approved by the Animal Ethic Committee at Shenzhen University. Mice were immunized intraperitoneally with TP or BME (100 µg/mouse) in 0.1 mL of 2% aluminum hydroxide on days 0, 3, and 7, respectively. The mice were challenged with TP or BME (50 µg/mouse) in 50 µl PBS via nostril drop daily for 7 consecutive days. On day 22, the penh was measured with inhaling the incremental doses of methacholine (0, 6.25, 12.5, 25, 50 and 100 mg/ml). On day 23, the mice were sacrificed. Lung tissues were fixed in 4% formalin, and embedded in paraffin wax for pathology analysis. The BALF, serum and spleen cells were collected. The levels of IL-4 and IFN-γ in BALF were measured by ELISA with commercial reagent kits (Ebioscience) according to the manufacturer's instructions. Splenocytes ( $5 \times 10^6$  cells per well) were incubated in presence of TP or BME for 96 hours, and the proliferation was tested by CCK8 kits, and the levels of IL-4 and IFN-γ were also detected by commercial ELISA kits (Ebioscience). The levels of specific IgE in serum were also assessed by indirect ELISA.

### *Cell culture*

The components of cell culture media, fetal bovine serum (FBS) and Trypsin were from Gibco. RAW264.7 (ATCC TIB71) and DC2.4 were maintained at 37 °C in 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, and 10 mM HEPES (C-DMEM).

### *Assessment of apoptotic cells*

RAW264.7 cells were seeded into 6 wells plates, and stimulated with TP or heat-inactivated TP at 20 µg/ml. After 48 hours, the cells were dyed with hoechst 33258 (Beyotime, C1018), and observed by fluorescence microscope. For further flow cytometer analysis, drained the medium and added 200 µl trypsin at 37°C. After 5 minutes, 800 µl C-DMEM were added to each well and transferred to a clean EP tube. The medium was drained by centrifugation at 1200 rpm. Propidium iodide (PI) and Annexin v reagents (4abio Company) were added to the culture and incubated in the dark. The supernatant was then removed and 300 µl PBS was added. The cells were analyzed by flow

cytometer. The proteins of apoptosis were measured by Western blot [16].

### *Neutralization of TLR4 receptor*

DC2.4 cells were seeded in 96 well plates ( $0.5 \times 10^6$  cells/well) and cultured for 20 hours. The cells were treated with or without TLR4 signaling inhibitor for 6 hours at 37°C, and then stimulated with TP for 24 hrs. The supernatants were collected and subjected to the assessment of TNF-α levels by ELISA with a commercial reagent kit (Ebioscience) according to the manufacturer's instructions.

### *Flow cytometry*

DC2.4 cells were seeded into 6-well dishes at a density of  $2 \times 10^5$  cells per well and maintained at 37°C in 5% CO<sub>2</sub>. After overnight growth, DC2.4 cells were treated with or without TLR4 signaling inhibitor for 6 hours at 37°C, and then stimulated by TP at a concentration of 20 µg/ml for 24 hrs. The cells were collected and incubated with antibodies of interest (Ebioscience) for 2 hours in the dark, and then analyzed with a flow cytometer.

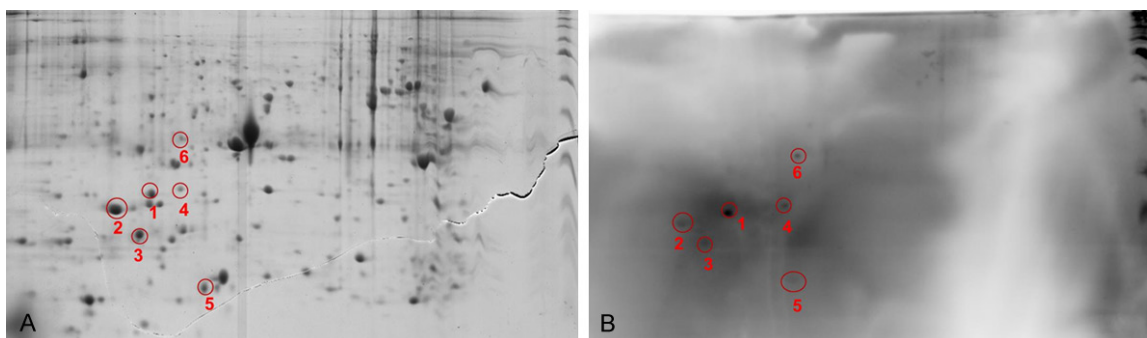
### *Statistical analysis*

All data were performed as means ± SEM by GraphPad Prism 5.0. Two-tailed Student's t-test was employed to test significant difference between two groups. \*P<0.05. \*\*P<0.01. \*\*\*P<0.001. ns, no significant difference.

## **Results**

### *Identification of silkworm pupa allergens by proteomics*

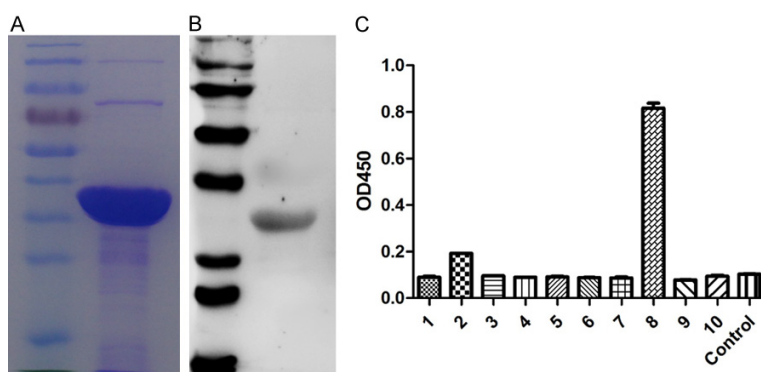
To identify new allergens of silkworm pupa, its water-soluble proteins were separated by 2-DE with IPGs and SDS-PAGE, and then stained with CBB. **Figure 1A** shows the protein maps of 2-DE. Then, the gel was incubated with the pooled sera of patients sensitized to the silkworm to be detected by western blotting. The positive spots were selected for new allergen identification. As shown by **Figure 1B**, There were 6 spots showing specific-IgE reactivity. These spots were selected for further analysis by mass spectrometry. As shown in **Table 1**, six potential allergens were discovered, and the TP was one of them. To further confirm the allergenicity, TP was cloned and purified (**Figure 2A**).



**Figure 1.** Identification of allergens from silkworm pupa via 2-DE. Silkworm pupa extracts were separated by 2-DE, and then new allergens were identified by western blot with patients' serum which were sensitive to silkworm. A: Separation of the silkworm pupa extracts via 2-DE. B: The results of western blot with patients' serum.

**Table 1.** The potential allergens of silkworm pupa

No	GI number	Protein name	MW
B1	gi 112983152	Heat shock protein 20.4	20.4 KD
B2	gi 290560816	Cuticular protein hypothetical 30 precursor	21.5 KD
B3	gi 290563229	Cuticular protein RR-2 motif 63 precursor	18.5 KD
B4	gi 112982996	Thiol peroxiredoxin	22 KD
B5	gi 112983054	Chemosensory protein 5 precursor	14.5 KD
B6	gi 512917985	PREDICTED: uncharacterized protein LOC101743840 isoform X1	28.4 KD



**Figure 2.** The allergenicity of TP. According to the protein sequence, TP was cloned, expressed and purified in vitro. A: The results of TP purification. B: The pooled serum specific-IgE against TP was detected by western blot. C: The specific IgE against TP was measured by ELISA. 1-10, the serum from BME positive patients; control, the serum from healthy subject.

As shown by **Figure 2B**, specific-IgE antibody against TP existed in the sera from patients sensitized to the silkworm. The positive rates were about 20% (**Figure 2C**). Therefore, TP is a new allergen of the silkworm.

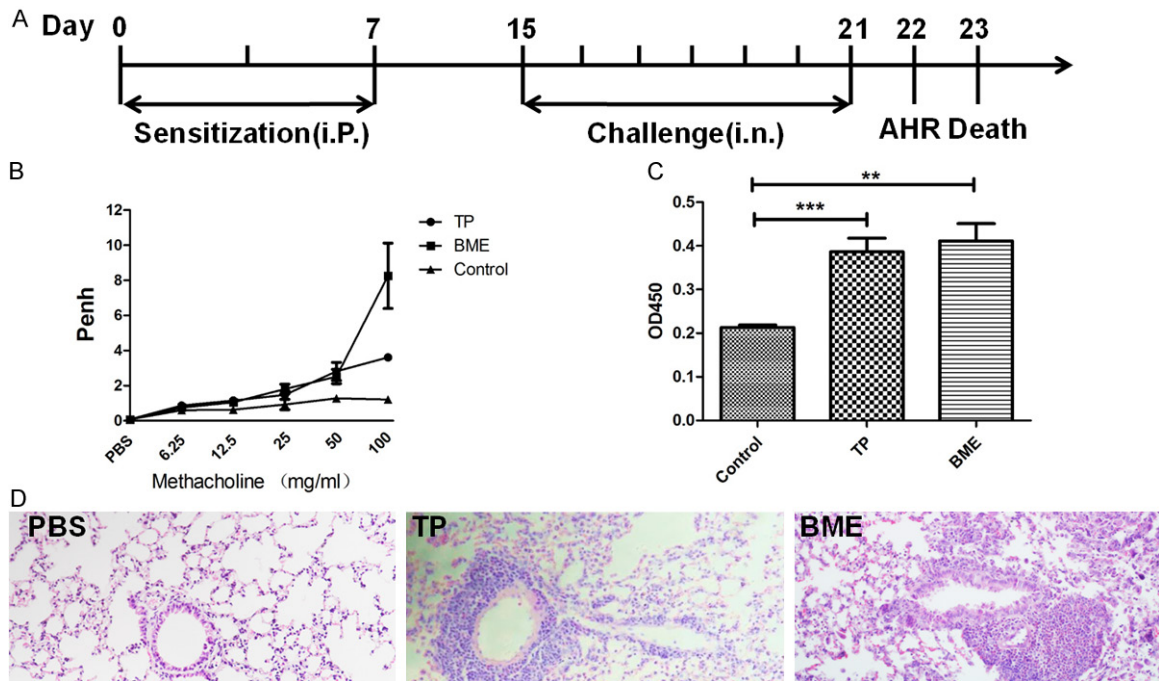
#### *Airway allergy is induced by TP in the mouse model*

To further characterize the allergenicity of TP, according to our previous procedures, mice

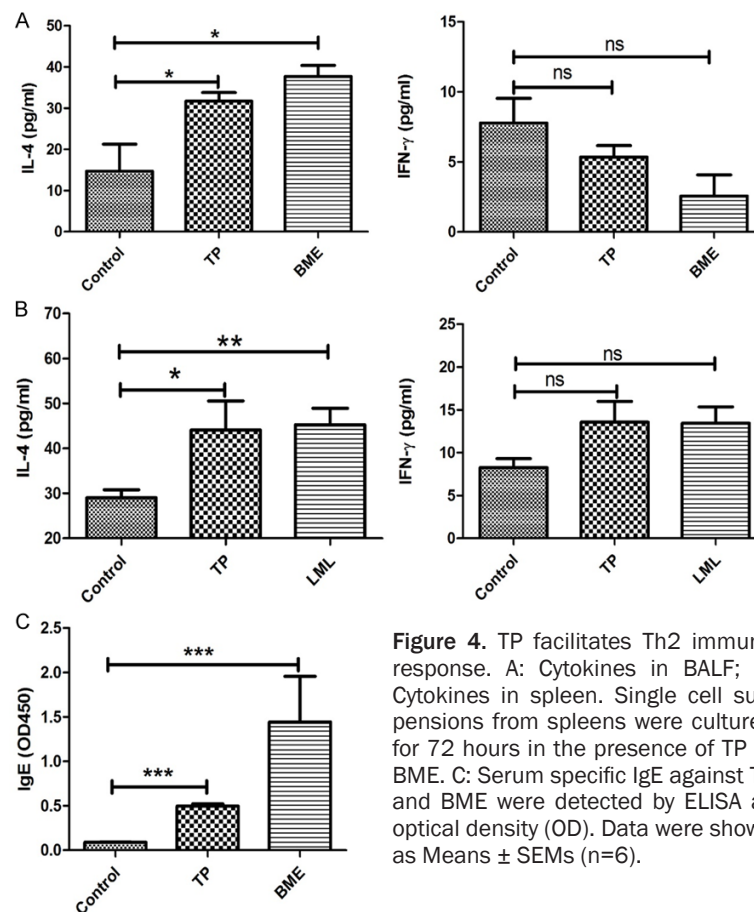
were immunized by PBS, TP and BME, respectively (**Figure 3A**) [17]. Methacholine induced AHR was detected on day 22. As shown by **Figure 3B**, the mice challenged with PBS showed little increase in Penh; the Penh in the TP group and the BME group were significantly higher than the PBS group. Further analysis showed that the spleen cell proliferation was significantly increased in mice immunized with TP or BME compared to mice exposed to PBS (**Figure 3C**). As shown by **Figure 3D**, lung pathology induced

by TP and BME were significantly severe than PBS. Meanwhile, mice challenged with TP and BME produced significantly higher levels of specific IgE than PBS group (**Figure 4C**). The cytokine levels in BALF were also assessed. As shown by **Figure 4A**, IL-4 levels in BALF of TP and BME were significantly higher compared to the PBS group. The IFN- $\gamma$  levels in the TP group and the BME group had no significant difference with the PBS group. The levels of IL-4 and





**Figure 3.** Allergy-like asthma induced by TP in the mouse model. Mice were immunized by PBS, TP and BME, respectively. A: Schematic representation of establishment of a mouse model. B: Airway hyperresponsiveness (AHR). C: Spleen cells proliferation was measured by CCK8 experiments. D: Lung pathology. Data were shown as Means  $\pm$  SEMs (n=6).

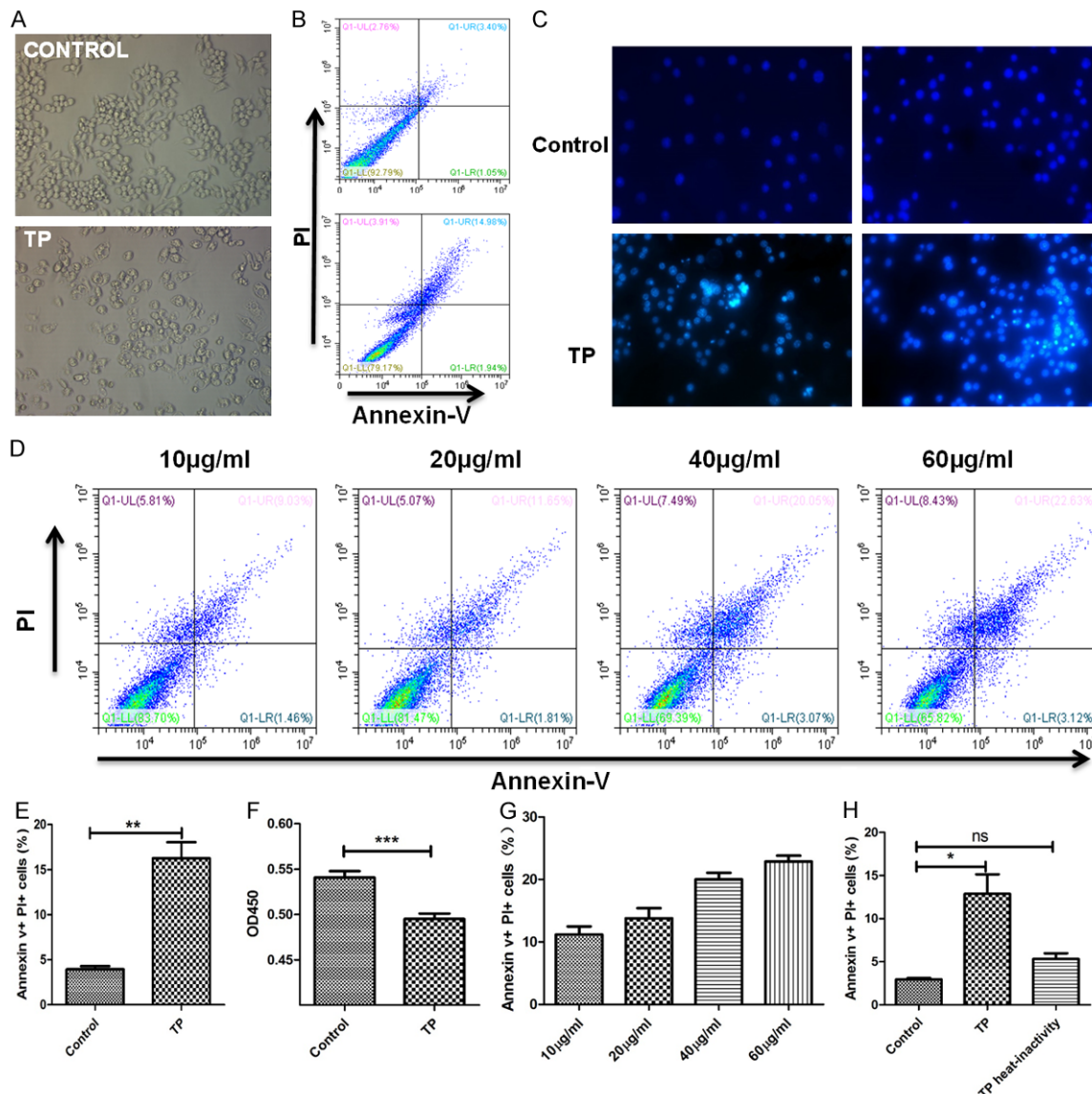


**Figure 4.** TP facilitates Th2 immune response. A: Cytokines in BALF; B: Cytokines in spleen. Single cell suspensions from spleens were cultured for 72 hours in the presence of TP or BME. C: Serum specific IgE against TP and BME were detected by ELISA as optical density (OD). Data were shown as Means  $\pm$  SEMs (n=6).

IFN- $\gamma$  in the supernatant of the spleen cell culture were consistent with that in BALF (**Figure 4B**). The data suggested that TP is an allergen, can induce a specific Th2 immune responses.

#### TP induces macrophages apoptosis

The apoptosis of immune cells play an important role in allergic disease [18]. Thus, we detected the apoptosis of macrophages and DCs after exposure to TP. As shown by **Figure 5A, 5B** and **5E**, we found that the TP protein enforced macrophage apoptosis compared to the controls (**Figure 5C**). TP also induced the death of macrophages as shown by CCK8 experiments



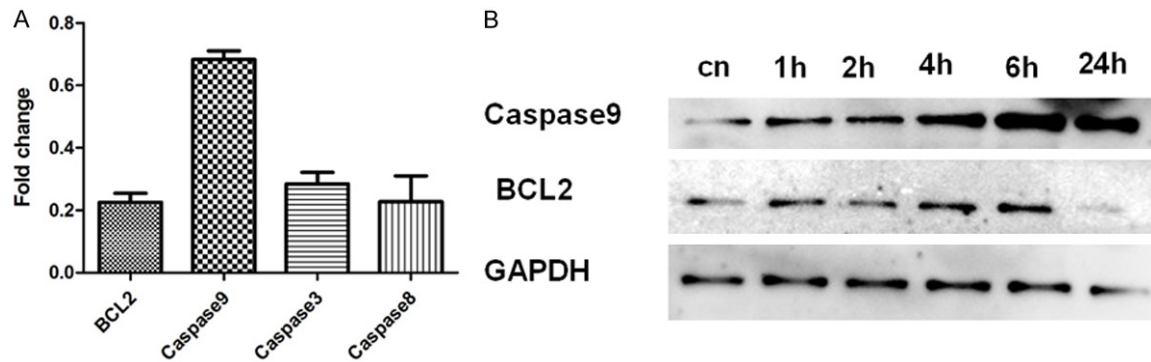
**Figure 5.** TP induces macrophages apoptosis. RAW264.7 were stimulated with TP for 48 hours. A: The phenotypes of macrophages in microscopy; B and D: Represented graph of flow cytometry; C: Observed macrophages dyed by hoechst33258 via fluorescence microscopy; E and G: Statistical Chart; F: The results of CCK8; H: The apoptosis induced by TP or heat-inactivity TP. Data are generated from three independent experiments.

(Figure 5F). Meanwhile, The apoptosis induced by TP had a TP concentration-dependent manner (Figure 5G). In addition, as shown by Figure 5H, heat-inactivated TP could not induce macrophage apoptosis. We also found that TP could upregulated Caspase9 and downregulated BCL2 (Figure 6A and 6B).

#### DCs are activated by TP via TLR4

To study the functions of TP, DC2.4 cells were stimulated with TP in the culture for 24 hrs. The supernatant was collected at the end of cul-

ture and analyzed the levels of CD40, CD80, MHC II, CD83 and TNF- $\alpha$  by ELISA. As shown by Figure 7A-C, the levels of CD40, CD80, CD83 and TNF- $\alpha$  in DC2.4 cells were increased significantly after exposure to TP. Previous researches have found that TLR4 plays an important role in allergic diseases [19]. In order to clarify whether TLRs are involved in the TP-induced allergy, the phenotypes of DCs were detected in the presence of TLR4 blocking agents. As shown by Figure 7C and 7D, the levels of CD40, CD80, CD83 and TNF- $\alpha$  were suppressed in DCs in the presence of TP and TLR4 signaling



**Figure 6.** The pathway of apoptosis induced by TP. RAW264.7 were stimulated with TP at 20  $\mu$ g/ml for 4 hours, and then detected the gene expression by RT-PCR. A: Statistic charts of the RT-PCR. Meanwhile, whole cell lysates were collected to detect the protein levels by western blot at 1, 2, 4, 6 and 24 hours. B: The results of western blot.

inhibitors. The results implicate that TLR4 is involved in the TP activating DCs.

### Discussion

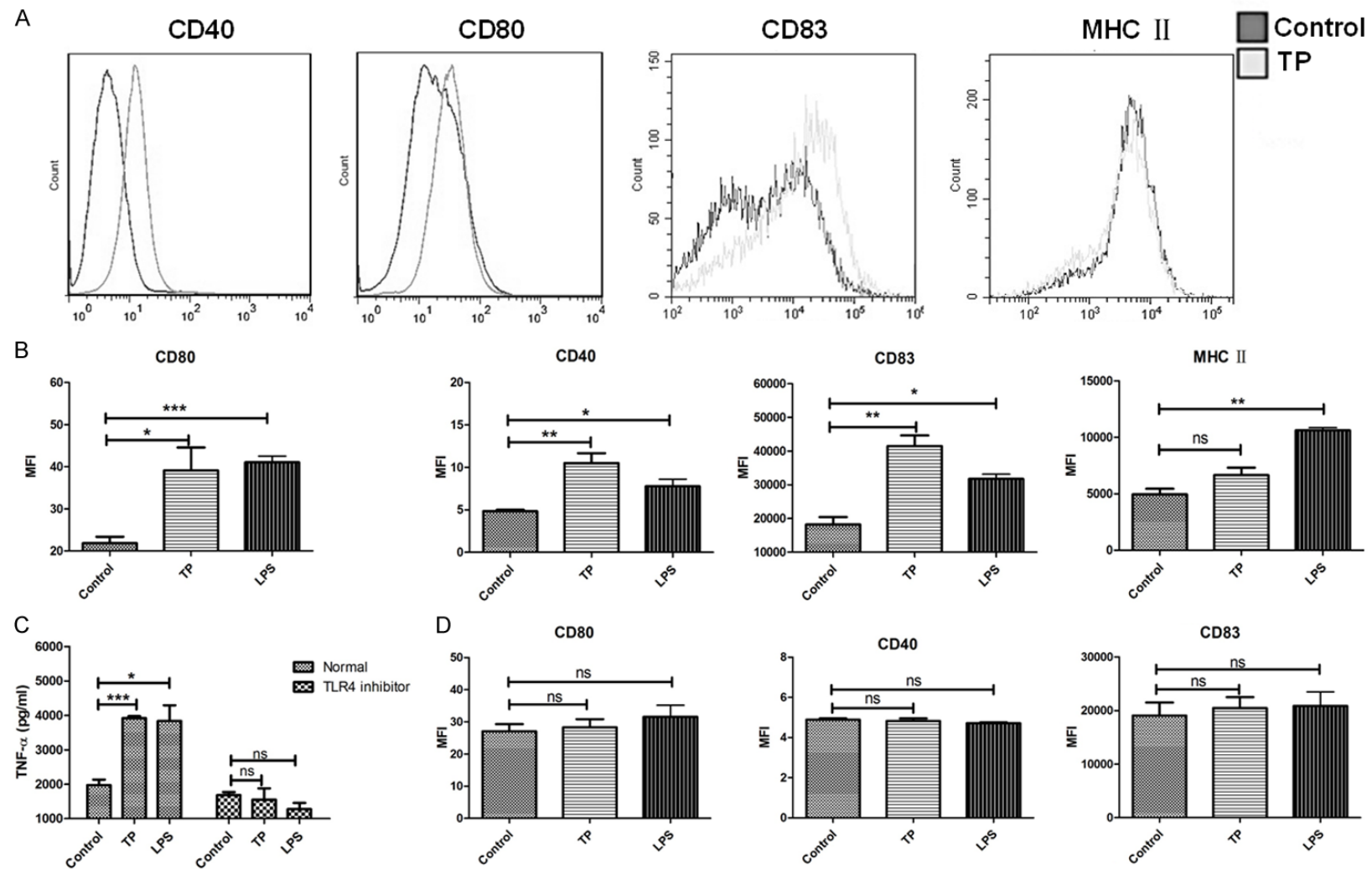
The silkworm is one of the commercial insects in China, India and some other developing countries [1]. The silkworm pupa has been used as food and medicine in China for a long time [15]. Published data also show that the silkworms are important sources of allergens [20, 21]. However, to date, little allergens have been characterized from silkworms. In our work, the silkworm pupa extracts were resolved by using 2-DE. Subsequently, the potential allergens were detected by immunoblotting using the sera from patients with silkworm allergy. 6 allergens were identified by mass spectrometry. Recombinant TP can react to the silkworm-allergic patients' sera, as shown by immunoblotting and ELISA, which is consistent with the results of proteomics.

The pathogenesis of allergic diseases is mainly due to aberrant Th2 polarization [22]. The Th2 responses include increases in Th2 cells and cytokines (such as IL-4, IL-5 and IL-13). They elevate the production of IgE, and then IgE sensitizes the effector cells to induce allergic inflammation [23]. In order to further investigate allergenicity of TP, we tried to ascertain whether it can induce asthma in a mouse model. We found that the penh of TP-sensitized mice was higher in TP-sensitized mice than the controls; the bronchial and vascular walls of lung were thickened and infiltrated by many inflammatory cells in TP-sensitized mice. The serum IgE and IL-4 from BALF and spleen

supernatants in the TP-sensitized mice were increased. Thus, the data demonstrate that TP is a new allergen from silkworm pupa.

Many immune cells, including eosinophils, mast cells, lymphoid cells and dendritic cells, are involved in allergic diseases. Kodama et al found that the apoptosis of eosinophils were increased significantly in the natural healing of allergic inflammation [24]. House dust mites are shown to inhibit the apoptosis of PMN by TLR4 pathways [25]. The BCL2 gene can inhibit the apoptosis. Jang et al found that the BCL2 gene of sputum eosinophils from patients with severe asthma was increased, and the eosinophil apoptosis was inhibited [26]. Therefore, the apoptosis of immune cells plays an important role in the pathogenesis of allergic diseases. In the present study, we found TP could induce macrophage apoptosis. Previous research has found that severe allergic asthma patients have more macrophage apoptosis, indicating that the macrophage apoptosis is conducive to the development of allergic asthma [9]. It suggests that macrophage apoptosis is part of the reason how TP induces allergic asthma.

DCs are well-known APCs. Mice with CD11c+ cell-deficiency cannot be induced with eosinophilia, goblet cell hyperplasia and bronchial hyperreactivity, indicating that DCs played a critical role in the initiating Th2 responses [27]. The activation of CD4+ T cells requires antigen signals and the costimulatory signals; the latter may be the major determinants [28, 29]. In this study, the levels of CD80, CD40, CD83 and TNF- $\alpha$  of DCs were upregulated by TP; indicat-



**Figure 7.** TP activates the function of DCs via TLR4. DC2.4 cells were stimulated with TP or LPS at 20  $\mu$ g/ml for 24 hours, and then detected CD80, CD83, CD40 and MHC II levels by flow cytometry. A: The representative diagrams of flow cytometer. B: Statistic charts of the flow cytometer. Detected the TNF- $\alpha$  levels by ELISA. C: The TNF- $\alpha$  levels in the culture supernatants with or without TLR4 signaling inhibitors. D: Statistic charts of the flow cytometer with TLR4 signaling inhibitors. Data were generated from three independent experiments.



ing that TP activates DCs. Some reports indicate that TLR2 and TLR4 participate in the human allergic asthma [30, 31]. In our study, the phenotypes of CD80, CD40, CD83 and TNF- $\alpha$  were disappeared in DCs after blocking TLR4. Thus, the data suggest that TLR4 is the receptor of TP.

In summary, we identified 6 different silkworm pupa proteins by the proteomic approach. Further research showed that TP was a new allergen of silkworm pupa; it could induce allergic asthma in a murine model. TP modulated the functions of DCs, and induced the apoptosis of macrophages.

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## Disclosure of conflict of interest

None.

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