



PRELIMINARY REPORT

Differential role of peroxiredoxin II (PrxII) on the expression of toll-like receptor 4 (TLR4) and B-cell activating factor (BAFF) in ovalbumin (OVA)-induced mouse asthma

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KEYWORDS

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Abstract

Peroxiredoxin II (PrxII) is one of reactive oxygen species (ROS)-degrading enzyme. Here, we investigated the role of PrxII on toll-like receptor 4 (TLR4) and B-cell activating factor (BAFF) expression in ovalbumin (OVA)-induced mouse asthma. We used ROS-producing PrxII^{-/-} mice of which cells up-regulate BAFF expression. As significant changes were detected in TLR4 mRNA with real-time quantitative RT-PCR analysis, TLR4 protein was decreased in PrxII^{-/-} mouse splenocytes and peritoneal macrophages, compared to wild type cells. Airway hyper-responsiveness (AHR) was more severe in PrxII^{-/-} mice than wild type mice, which was measured by the level of various parameters, number of eosinophils, IgE level, airway thickness, and mucous secretion. BAFF was detected in cells surrounding airways of OVA-induced mouse and it

Abbreviations: PrxII, peroxiredoxin II; BAFF, B-cell activating factor; ROS, reactive oxygen species; OVA, ovalbumin; TLR, toll-like receptor; AHR, airway hyper-responsiveness; PAS, periodic-acid schiff; BAL, bronchoalveolar lavage; MEF, mouse embryonic fibroblast; DAB, diaminobenzidine1.

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was highly augmented in $\text{PrxII}^{-/-}$ mice. BAFF promoter activity was also higher in $\text{PrxII}^{-/-}$ mouse embryonic fibroblast (MEF) than in wild type MEF. Collectively, results show that PrxII may have benefits in asthma through reducing ROS. It suggests that BAFF and TLR4 expressions are differentially regulated by PrxII and TLR4 protein level may not be crucial in OVA-induced asthma.

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1. Introduction

Asthma is a chronic inflammatory disease of the lower airways, characterized clinically by reversible airway obstruction and airway hyper-responsiveness (AHR). The characteristic features of asthma are airway inflammation (including the infiltration of inflammatory cells such as eosinophils and lymphocytes), epithelial damage, and airway remodeling. Oxidative stress and oxidants have been implicated in the pathogenesis of asthma [1,2]. Asthma induction with OVA is resulted in the increase of ROS [3–5]. A reducing agent, L-2-oxothiazolidine-4-carboxylic acid (OTC), a pro-drug of cysteine, increases intracellular GSH and it may reduce airway inflammation and AHR through the regulation of NF- κ B activity [5].

Mature B-cell generation and maintenance are regulated by B-cell activating factor (BAFF). BAFF is produced by macrophages or dendritic cells (DCs) upon stimulation with lipopolysaccharide (LPS) or interferon- γ . BAFF belongs to the tumor necrosis factor (TNF) family. Its biological role is mediated by the specific receptors, B-cell maturation antigen (BCMA), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and BAFF receptor (BAFF-R) [6,7]. BAFF-deficient mice exhibit an almost complete loss of follicular and marginal-zone B lymphocytes [8]. BAFF is associated with autoimmune disorders and induces rheumatic arthritis in a transgenic mouse model [9,10]. These disorders are well-known Th1-mediated inflammatory diseases. In addition, BAFF induces class-switch DNA recombination to produce IgG, IgA and IgE [11], which is in relation to Th2-mediated immune reaction-mediated allergic disorder such as asthma [12–14]. BAFF expression was increased by ROS treatment and PrxII gene deletion [15]. Peroxiredoxins (Prxs) play a role in ROS degradation. ROS production increased in $\text{PrxII}^{-/-}$ mice compared to wild type mice [15–18]. Asthma induction with OVA resulted in the increase of BAFF [19]. Treatment of asthma with TACI:Fc scavenging soluble BAFF reduced asthmatic symptom in mouse model [19].

Several reports have shown that toll-like receptor 4 (TLR4) agonist, LPS induced ROS generation and ROS are involved in TLR4-associated activation of NF- κ B [20,21]. ROS that is produced by LPS-stimulation or PrxII gene deletion, augments BAFF expression through NF- κ B activation [15]. BAFF protein level was also increased in asthmatic patient serum [22] and OVA-induced mouse lung tissues [19]. The requirement of TLR4 for pulmonary inflammation depends on the nature of the toxin and appears specific to toxin and exposure conditions [23]. In addition, no differences were seen in the abilities of naive C57BL/6 and $\text{TLR4}^{-/-}$ mice to develop allergen-specific tolerance after exposure to similar preparations of OVA [24]. However, little has been known about the requirement of TLR4 in $\text{PrxII}^{-/-}$ mouse asthma and

the regulation of ROS-mediated TLR4 expression compared to the augmentation of ROS-mediated BAFF expression.

In this study, we investigated the role of PrxII on TLR4 expression and the relationship between OVA-induced asthma and PrxII expression. Here, we found the reciprocal expression of TLR4 and BAFF in ROS-producing cells that were experimentally modified by serum deprivation and in constitutively ROS-producing $\text{PrxII}^{-/-}$ spleen cells. We also found that asthma induction was more severe and BAFF expression was higher in $\text{PrxII}^{-/-}$ mice than wild type mice. It suggests that TLR4 expression is differentially regulated from BAFF and TLR4 expression level may not be crucial in OVA-induced asthma.

2. Materials and methods

2.1. Mice and reagents

Wild type and peroxiredoxin $\text{II}^{-/-}$ 129 sv/J mice were obtained by breeding male $\text{PrxII}^{+/+}$ and female $\text{PrxII}^{+/+}$ mice and by performing the genotyping of animals as described [18]. Animals were maintained in the pathogen-free authorized facility in the Korea Research Institute of Bioscience and Biotechnology (KRIBB) where the temperature is at 20–22 °C, the humidity is 50–60%. The dark/light cycles were 12 h. All animal procedures were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee, KRIBB. Periodic-Acid Schiff (PAS) staining kit, ovalbumin and aluminum hydroxide were purchased from Sigma-Aldrich (St. Louis, MO). Except where indicated, all other materials were obtained from the Sigma-Aldrich (St. Louis, MO).

2.2. Bronchial asthma induction with ovalbumin (OVA)

Wild type and $\text{PrxII}^{-/-}$ 129 sv/J mice were sensitized by intraperitoneal injection of 20 μ g OVA emulsified in 2 mg aluminum hydroxide in a total volume of 100 μ l phosphate-buffered saline (PBS) on days 0 and 14. On days 28, 29, and 30, mice were challenged by ultrasonic nebulization exposure to an aerosol of 1% (w/v) OVA in PBS for 30 min. Twenty-four hours after the last challenge (day 31), the mice were sacrificed, and airway inflammation was characterized by histological examination.

2.3. Bronchoalveolar lavage (BAL)

The mice were killed with an overdose of pentobarbital. BAL fluid (BALF) was collected from each animal via cannulation of the exposed trachea and gentle flushing of the lungs with 0.5 ml of PBS three times (total 1.5 ml). BALF was used to analyze the number of eosinophils, and inflammatory cells.

2.4. Histological analysis

To count the number of eosinophils, cells in BALF were cyto-spinned on slide glass, stained with Giemsa, and observed under light

microscope. Immediately after the collection of BALF, the lung was then inflated with 1 ml of 10% neutral buffered formalin. After the fixation overnight in 10% formalin solution, lung tissues were embedded in paraffin. Paraffin-embedded tissues were sectioned into 4 μ m thickness and stained with hematoxylin and eosin Y. The bronchoalveolar changes were observed under light microscope with 200 $\times$ magnification.

2.5. Immunostaining

Immunohistochemical studies to detect BAFF protein were performed on formalin-fixed and paraffin-embedded lung tissue sections (4 μ m thick). Briefly, antigen was retrieved with Target Retrieval solution (DAKO Cytomation, Denmark). Primary biotinylated rat anti-mouse BAFF monoclonal antibody (IC9) (ALEXIS, San Diego, CA) and secondary streptavidin-conjugated horseradish peroxidase were used to detect BAFF in lung tissues.

Wild type and PrxII^{-/-} peritoneal macrophages were collected and plated on cover glass. Cells were stimulated with LPS and fixed in 3% paraformaldehyde solution. Then, cells were stained with primary anti-TLR4 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary immunoglobulin-conjugated horseradish peroxidase (HRP). The antigen-antibody reaction was visualized using diaminobenzidine (DAB) as a chromogen, with Mayer's hematoxylin counter staining. Cells were observed with 400 $\times$ magnification. Number of TLR4-positive cells was counted under light microscope using image analyzer (Media Cybernetics, Silver Spring, MD).

2.6. Periodic-acid Schiff (PAS) staining

PAS staining was performed following the instruction's manual (Sigma, St. Louis, MO). Briefly, tissues were de-paraffinized and hydrated to deionized water. Glycol-containing cellular elements in bronchi were visualized by the incubation in periodic-acid solution and Schiff's reagent with Mayer's hematoxylin counter staining. When treated with periodic acid, glycols are oxidized to aldehydes. After reaction with Schiff's reagent (a mixture of pararosaniline and sodium metabisulfite), a pararosaniline adduct is released that stains the glycol-containing cellular elements pink to red. Cellular elements which may be demonstrated with the PAS procedure include glycogen, fungal walls, basement membrane, certain epithelial sulfomucins and sialomucins, neutral mucosubstances.

2.7. Cell preparation

Spleens were dissected from control and OVA-induced mice in RPMI 1640 supplemented 2% fetal bovine serum (FBS). Splenocytes were obtained by teasing spleen gently with plunger of 3 ml syringe and by sieving through 70 μ m cell strainer (Falcon, Bedford, MA). Red blood cells were removed by incubating with RBC lysis buffer (Sigma Chemical Co., St. Louis, MO) for 5 min. Spleen cells were washed twice with RPMI 1640 medium. Cell numbers were adjusted to appropriate concentrations.

2.8. Preparation of the mouse embryo fibroblast (MEF)

MEF (mouse embryo fibroblast) were prepared with 13.5-day embryos derived from PrxII^{+/-} mice mated with each other. Head, tail and viscera were removed, the remain body was minced and dispersed in 0.25% trypsin/EDTA and incubated in a 5% CO₂ at 37 °C for 30 min. Then, the large fragments were removed and the cell suspension was plated in 100 mm plates and incubated at 37 °C until confluent.

2.9. Measurement of mBAFF promoter activity

Wild type and PrxII^{-/-} MEF cells were transfected with mBAFF promoter (Genebank # AC138397) [25], pGL3-mBAFF-Luc and pcDNA-

lacZ for monitoring transfection efficiency by β -galactosidase assay. Luciferase activity was determined by incubating cell extracts with luciferase substrate (Promega). Luminescence was measured using a luminometer (Perkin Elmer, Wellesley, MA). Luciferase units of experimental vector were normalized to the control vector in each sample.

2.10. Enzyme-linked immunosorbent assay (ELISA) for mouse immunoglobulins (Igs)

IgG and IgE concentration was measured by ELISA quantitation kit (Bethyl, Montgomery, TX), according to manufacturer's instruction. 100 μ l of polyclonal anti-IgE or anti-IgG antibodies diluted in NaHCO₃ buffer (pH 9.6) were transferred to an ELISA plate and incubated overnight at 4 °C refrigerator. Plate was washed twice with washing buffer (0.05% Tween 20 in PBS, pH 7.4) and blocked with blocking solution (1% BSA, 5% sucrose and NaN₃ in PBS) for 1 h. Mouse serum diluted 5 times was used to measure IgG and IgE. Anti-IgE or anti-IgG antibodies-conjugated horseradish peroxidase (HRP) were added and incubated for 1 h. Substrate was 1:1 mixture of color reagent A (H₂O₂) and color reagent B [tetramethylbenzidine (TMB)] (R&D system, Minneapolis, MN). 50 μ l of 1 M H₂SO₄ was added to stop reaction. Absorbance was measured at 450 nm.

2.11. RT-PCR

RNA was isolated from cells using TRIzol (Invitrogen Life Technology, Carlsbad, CA). cDNA was synthesized from 1 μ g of total RNA, using oligo (dT)₁₅ primers and superscript reverse transcriptase in a final volume of 21 μ l (Bioneer, Taejeon, Korea). For standard PCR, 1 μ l of the first strand cDNA product was then used as a template for PCR amplification with Taq DNA polymerase (Bioneer, Taejeon, Korea). PCR amplification proceeded as follows: 28 thermocycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, using oligonucleotides specific for TLR2 (forward: cag ctt aaa ggg cgg gtc aga g, reverse: tgg aga cgc cag ctc tgg ctc a), TLR4 (forward: agt ggg tca agg aac aga agc a, reverse: ctt tac cag ctc att tct cac c), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: tcc acc acc ctg ttg ctg ta; reverse: acc aca gtc cat gcc atc ac) and mBAFF (forward: gct ttc cag gga cca gag gaa a; reverse: tta cag cag ttt tag ggc acc aa).

2.12. Real-time quantitative RT-PCR

Real-time PCR for TLR4 was performed using iQTM SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and primers used in RT-PCR. For TLR4 amplification, samples were heated to 94 °C for 8 min and cycled 45 times at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s. iCycler iQTM Real-time PCR for mBAFF was performed using TaqMan® Universal PCR Master mix (2X) and primers (forward: cgc cga cta tac gaa aag gaa ctt a; reverse: ctc ctc caa ggc att tcc tct tt) and FAMTM dye-labeled TaqMan® MGB probe (cca tgg ctt ctc agc tt). For mBAFF amplification, samples were heated to 95 °C for 10 min and cycled 40 times at 95 °C for 15 s and 60 °C for 1 min. Real-time PCR analysis for TLR4 and mBAFF was carried out in duplicate using real-time PCR Detection System from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) and Applied Biosystems (Foster City, CA, USA), respectively. The amount of TLR4 and mBAFF mRNA was determined and normalized by the amount of GAPDH mRNA as an internal control.

2.13. Western blot analysis

Cells were lysed in ice-cold lysis buffer containing 0.5% Nonidet P-40 (vol/vol) in 20 mM Tris-HCl (pH 8.3); 150 mM NaCl; protease inhibitors (2 μ g/ml aprotinin, pepstatin, and chymostatin; 1 μ g/ml leupeptin and pepstatin; 1 mM phenylmethyl sulfonyl fluoride (PMSF); and 1 mM Na₄VO₃). Lysates were incubated for 30 min on

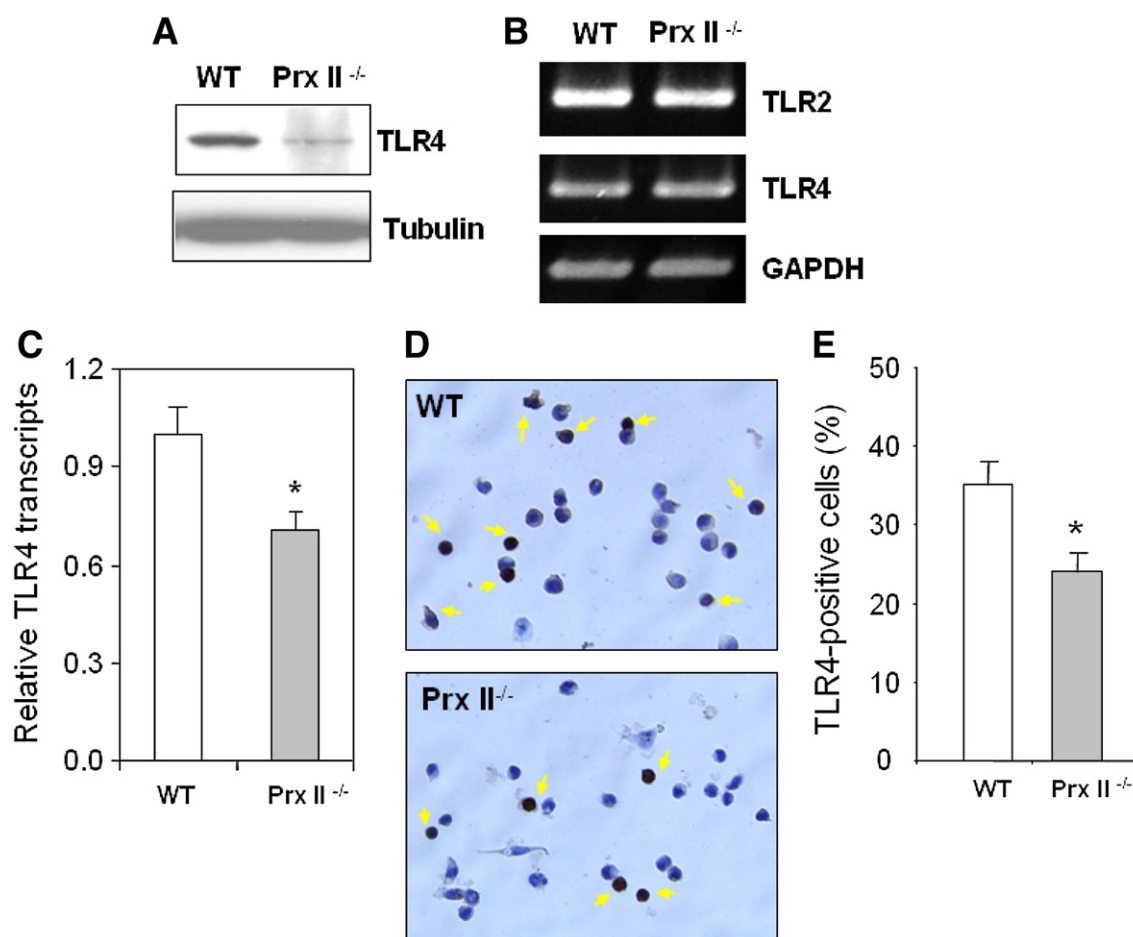


Figure 1 Inhibition of TLR4 protein level by the deletion of peroxiredoxin II (PrxII). Splenocytes from wild type and PrxII^{-/-} mice were prepared and TLR4 proteins were detected by Western blot analysis (A). RT-PCR was performed for the measurement of TLR2 and TLR4 transcripts (B). Relative TLR4 transcripts in PrxII^{-/-} to wild type mouse splenocytes were measured by real-time quantitative RT-PCR analysis described in Materials and methods. Data in bar graph represent mean ± SED. **p* < 0.05; Significantly different from wild type control (C). TLR4 was immunohistochemically detected in peritoneal macrophages from wild type and PrxII^{-/-} mice. Macrophages were fixed with 3% paraformaldehyde and incubated with anti-TLR4 antibodies. Antigen-antibody complexes were visualized with diaminobenzidine (DAB). TLR4-positive cells were indicated by yellow arrows (D). TLR4-positive cells were counted under light microscope with 400× magnification. Data in bar graph represent mean ± SED. **p* < 0.05; Significantly different from wild type control (E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

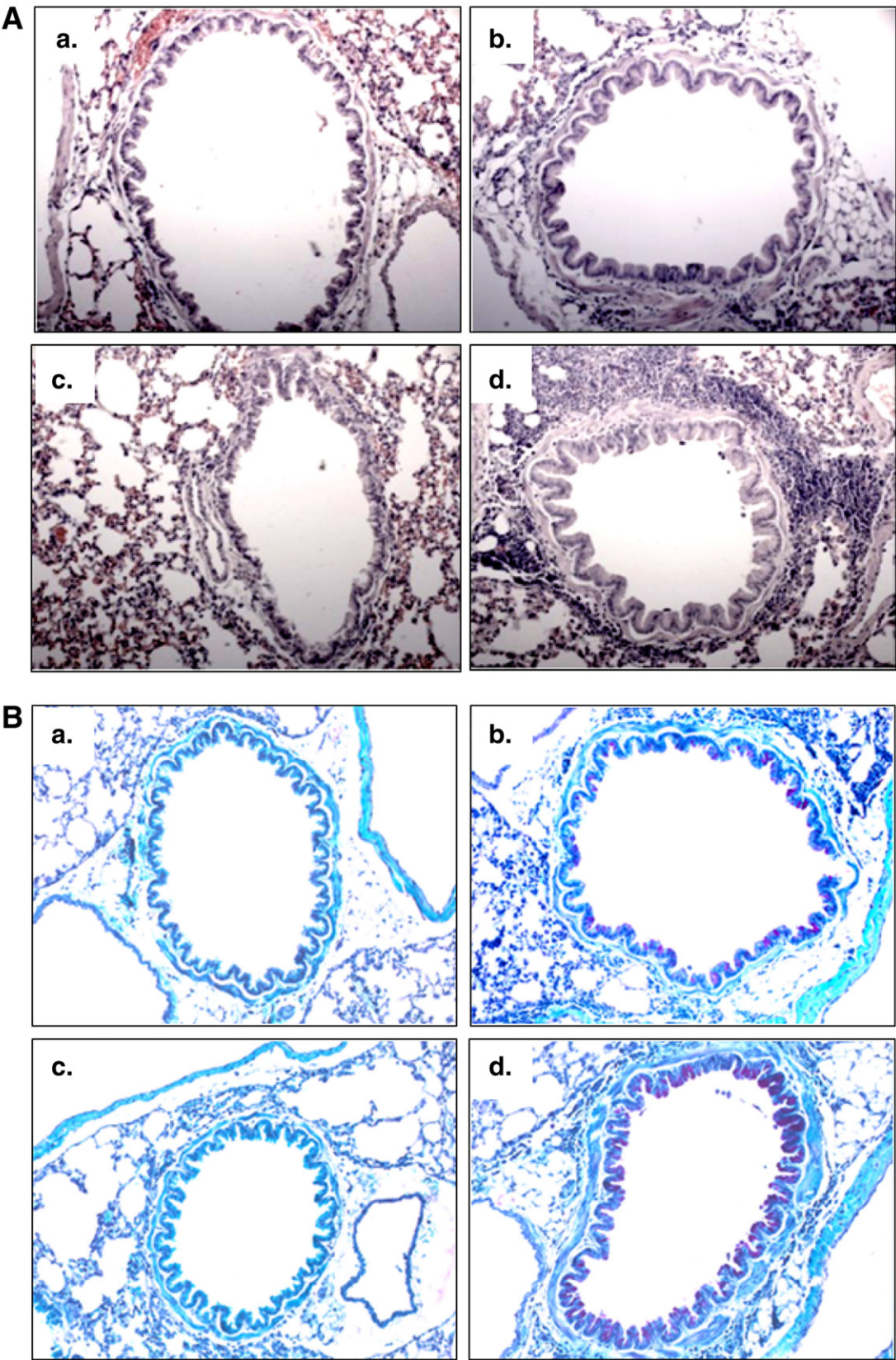
ice before centrifugation at 14,000 rpm for 5 min at 4 °C. Proteins in the supernatant were denatured by boiling for 5 min in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes. Following transfer, equal loading of protein was verified by Ponceau staining. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.5% Tween 20) and incubated with the indicated antibodies, monoclonal tubulin (1:5000) from Sigma-Aldrich (St. Louis, MO), and polyclonal TLR4

(1:1000) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Bound antibodies were visualized with HRP-conjugated secondary antibodies with the use of enhanced chemiluminescence (ECL) (Pierce, Rockford, IL).

2.14. Statistical analyses

Experimental differences were tested for statistical significance using ANOVA and Students' *t*-test. *p* value of < 0.05 or 0.01 was considered to be significant.

Figure 2 Histopathological observation of control and OVA-challenged mouse lung tissues. Mice were injected with OVA in aluminum hydroxide at day 1 and day 14. Then, mice were challenged by inhalation with OVA at days 28, 29, and 30. Lung tissues were fixed, paraffin-embedded, micro-sectioned and stained with hematoxylin/eosin (A). Periodic acid-Schiff staining (PAS) was performed. Glycol-containing cellular elements in bronchi were visualized by the incubation in periodic-acid solution and Schiff's reagent with Mayer's hematoxylin counter staining (B). Photographs were obtained with 200× magnification from wild type (a and e), OVA-induced wild type (b and f), PrxII^{-/-} (c and g), and OVA-induced PrxII^{-/-} (d and h) lung tissues. All pictures were the representative of 3 experimental tissue samples.



3. Results

3.1. Reduction of toll-like receptor 4 (TLR4) by the deletion of *PrxII* gene

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns and evoke various cell signaling pathways [23]. We investigated the TLR4 expression in *PrxII*^{-/-} mouse cells. ROS level was elevated by the deletion of *PrxII* gene [18,26]. As shown in Fig. 1A, TLR4 protein level was reduced in *PrxII*^{-/-}

spleen cells, compared to wild type cells. While no changes of TLR4 transcriptional level were detected by RT-PCR (Fig. 1B), the decrease of TLR4 expression was about 30%, which was measured by real-time quantitative RT-PCR analysis (Fig. 1C). The decrease of TLR4 protein level was confirmed in *PrxII*^{-/-} peritoneal macrophages that were immunohistochemically stained with anti-TLR4 antibodies (Fig. 1D). The percentage of TLR4-positive peritoneal macrophages was reduced by the deletion of *PrxII* gene (Fig. 1E). It suggests that TLR4 expression could be regulated by *PrxII*-degradable ROS.

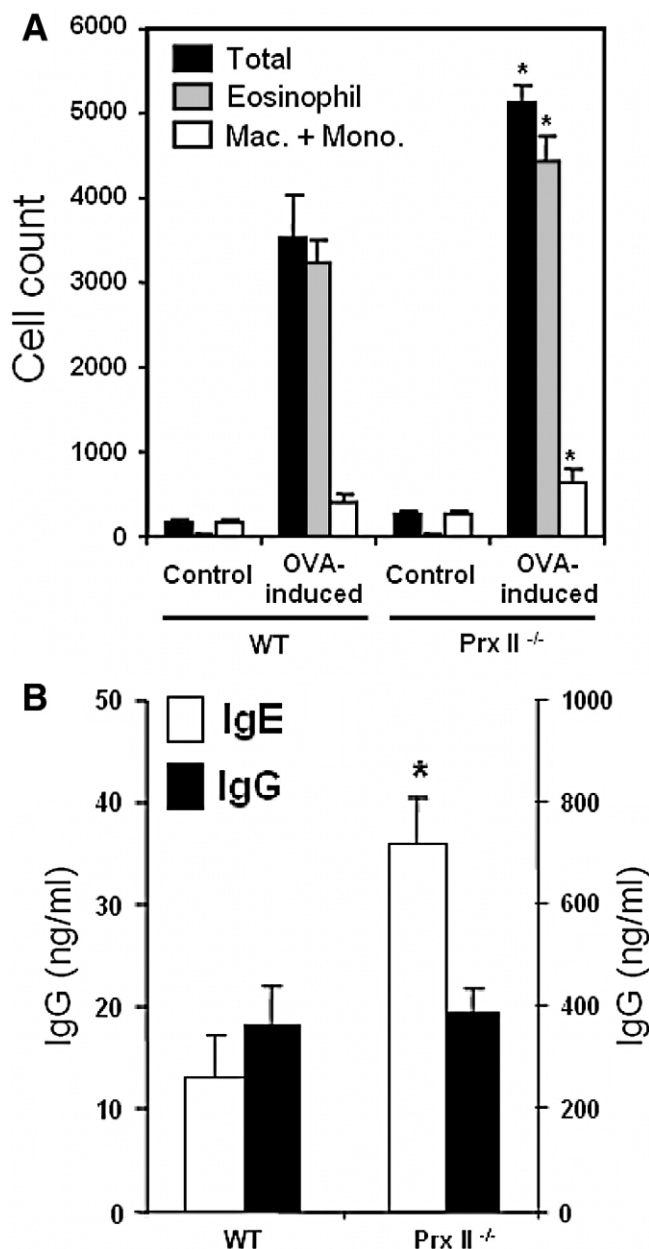


Figure 3 Assessment of various asthmatic parameters in OVA-induced airway hyper-responsiveness. Mice were injected with OVA in aluminum hydroxide at day 1 and day 14. Then, mice were challenged by inhalation with OVA at days 28, 29, and 30. Bronchoalveolar lavage fluid (BALF) was collected from each *PrxII*^{-/-} and wild type mice. Macrophages (Mac.), monocytes (Mono.) and eosinophils were cyto-spinned and stained with Giemsa. Number of cells was counted under light microscope (A). ELISA was used to measure baseline of IgE and IgG concentration in plasma from *PrxII*^{-/-} and wild type mice (B). All data were the representative of three experiments. Data in bar graph represent mean $\pm$ SED. * $p < 0.05$; Significantly different from wild type control.

3.2. High allergic responses were invoked in $\text{PrxII}^{-/-}$ mice

Given that oxidative stress and oxidants have been implicated in the pathogenesis of asthma [1,2] and PrxII regulates pro-inflammatory responses [27], we investigated whether OVA-

induced asthma could be affected by the deletion of PrxII gene and the reduced TLR4 production. Fig. 2 showed that asthmatic symptom was more severe in $\text{PrxII}^{-/-}$ mouse bronchi in OVA-induced asthma model, compared to wild type mouse bronchi. Bronchial thickness and mucous secretion were increased by

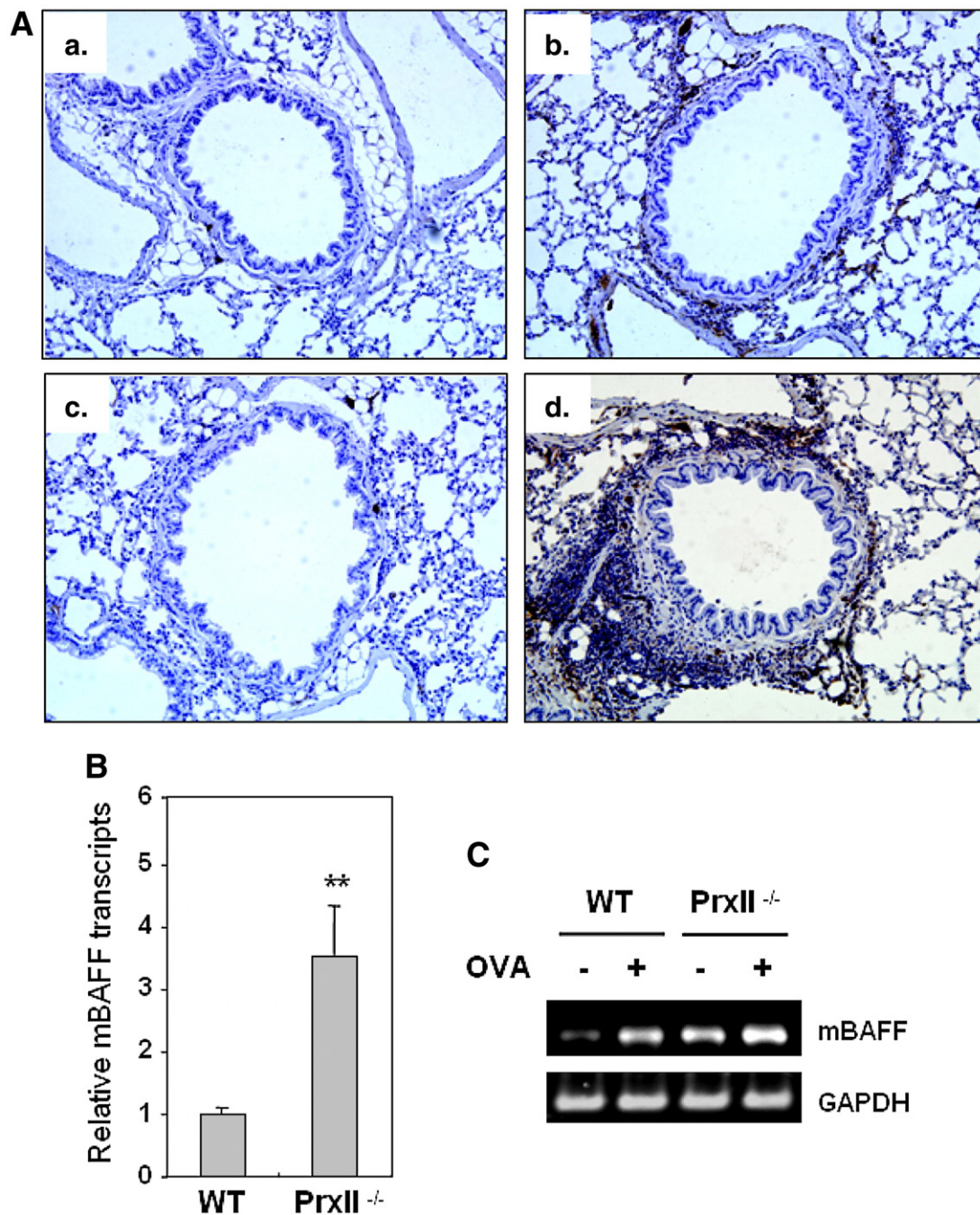


Figure 4 Increased BAFF expression in OVA-challenged mouse asthma. BAFF in airways from OVA-induced mice was detected by immunohistochemical staining. Lung tissues were incubated with biotinylated anti-BAFF antibodies. Antigen-antibody complexes were visualized with diaminobenzidine (DAB). Photographs were obtained from wild type (a), OVA-induced wild type (b), $\text{PrxII}^{-/-}$ (c), and OVA-induced $\text{PrxII}^{-/-}$ (d) lung tissues with 200 $\times$ magnification. All pictures were the representative of 3 experimental tissue samples (A). Relative mBAFF transcripts in $\text{PrxII}^{-/-}$ to wild type mouse splenocytes were measured by real-time quantitative RT-PCR analysis described in Materials and methods. Data in bar graph represent mean $\pm$ SED. ** $p < 0.01$; Significantly different from wild type control (B). Control and OVA-challenged mouse splenocytes were prepared from wild type and $\text{PrxII}^{-/-}$ mice. Real-time PCR was performed by the method described in Materials and methods (C).

PrxII deletion (Fig. 2A and B). The number of eosinophils and inflammatory cells including macrophages and monocytes were higher in OVA-induced PrxII^{-/-} mice compared to those in OVA-induced wild type mice (Fig. 3A). IgE level was higher and IgG level was comparable in PrxII^{-/-} mice, compared to wild type mice (Fig. 3B). It suggests that PrxII may have benefits in asthma through reducing ROS and TLR4 protein level may not be crucial in OVA-induced asthma.

3.3. BAFF expression was increased in PrxII^{-/-} mouse cells surrounding bronchi

Given that BAFF expression was elevated by ROS in cellular model [15] and by OVA in mouse asthma model [19], we investigated whether OVA-induced BAFF expression increased by the deletion of PrxII. BAFF protein was detected by immunohistochemical staining with anti-BAFF antibodies. As shown in Fig. 4A, BAFF protein expression was detected in OVA-induced wild type mouse cells surrounding bronchi. In addition, BAFF transcripts in splenocytes were increased by OVA-induction (Fig. 4C). When assayed by real-time quantitative RT-PCR, BAFF transcripts in splenocytes were highly increased by the deletion of PrxII gene (Fig. 4B). More BAFF transcripts were also detected by RT-PCR in PrxII^{-/-} mouse splenocytes compared to wild type (Fig. 4C). These data are consistent with our previous data that ROS augmented BAFF expression [19].

To confirm the higher BAFF level in PrxII^{-/-} bronchial tissues than that in wild type tissues, we measured BAFF promoter activity [25]. When BAFF promoter was transfected into wild type and PrxII^{-/-} MEF cells, promoter activity was higher in PrxII^{-/-} MEF than wild type MEF. When cells were incubated in low percentage of serum which is a well-known ROS-producing condition, promoter activity was elevated much more than that in 10% of serum (Fig. 5). It suggests that BAFF expression could be up-regulated by ROS and it could be differently regulated from TLR4 in PrxII^{-/-} mice.

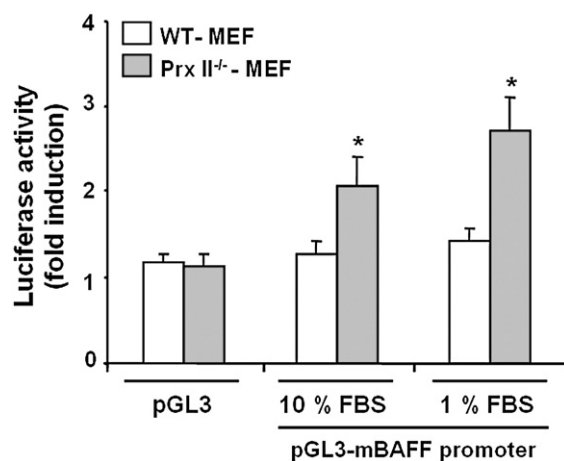


Figure 5 Measurement of BAFF promoter activity in mouse embryonic fibroblast (MEF). Wild type and PrxII^{-/-} MEF cells were transfected with pGL3-mBAFF-Luc. mBAFF promoter activity was measured as luciferase activity using luminometer. BAFF promoter activities were increased by the deletion of PrxII gene. Data were the representative of four experiments. Data in the bar graph represent the mean $\pm$ SED. * $p < 0.05$; Significantly different from pGL3-Luc basic vector-transfected control.

4. Discussion

Toll-like receptor 4 agonist, LPS-induced ROS generation and ROS are involved in TLR4-associated activation of NF- κ B [20,21]. Peroxiredoxins (Prxs) play a role in ROS degradation. ROS production increased in PrxII-deficient mice compared to wild type mice. Yang et al. reported that TLR4-stimulated inflammation was elevated in PrxII-deficient mice [15] but they did not mention the expression level of TLR4. BAFF expression in splenocytes was regulated by ROS-producing conditions such as LPS-stimulation, serum deprivation and PrxII deletion [15]. Serum BAFF level in asthmatic patients was above normal [22]. Little is known about the regulation of ROS-mediated TLR4 expression compared to ROS-mediated BAFF expression. Here, we investigated the role of PrxII on ROS-mediated expression of TLR4 and BAFF in OVA-induced mouse asthma model. We found that TLR4 proteins were decreased by PrxII deletion (Fig. 1). We also found that BAFF expression was increased in surrounding bronchi in OVA-induced asthmatic mice, which was higher in PrxII^{-/-} mice than wild type mice. It suggests that BAFF and TLR4 expressions are differentially regulated by PrxII-degradable ROS and TLR4 protein level may not be crucial in OVA-induced asthma.

Usually, inflammatory responses were accompanied in asthma, which was explained by the activation in toll-like receptor-mediated signaling [28]. However, it is yet to be defined whether the stimulation of TLR4 can protect, have no effect, or exacerbate allergic asthma [24]. The requirement of TLR4 for pulmonary inflammation depends on the nature of the toxin and appears specific to toxin and exposure conditions [23]. Wild type and TLR4-deficient mice had similar responses to allergen for short periods [24].

A few explanations are possible to interpret our data, up-regulation of BAFF gene and down-regulation of TLR4 protein. In the first explanation, BAFF expression could be independent of TLR4 and it might be dependent on ROS production, in part. Although the deletion of PrxII gene may not only be resulted in the elevation of ROS but also in the much complex consequences, ROS production could contribute to BAFF expression by the stimulation of other target genes or proteins, rather than TLR4. It is supported by previous reports that BAFF is induced by TLR3-activation in airway epithelial cells [29].

In the second explanation, PrxII could differentially regulate TLR4 and BAFF. While TLR4 production was translationally regulated by ROS production in PrxII^{-/-} cells, BAFF expression was transcriptionally controlled. It is well-known that ROS activate NF- κ B [20,30–33]. ROS and other free radicals can also activate AP-1 and NF- κ B transcription coordinately [34]. BAFF gene was regulated by NF- κ B activation [35,36] and by the involvement of co-activator, p300 [25]. The TLR4 promoter has a binding site for AP-1 and hypoxia-induced TLR4 down-regulation may be due to an ROS-mediated decrease in AP-1-binding activity [37]. This is required to be cleared in the next study.

The third explanation is that the down-regulation of TLR4 in PrxII^{-/-} cells could be a negative feedback regulation to consistent stimulatory effect of ROS on BAFF expression. It may be one of the compensatory effect to prevent hyper-activation and then to maintain cellular homeostasis. In immune responses, PrxII is an essential negative regulator of

LPS-induced inflammatory signaling through modulation of ROS synthesis via NADPH oxidase activities and therefore, is crucial for the prevention of excessive host responses to microbial products [27]. It would be defined whether BAFF and TLR4 are regulated by PrxII or ROS produced by the deletion of PrxII, directly or indirectly.

In conclusion, ROS produced by PrxII deletion augment BAFF expression and inhibit TLR4 production. OVA-induced asthma was more severe in ROS-producing PrxII^{-/-} mice than wild type mice. It suggests that BAFF and TLR4 expressions are differentially regulated by PrxII and TLR4 expression level may not be crucial in OVA-induced asthma.

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References

- [1] Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol* 2003;3(9):721–32.
- [2] El Biaze M, Boniface S, Koscher V, Mameessier E, Dupuy P, Milhe F, et al. T cell activation, from atopy to asthma: more a paradox than a paradigm. *Allergy* 2003;58(9):844–53.
- [3] Lee KS, Park HS, Park SJ, Kim SR, Min KH, Jin SM, et al. An antioxidant modulates expression of receptor activator of NF-kappaB in asthma. *Exp Mol Med* 2006;38(3):217–29.
- [4] Lee KS, Park HS, Park SJ, Kim SR, Min KH, Jin SM, et al. A prodrug of cysteine, L-2-oxothiazolidine-4-carboxylic acid, regulates vascular permeability by reducing vascular endothelial growth factor expression in asthma. *Mol Pharmacol* 2005;68(5):1281–90.
- [5] Lee YC, Lee KS, Park SJ, Park HS, Lim JS, Park KH, et al. Blockade of airway hyperresponsiveness and inflammation in a murine model of asthma by a prodrug of cysteine, L-2-oxothiazolidine-4-carboxylic acid. *Faseb J* 2004;18(15):1917–9.
- [6] Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol* 2003;21:231–64.
- [7] Mak TW, Yeh WC. Signaling for survival and apoptosis in the immune system. *Arthritis Res* 2002;4(Suppl 3):S243–52.
- [8] Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 2001;293(5537):2111–4.
- [9] Schneider P, MacKay F, Steiner V, Hofmann K, Bodmer JL, Holler N, et al. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med* 1999;189(11):1747–56.
- [10] Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, et al. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J Exp Med* 1999;190(11):1697–710.
- [11] Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat Immunol* 2002;3(9):822–9.
- [12] Rackeman FM. A working classification of asthma. *Am J Med* 1947;33:601–6.
- [13] Ostergaard PA. Non-IgE-mediated asthma in children. *Acta Paediatr Scand* 1985;74(5):713–9.
- [14] Corrigan C. Mechanisms of intrinsic asthma. *Curr Opin Allergy Clin Immunol* 2004;4(1):53–6.
- [15] Moon EY, Lee JH, Oh SY, Ryu SK, Kim HM, Kwak HS, et al. Reactive oxygen species augment B-cell-activating factor expression. *Free Radic Biol Med* 2006;40(12):2103–11.
- [16] Han YH, Kim HS, Kim JM, Kim SK, Yu DY, Moon EY. Inhibitory role of peroxiredoxin II (Prx II) on cellular senescence. *FEBS Lett* 2005;579(21):4897–902.
- [17] Han YH, Kwon JH, Yu DY, Moon EY. Inhibitory effect of peroxiredoxin II (Prx II) on Ras-ERK-NFkappaB pathway in mouse embryonic fibroblast (MEF) senescence. *Free Radic Res* 2006;40(11):1182–9.
- [18] Moon EY, Han YH, Lee DS, Han YM, Yu DY. Reactive oxygen species induced by the deletion of peroxiredoxin II (PrxII) increases the number of thymocytes resulting in the enlargement of PrxII-null thymus. *Eur J Immunol* 2004;34(8):2119–28.
- [19] Moon EY, Ryu SK. TACI:Fc scavenging B cell activating factor (BAFF) alleviates ovalbumin-induced bronchial asthma in mice. *Exp Mol Med* 2007;39(3):343–52.
- [20] Asehnoun K, Strassheim D, Mitra S, Kim JY, Abraham E. Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B. *J Immunol* 2004;172(4):2522–9.
- [21] Sanlioglu S, Williams CM, Samavati L, Butler NS, Wang G, McCray Jr PB, et al. Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-alpha secretion through IKK regulation of NF-kappa B. *J Biol Chem* 2001;276(32):30188–98.
- [22] Kang JS, Yoon YD, Ahn JH, Kim SC, Kim KH, Kim HM, et al. B cell-activating factor is a novel diagnosis parameter for asthma. *Int Arch Allergy Immunol* 2006;141(2):181–8.
- [23] Hollingsworth II JW, Cook DN, Brass DM, Walker JK, Morgan DL, Foster WM, et al. The role of Toll-like receptor 4 in environmental airway injury in mice. *Am J Respir Crit Care Med* 2004;170(2):126–32.
- [24] Hollingsworth JW, Whitehead GS, Lin KL, Nakano H, Gunn MD, Schwartz DA, et al. TLR4 signaling attenuates ongoing allergic inflammation. *J Immunol* 2006;176(10):5856–62.
- [25] Moon EY, Park H. B cell activating factor (BAFF) gene promoter activity depends upon co-activator, p300. *Immunobiology* 2007;212(8):637–45.
- [26] Moon EY, Noh YW, Han YH, Kim SU, Kim JM, Yu DY, et al. T lymphocytes and dendritic cells are activated by the deletion of peroxiredoxin II (Prx II) gene. *Immunol Lett* 2005;102(2):184–90.
- [27] Yang CS, Lee DS, Song CH, An SJ, Li S, Kim JM, et al. Roles of peroxiredoxin II in the regulation of proinflammatory responses to LPS and protection against endotoxin-induced lethal shock. *J Exp Med* 2007;204:583–94.
- [28] Nigo YI, Yamashita M, Hirahara K, Shinnakasu R, Inami M, Kimura M, et al. Regulation of allergic airway inflammation through Toll-like receptor 4-mediated modification of mast cell function. *Proc Natl Acad Sci U S A* 2006;103(7):2286–91.
- [29] Kato A, Truong-Tran AQ, Scott AL, Matsumoto K, Schleimer RP. Airway epithelial cells produce B cell-activating factor of TNF family by an IFN-beta-dependent mechanism. *J Immunol* 2006;177(10):7164–72.
- [30] Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol* 1994;12:141–79.
- [31] Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L. Redox regulation of NF-kappa B activation. *Free Radic Biol Med* 1997;22(6):1115–26.
- [32] Toledano MB, Leonard WJ. Modulation of transcription factor NF-kappa B binding activity by oxidation-reduction in vitro. *Proc Natl Acad Sci U S A* 1991;88(10):4328–32.
- [33] Zhang H, Spapen H, Nguyen DN, Benlabeled M, Buurman WA, Vincent JL. Protective effects of N-acetyl-L-cysteine in endotoxemia. *Am J Physiol* 1994;266(5 Pt 2):H1746–54.

- [34] Dhar A, Young MR, Colburn NH. The role of AP-1, NF-kappaB and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. *Mol Cell Biochem* 2002;234–235(1–2):185–93.
- [35] Fu L, Lin-Lee YC, Pham LV, Tamayo A, Yoshimura L, Ford RJ. Constitutive NF-kappaB and NFAT activation leads to stimulation of the BLyS survival pathway in aggressive B-cell lymphomas. *Blood* 2006;107(11):4540–8.
- [36] Tai YT, Li XF, Breitkreutz I, Song W, Neri P, Catley L, et al. Role of B-cell-activating factor in adhesion and growth of human multiple myeloma cells in the bone marrow microenvironment. *Cancer Res* 2006;66(13):6675–82.
- [37] Ishida I, Kubo H, Suzuki S, Suzuki T, Akashi S, Inoue K, et al. Hypoxia diminishes toll-like receptor 4 expression through reactive oxygen species generated by mitochondria in endothelial cells. *J Immunol* 2002;169(4):2069–75.