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The Effect of *Lactobacillus actobacillus* Peptidoglycan on Bovine β-Lactoglobulin-Sensitized Mice via TLR2/NF-κB Pathway

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ABSTRACT

Our previous study reported that *Lactobacillus acidophilus* (*L.acidophilus*) key laboratory of dairy science (KLDS) 1.0738 had an effective impact on inhibiting β-lactoglobulin (β-lg) allergy. This study further investigated the anti-allergic activity of peptidoglycan (PGN) isolated from KLDS 1.0738. This study aimed to assess whether toll-like receptor 2 (TLR2)/NF-kappaB (NF-κB) signaling activated by PGN was responsible for reducing allergic inflammation.

We first examined the role of PGN on cytokine production and TLR2 signaling expression of macrophages. Then the immunoregulatory capacity of PGN was further evaluated by adopting the β-lg-sensitized mice model. The levels of sera IgE, regulatory T cells (Treg) and T-helper (Th) 17-related cytokine were detected by ELISA. TLR2 signaling mRNA and protein expression in colon tissues were measured by quantitative RT-PCR and western blot, respectively.

Our data showed that administration of L. *acidophilus* PGN inhibited IgE production and improved the Treg/Th17 balance toward a Treg response in a mouse model of β-lg allergy. In addition, treating different doses *L. acidophilus* PGN to sensitized mice significantly increased TLR2 levels, along with enhancing NF-κB expression, especially in medium and high concentration ($p<0.05$). Further analysis revealed that the mRNA expression of TLR2 and NF-κB were positively correlated with the Foxp3 mRNA expression ($p<0.05$), but were negatively correlated with the RORγt mRNA expression in *L. acidophilus* PGN-treated group compared to allergy group ($p<0.05$).

This study suggests PGN was similar to probiotics in preventing β-lg allergy through regulating Treg/Th17 imbalance, and activation of TLR2/NF-κB signaling may be involved in this process.

Keywords: β-lactoglobulin allergy; Peptidoglycan; Regulatory; TLR2/NF-κB signaling; Th17 cells; T-lymphocytes

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INTRODUCTION

Cow's milk protein allergy (CMA) is an abnormal reaction of the immune system, which commonly

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develops in infants and young children, resulting In chronic inflammation and intestinal microecology disorders.[1,2] Many experimental data showed that *Lactobacilli* present inhibitory effects on the allergic and autoimmune diseases.[3, 4] However, further studies are required to dissect its underlying mechanism.

Recently, the immunomodulatory activity of *Lactobacillus* were considered to be exerted via toll-like receptors (TLRs). TLRs played a fundamental role in innate immunity by recognizing pathogen-associated molecular patterns (PAMPs).[5, 6] Peptidoglycan (PGN), an unique component of *Lactobacilli*, is also the PAMP and ligand of TLR2. [7, 8] Previous studies revealed that PGN reacted with TLR2, which was partly responsible for Th1 cell activation and differentiation.[9,10,11] The current studies provided that, in addition to the general paradigm of Th1/Th2 immune imbalance, allergic symptoms were also associated with a reduced Treg/Th17 ratio. [12, 13] Furthermore, activation of TLRs signaling has shown to be involved in the pathogenesis of inflammatory diseases. For instance, Loures et al [14] and Nichols et al [15] reported that TLR2 deficiency led to induction of Th17-dominant inflammation and diminish of the Treg immunity development. Filippi et al [16] indicated that TLR2 signaling had a protective effect on preventing type 1 diabetes due to expanding $CD4^+CD25^+$Tregs. Lesiak et al [17]noticed that atopic dermatitis patients had lower percentages of Treg lymphocytes and higher ratio of Th17 cells in PBMC, accompanied with reduced TLR2 and TLR4 expression. Therefore, a better understanding of the relationship between TLR2/NF-κB signaling and Treg/Th17 cells balance might provide a new target for effective CMA control.

We previously reported that β-lactoglobulin (β-lg) allergy exhibited a Treg/Th17 imbalance toward Th17 dominance, while oral administration of *Lactobacillus acidophilus* (*L.acidophilus*) strain key laboratory of dairy science (KLDS) 1.0738 effectively suppressed β-lg-induced Th17 immune responses in allergic murine model. [18, 19] Since *L. acidophilus* PGN is an active component of probiotics, we wonder whether PGN-regulated TLR2 signaling is beneficial to suppression of Th17-mediated allergic inflammation. Therefore, this study further investigated the TLR2 activation in allergic mice stimulated with varying doses of PGN. These results might reflect a new protective mechanism of the *Lactobacilli* to limit excessive inflammation and collateral tissue damage during allergy.

MATERIALS AND METHODS

Preparation and Analysis of PGN

The strain of *L. acidophilus* KLDS 1.0738 (obtained from Key Laboratory of Dairy Science, Ministry of Education, Northeast Agriculture University, China) was grown in Man-Rogosa-Sharpe medium at 37°C for 18 h without stirring. After washing several times with cold saline, the bacteria cells were collected by centrifugation (5000 g for 20 min at 4°C). Then the organisms were heat-treated at 100 °C for 30 min then stored at -20°C. [20]

The PGN derived from *L. acidophilus* were prepared using the method of Sekine et al [21] and Shida et al [22]. First, the heat-killed whole cells were boiled in 2 % sodium dodecyl sulphate for 15 min. After washing with water and dehydrated alcohol, proteins were enzymatically digested with 50 mM Tris-HCl buffer (pH 7.2-7.4) at 37°C for 14 h, and lipids were removed by continuous refluxing with methanol, methanol/chloroform (1:1) and chloroform. After acidification with 0.01M H_2SO_4, the sediment was then lyophilized and stored at -20°C.

PGN extraction from *L. acidophilus* were proved by measuring lysozyme solubility, chemical composition (contents of amino acids, fat, protein, neutral suga, amino hexose), and molecular weight.[23, 24]

Animals

6-week-old female BALB/c mice weighting 22-26 g were obtained from Harbin Veterinary Research Institute (Harbin, China). Mice were bred in a pathogen-free facility and provided milk-free chow and water. Animal protocol was performed in accordance with the guidelines of Laboratory Animals of Northeast Agricultural University.

Murine Peritoneal Macrophages Preparation and PGN Stimulation

The peritoneal macrophages from BALB/c mice were isolated as described previously.[25] These collected cells were seeded at 2.5×10^6 cells/mL in 96-well tissue culture plates and cultured with 200 µL different doses of *L. acidophilus* PGN (0.1, 0.5, 1 mg/mL) for 48 hr at 37°C in 5% CO_2 concentration. Control group was treated with 200 µL RPMI-1640 medium. The cells were harvested to analyze the mRNA expression of TLR2 and NF-κB.

In blocking studies, the macrophages were pretreated with 20 µg/mL anti-TLR2 (Santa Cruz

Biotechnology, USA) for 1 h at 37°C and then stimulated with 100 μg/mL *L. acidophilus* PGN for 12 h. The supernatants of these cultures were collected to determine the levels of IFN-γ, TGF-β and IL-10.

Sensitization and Challenge with β-lg and PGN Treatment

Animals experiments (n=6 per group) were performed according to previously described methods with minor modifications.[19, 26] Briefly, the anti-allergic properties of PGN (low dose (L): 5 mg/kg wt, medium dose (M): 10 mg/kg wt and high dose (H): 20 mg/kg wt) were evaluated by intraperitoneal injection in mice once daily for six consecutive days before allergen induction. Then the mouse model of β-lg allergy were established by intraperitoneal injection with 50 μg β-lg (Sigma, USA) adsorbed in 2 mg of $Al(OH)_3$ (Sigma, USA) in 200 μL phosphate buffer saline（PBS） each week for three weeks, negative control mice were treated with sterile PBS. Five days after last injection, different groups were orally challenged with two times of β-lg (20 mg/mouse) or PBS. Two hours after antigen challenge, the serum and tissue samples from each mouse were harvested for further analysis.

Measurement of IgE and Cytokine Secretion Levels

Serum total IgE was measured by mouse IgE enzyme-linked immunosorbent assay (ELISA) quantitation kits (Bethyl Laboratories, USA). The amount of β-lg-specific IgE antibody in serum was also measured by ELISA as previously reported.[27] IL-17, IL-6, IL-23, and TGF-β levels in serum were also evaluated using ELISA according to the manufacturer's manual (R&D Systems, USA). Optical density was measured at 450 nm. Cytokine concentrations were determined by comparison with known standards.

Quantification of mRNA Expression

Total RNA from colon tissues specimen were isolated using RNA simple Total RNA kit (Tiangen, China), and then reverse transcribed into cDNA using PrimeScript RT reagent kit (Takara, China). Expressions of genes encoding Treg/Th17 related transcription factors (CD25, IL-17A IL-6, IL-23, TGF-β, Foxp3 and RORγt) and TLR2 pathway related factors (TLR2, myeloid differentiation protein 88

Table 1. List of primers used in real-time PCR for evaluating the effect of lactobacillusl. actobacillus peptidoglycan on bovine β-lactoglobulin-sensitized mice.The primers were designed by the Beijing Genomics Institute.

Gene name	Primer sequence (5'-3')	
CD25	Forward primer	5-ACACCTGTAAGCCCAGCTCT-3
	Reverse primer	5-TGGAAAGGTTGAGGGGTAAG-3
IL-17A	Forward primer	5-AGGGAGAGCTTCATCTGTGG-3
	Reverse primer	5-AGATTCATGGACCCCAACAG-3
IL-6	Forward primer	5-GATGCTACCAAACTGGATATAATC-3
	Reverse primer	5-GGTCCTTAGCCACTCCTTCTGTG-3
IL-23	Forward primer	5-TGCTGGATTGCAGAGCAGTAA-3
	Reverse primer	5-GCATGCAGAGATTCCGAGAGA-3
TGF-β	Forward primer	5-ACCATGCCAACTTCTGTCTG-3
	Reverse primer	5-CGGGTTGTGTTGGTTGTAGA-3
Foxp3	Forward primer	5-TTTCCAAGAACGGGCATTA-3
	Reverse primer	5-TGTGGCTGACTGAGGGTGT-3
RORγt	Forward primer	5-AGAAAGAAAAGGGGAACTGG-3
	Reverse primer	5-CTATTGTGGCTGCTGAGTTC-3
TLR2	Forward primer	5- GCCACCATTTCCACGGACT-3
	Reverse primer	5- GGCTTCCTCTTGGCCTGG-3
Myd88	Forward primer	5-CATGGTGGTGGTTGTTTCTGAC-3
	Reverse primer	5-TGGAGACAGGCTGAGTGCAA-3
NF-κB	Forward primer	5-AGTTGAGGGGACTTTCCCAGGC-3
	Reverse primer	5-GCC TGGGAAAGTCCCCTCAACT-3
β-actin	Forward primer	5-CGCAAAGACCTGTATGCCAAT-3
	Reverse primer	5-GGGCTGTGATCTCCTTCTGC-3

Table 2. Analysis of the chemical composition of lactobacillus actobacillus peptidoglycan (PGN) for evaluating its effect on bovine β-lactoglobulin-sensitized mice (mg/g)

Constituent	Bacteria	PGN-Sekine
Neutral sugar	207.86	350.60
Amino hexose	12.76	26.60
Fat	104.21	32.41
Protein	223.49	409.78

Table 3. The composition and contents of amino acids (mmol/g) for evaluating the effect of lactobacillus actobacillus peptidoglycan on bovine β-lactoglobulin-sensitized mice.

Amino acids	Bacteria	PGN-Sekine
Ala	0.702	0.916
Glu	0.640	0.53
Lys	0.413	0.399
Met	0.118	0.256
Thr	0.133	0. 150
Ser	0.202	0. 110
Leu	0.310	0.084
Asp	0.398	0.363
Gly	0.378	0.071
Val	0.366	0.073
Ile	0.203	0.067
Phe	0.149	0.035
Arg	0.181	0.076
Cys	0.132	0.054
Prg	0.152	0.116
Tyr	0.114	0.035
His	0.094	0.010

[Myd88], NF-κB) were performed by the *SYBR Premix Ex Taq*(Takara, China) using ABI 7500 (Applied Biosystems, USA). Designed primers (the Beijing Genomics Institute, China) for real-time PCR, are listed in Table 1. β-actin was used as a housekeeper to normalize the levels of target gene using the $2^{-\triangle\triangle Ct}$ method and represented as fold induction.

Western Blot Analysis

The cytosolic and nuclear proteins from colon tissues for analysis of TLR2, Myd88 and NF-κB p65 were extracted with nuclear and cytoplasmic extraction reagent kit (NE-PER)according to the manufacturer's instruction (Pierce, USA).

Protein concentration was determined by the BCA protein assay kit (Solarbio, China). Protein extracts (40 μg) were boiled and fractionated on 8-10% SDS-polyacrylamide gel, and then blotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was immunoblotted with rabbit anti-TLR2 (Abcam, USA, 1:1000), NF-κB p65 and Myd88 (Santa Cruz Biotechnology, USA, 1:500) ,which were incubated concomitantly with horse-radish peroxidase-conjugated [HRP]-conjugated anti-rabbit antibody (Zhongshan, China, 1:1000) as a secondary antibody. An enhanced chemiluminescene (ECL) chemiluminescene reagent (TransGen Biotech, China) was used for membrane exposure according to the manufacturer's protocol. Semi-quantification was valued with Gel-Pro Analyzer software and assessed for GAPDH as an internal control.

Statistical Analysis

The results were expressed as mean ± *SD*. One-way ANOVA was performed to determine the statistical significance using SPSS 17.0 (IBM SPSS, USA).. The Spearman test was used to determine correlations. A value of $P < 0.05$ was considered significant.

RESULTS

Extraction and Analysis of *L.acidophilus* PGN

Because lysozyme could exclusively degrade β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid structure in PGN, lysozyme assay generally was considered as a qualitative test for peptidoglycan. As shown in Figure 1, the absorbance values (A450 nm) of *L.acidophilus* PGN declined faster than that of *L.acidophilus* strain. We further analyzed the chemical composition of *L.acidophilus* PGN (Table 2, 3). The neutral sugar and protein contents of *L.acidophilus* PGN were 350.60 mg/g and 409.78 mg/g, the contents of Ala, Glu, Asp and Lys were higher than the others. Moreover, the molecular weight of PGN was around 30-14 kD. These results proved that the final extraction possessed the PGN characteristics.

L.acidophilus PGN Induce the TLR2 Signaling Pathways in Peritoneal Macrophages

The effect of *L. acidophilus* PGN on inducing the Treg response and TLR2 were evaluated by culturing it with peritoneal macrophages obtained from BALB/c mice.

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As shown in Figure 2 A and B, we found that treatment of *L. acidophilus* PGN activated the TLR2 and NF-κB mRNA expression in a dose-dependent manner. Moreover, *L. acidophilus* PGN administration effectively elevated the levels of Treg-related cytokines; whereas, pre-incubation of macrophages with anti-TLR2 antibodies significantly decreased the production of *L. acidophilus* PGN-stimulated IFN-γ, TGF-β, and IL-10 (Figure 2 C).

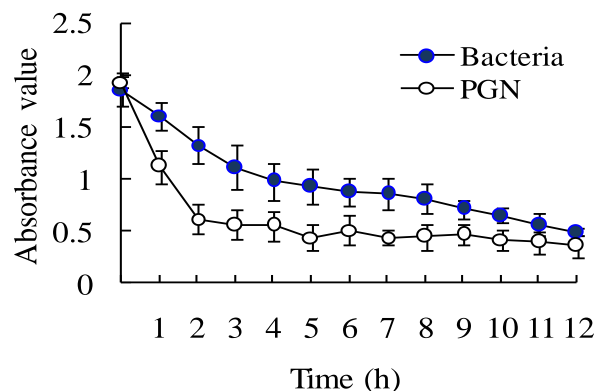

Figure 1. The degradation models of peptidoglycan (PGN) and bacteria when exposed to lysozyme. PGN and bacteria were treated with lysozyme and measured its UV absorbance at 450 nm in a UV-Vis detector (Waters, USA). Data are expressed as means ±S.D.

Effects of *L.acidophilus* PGN Treatment on Serum IgE levels of β-lg-sensitized Mice

As shown in Figure 3, consistent with our previous study, mice immunized with β-lg led to strongenhancement of the levels of total IgE and β-lg-specific IgE antibodies compared with those in the controls ($p<0.05$). In contrast, intraperitoneal administration of different doses of *L. acidophilus* PGN effectively reduced IgE production, especially in medium and high dose ($p < 0.05$).

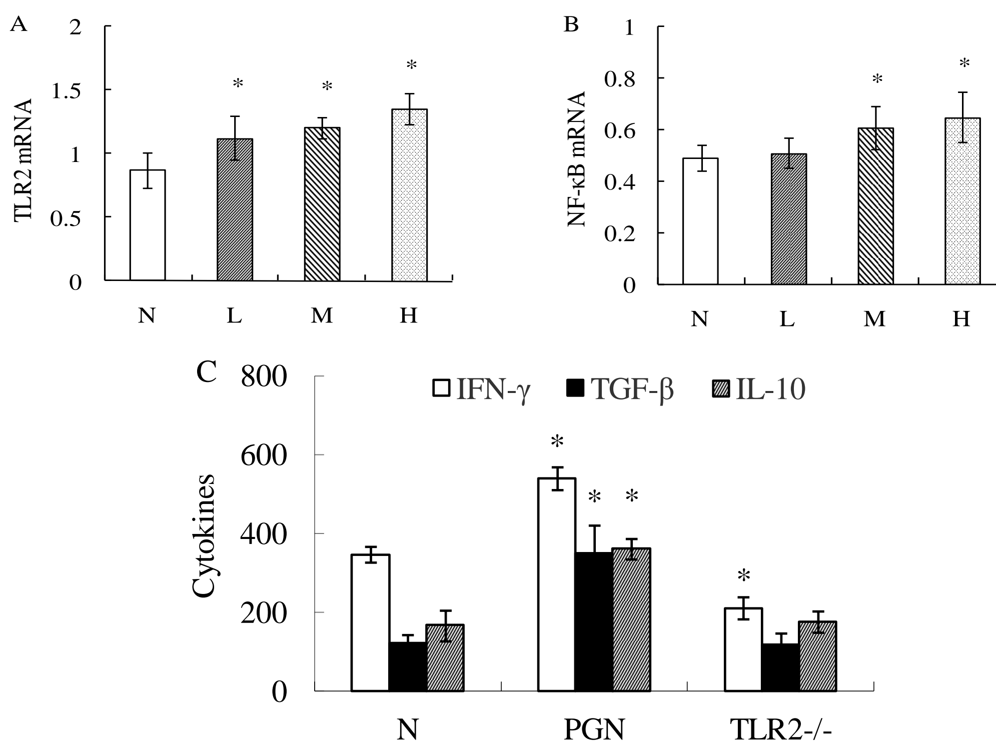

Figure 2. The effect of peptidoglycan (PGN) on cytokines concentrations and toll-like receptor 2 (TLR2)/NF-kappaB (NF-κB) expression in peritoneal macrophages. The macrophageswere stimulated with RPMI-1640 medium (N), 0.1 (L), 0.5 (M) or 1 mg/mL (H) *L. acidophilus* PGN. The cells were collected to measure the TLR2(A) and NF-κB (B) mRNA levels Then the macrophages were stimulated with PGN in the presence or absence of anti-TLR2. In both case, IFN-γ, TGF-β, and IL-10 production in supernatants were measured accordingly(C). Data are expressed as means ±S.D. (n=6); *$p<0.05$ versus control group

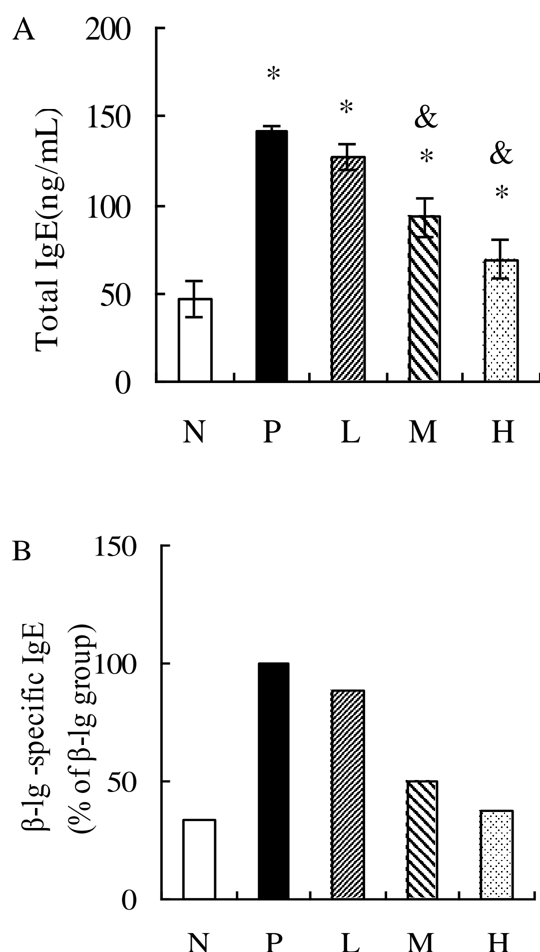

Figure 3. Intraperitoneal administration of peptidoglycan (PGN) reduces the total IgE and β-lg-sIgE levels in β-lg-sensitized mice. BALB/c mice were injected intraperitoneally with different doses of *L.acidophilus* PGN [low dose (L): 5 mg/kg wt, medium dose (M): 10 mg/kg wt and high dose (H): 20 mg/kg wt] before induction of allergy. Animals were sacrificed after allergen challenge, the expressions of serum total IgE (A) and β-lg-specific IgE (B) in all groups were determined by ELISA. Data are expressed as means ±S.D. (n=6) **p*<0.05 versus control group, &*p*<0.05 versus allergy group.

Effect of *L.acidophilus* PGN on the Treg/Th17 Reactions in β-lg-Sensitized Mice

In this study, we further investigated the capacity of *L. acidophilus* PGN for improving the Treg/Th17-related cytokines and signature transcription factors.

As shown in Figure 4, *L. acidophilus* PGN-induced TGF-β secretion and CD25, Foxp3 expression were increased in a concentration-dependent manner. Furthermore, no significant differences was found in the TGF-β production between high dose of PGN group and the controls (p>0.05). In contrast, we observed that treatment with *L. acildophilus* PGN markedly reduced not only the Th17 related cytokines (IL-17, IL-6 and IL-23) but also the mRNA expression of RORγt compared to the sensitized mice (p <0.05).

Effects of *L.acidophilus* PGN Treatment on the TLR2 Signal Gene Expression in Allergic Mice

Furthermore, we analyzed how the PGN induced TLR2 signaling in CMA allergy model. As shown in Figure 5, BALB/c mice treated with β-lg allergen weakly induced the mRNA levels of TLR2 in the colon tissue compared to the controls (p < 0.05). On the other hand, *L. acildophilus* PGN treatment had the ability to elevate TLR2 and NF-κB expression compared to the allergic mice, especially in medium and high dose group (p<0.05). Nevertheless, Myd88 mRNA level was not increased under *L. acildophilus* PGN treatment.

Consistent with the results of mRNA trends, we also found increased protein expression of TLR2, accompanied with the amplified NF-κB p65 protein expression in administered *L. acildophilus* PGN group compared to both the controls and allergy group (Figure 6). Similarly, the *L. acidophilus* PGN induced little change in the level of Myd88 protein expression.

Correlation between the mRNA Expression of Foxp3 and RORγt and TLRs Pathway Expression

Next, we analyzed the role of *L. acidophilus* PGN (high dose) on regulating the TLR2 signaling and the Treg/Th17 imbalance (Figure 7). As expected, after high dose of *L. acidophilus* PGN treatment, the mRNA expression of TLR2 and NF-κB were positively related to the mRNA expression of Foxp3 (p < 0.05), but were negatively related to the mRNA expression of RORγt (p < 0.05). But there was no significant relationship between the Myd88 mRNA expression and Treg/Th17 relative mRNA expression in high dose PGN group compared to allergy group (p > 0.05).

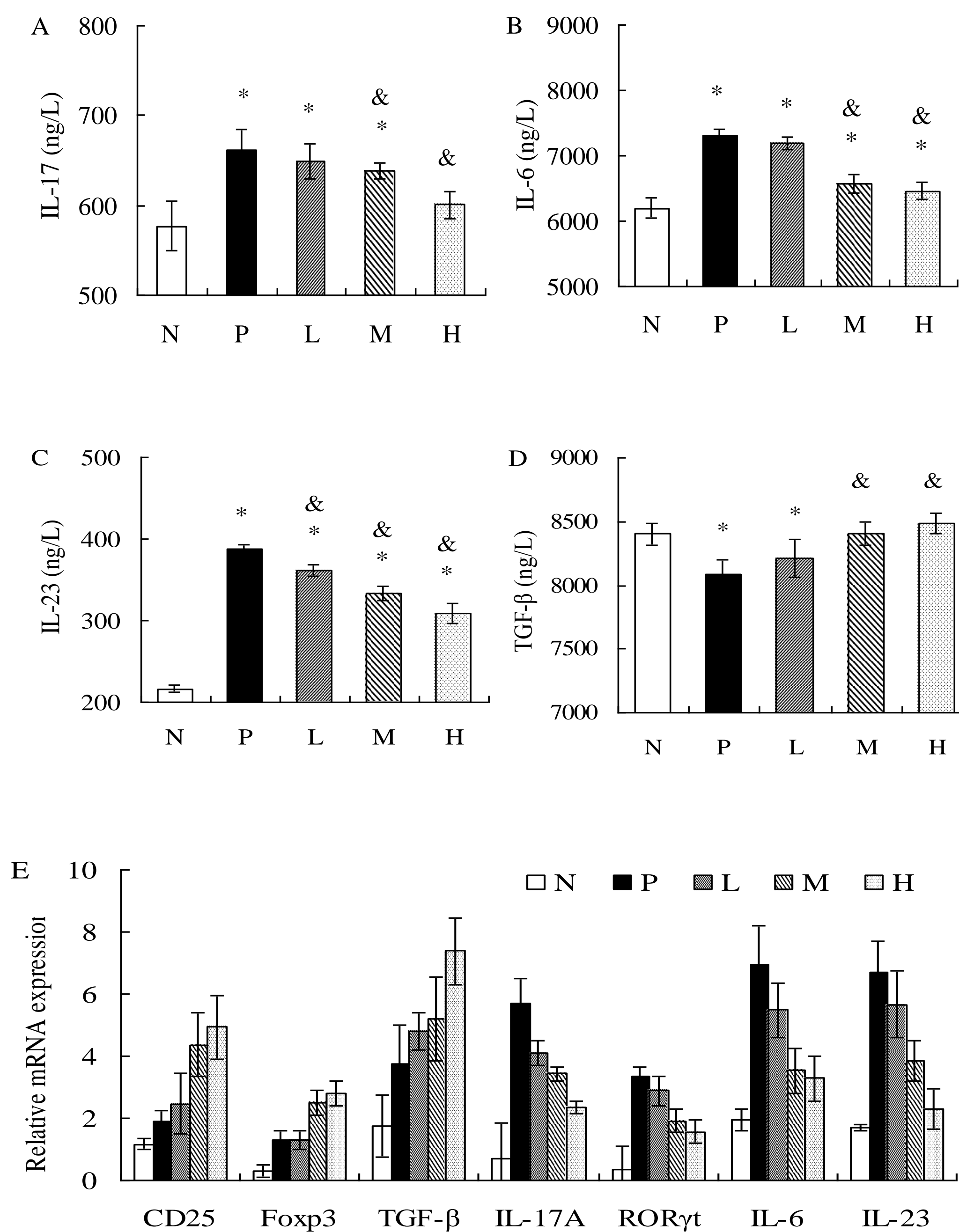

Figure 4. Effect of peptidoglycan (PGN) on Treg/Th17 balance in β-lg-sensitized mice. *L.acidophilus* PGN was given to mice at different doses [low dose (L): 5 mg/kg wt; medium dose (M): 10 mg/kg wt and high dose (H): 20mg/kg wt] before induction of allergy. The blood and colon tissue were collected after animals were sacrificed, the Treg and Th17 related cytokines IL-17(A), IL-6 (B), IL-23 (C), TGF-β (D) and relative mRNA expressions (E) were determined by ELISA and RT-PCR, respectively. Data are expressed as means ± SD (n=6). *$p < 0.05$ versus control group, $^{\&}p < 0.05$ versus positive group.

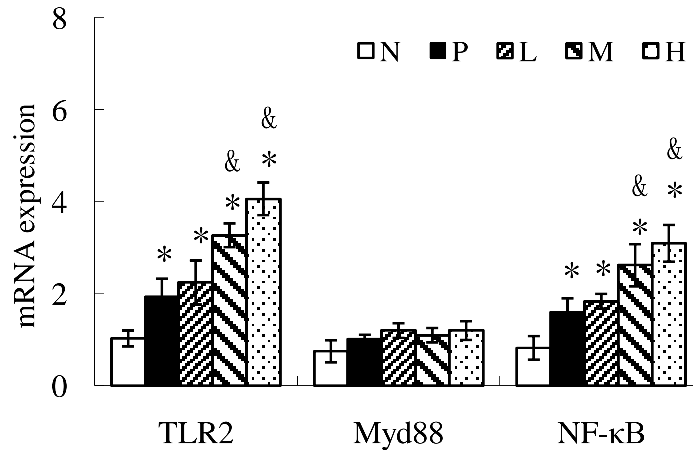

Figure 5. Effect of peptidoglycan (PGN) on toll-like receptor 2 (TLR2)/NF-κB signal gene expression in colon tissue of β-lg-sensitized mice. BALB/c mice were injected with *L.acidophilus* PGN at different doses [low dose (L): 5 mg/kg wt; medium dose (M): 10 mg/kg wt and high dose (H): 20mg/kg wt] before induction of allergy. After allergen challenge, the relative mRNA levels of TLR2, Myd88 and NF-κB in colon tissue of all groups were analyzed by RT-PCR, normalized to the level of β-actin and represented as fold induction. Data are expressed as means ± SD (n=6). * $p < 0.05$ versus control group, & $p < 0.05$ versus positive group.

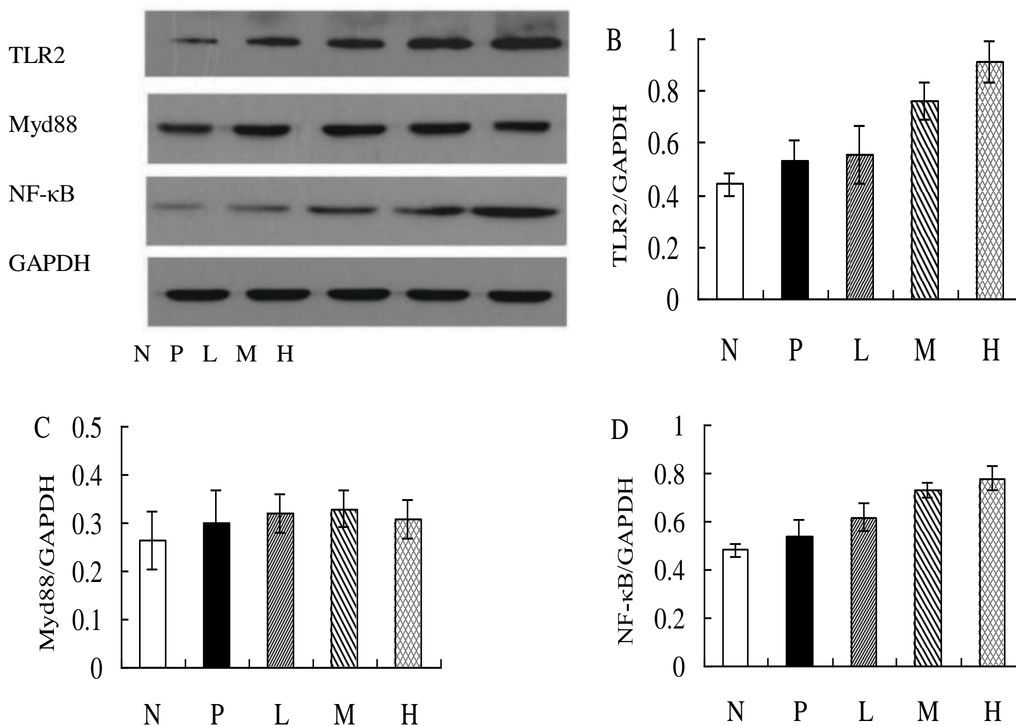

Figure 6. Effects of peptidoglycan (PGN) on protein expression of toll-like receptor 2 (TLR2)/ NF-κB in the colon tissue of β-lg-sensitized mice. BALB/c mice were intraperitoneally injected with PGN [low dose (L): 5 mg/kg wt, medium dose (M): 10 mg/kg wt and high dose (H): 20 mg/kg wt] before β-lg allergen induction. The protein samples were extracted from colon tissue and analyzed by western blot. (A) The protein levels of TLR2 (upper), Myd88 (middle 1), NF-κB p65 (middle 2) and GAPDH (lower) in the spleen; (B) The ratio of TLR2/GAPDH; (B) The ratio of Myd88/GAPDH; (C) The ratio of NF-κB/GAPDH. Data are expressed as means ±S.D (n=6).

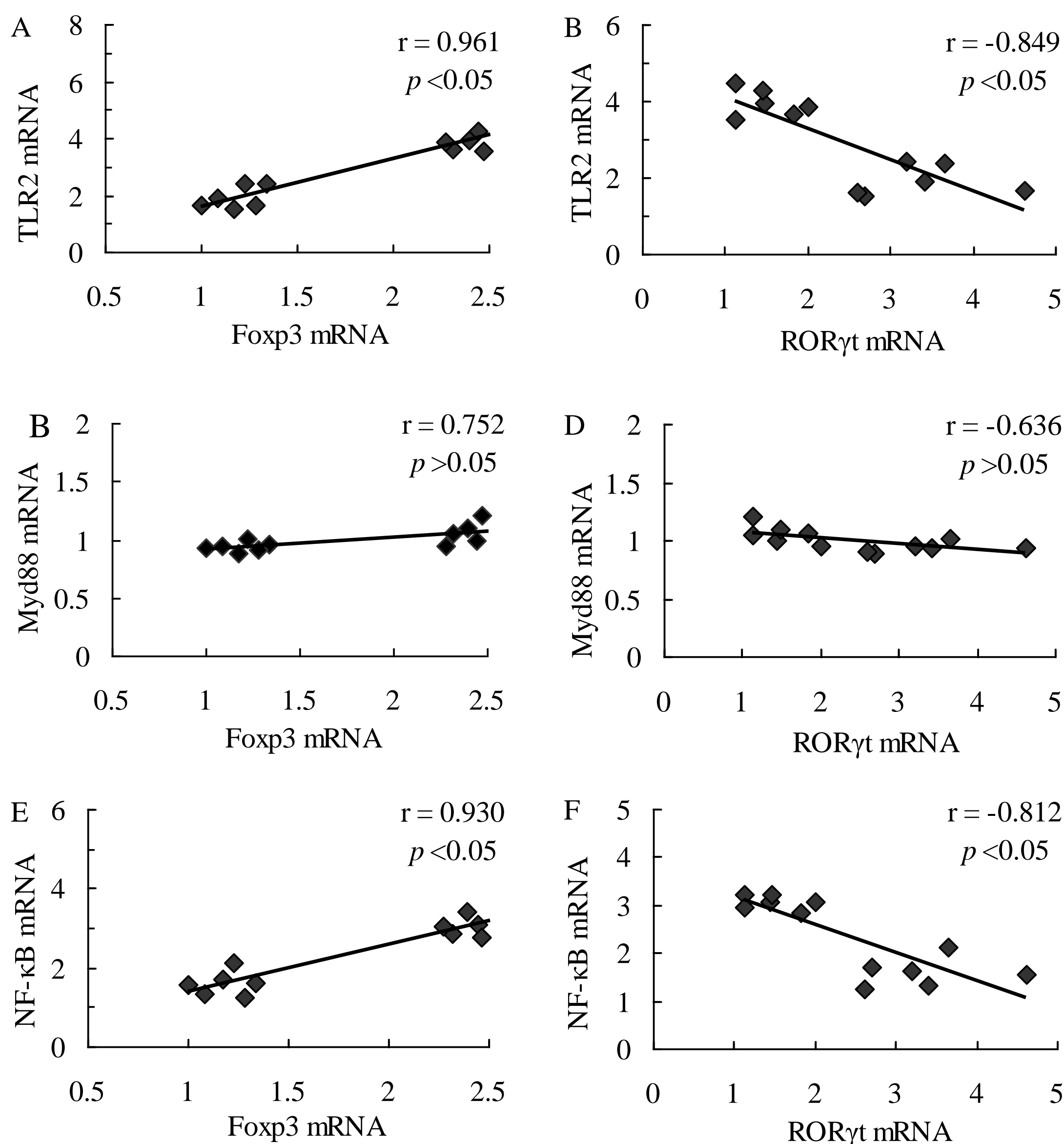

Figure 7. Correlation analysis of Foxp3, RORγt and toll-like receptor 2 (TLR2)/NF-κB signaling mRNA expression between high dose peptidoglycan (PGN) group and allergy group. Correlation of the gene expressions of TLR2 and Foxp3 (A), TLR2 and RORγt (B), Myd88 and Foxp3 (C), Myd88 and RORγt (D), NF-κB and Foxp3 (E), NF-κB and RORγt (F) in PGN group (scatter plot on the right side) compared to allergy group (scatter plot on the left side). A value of *p* <0.05 was considered significantly correlation.

DISCUSSION

The majority of evidence has demonstrated that similar to living probiotics, PGN isolated from lactobacilli cell wall also had beneficial anti-inflammation effects.[28, 29] Furthermore, numerous studies suggested that the healthful functions of PGN were closely related to the TLR2 signaling.[30, 31] This study initially tested the role of TLR2 in macrophages recognition of *Lactobacillus* PGN. The results showed that, PGN treatment can stimulate macrophages to activate the mRNA expression of TLR2 and downstream nuclear factors NF-κB. Moreover, blocking TLR2 had negative effect on IFN-γ, TGF-β

and IL-10 production, which is induced by PGN. Similarly, Rakoff-Nahoum et al [32] reported that intestinal microflora could induce protective and repair responses via activating TLR signaling in macrophages. Wang et al[33] demonstrated that MyD88 and NF-κB both participated in TLR2-dependent pathways in HEK293 cells induced by gram-positive bacteria PGN. Therefore, we demonstrated that *Lactobacillus* PGN increased Treg related cytokines via inducing TLR2/NF-κB pathway.

Subsequently, we investigated whether the immunoinhibitory effects of *Lactobacillus* PGN was dependent on TLR2 in a mouse model of β-lg allergy. Until now, the role of TLR2 signaling in allergic inflammatory disease has remained controversial. Some studies demonstrated that abnormal over-expression of TLRs were involved in inflammatory and reduced TLR stimulation might have protective effects. For instance, Sukkar et al [34], Dong et al [35], and Castoldi et al [36] reported that excessive TLR2 and TLR4 expression contribute to further aggravate infection and inflammation. While other studies showed that TLRs signaling were required to promote host defenses.[37] Finamore et al[38] revealed that TLR2 activated by *L. amylovorus* was critical for the down-regulation of TLR4 inflammatory induced by *Escherichia coli*. Castillo et al [39] found that *Lactobacillus casei* CRL 431 inhibited diarrheal infections caused by *Salmonella* through increasing the expression of TLR2, TLR4 and TLR9. In our mouse model, we observed that the TLR2 and inflammasome were involved in the pathogenesis of β-lg allergy. However, compared with both the control and allergy groups, pre-treatment with different doses of *L.acidophilus* PGN markedly attenuated allergic symptoms and enhanced TLR2, NF-κB p65 mRNA and protein expression. Meanwhile, we observed that binding of *Lactobacillus* PGN to TLR2 had little effect on the levels of MyD88. Though MyD88 was generally considered as an adapter protein responsible for the TLR2 recognition,[33] some studies provided evidence that, in addition to the MyD88-dependent signaling, TLR2 ligands also could trigger MyD88-independent pathways, which also led to NF-κB activation and cytokines production.[40, 41] Considering these observations, *L.acidophilus* PGN might induce protective immune defense responses against the development of β-lg allergy via TLR2-NF-κB pathways, but not MyD88-dependent signaling.

TLRs signaling stimulated by microbial components were highly associated with the Th cell differentiation during the inflammatory process. We have previously reported that CMA could be initiated by Th17 response and inhibited by Treg-related cytokines, [19] while oral administration of *L. acidophilus* could alleviate the severity of allergic inflammation through decreasing higher frequencies of Th17 cells.[18] In the present research, we also observed that intraperitoneal injection of *L. acidophilus* PGN to sensitized mice could cause concentration-dependent increase in the Treg-related cytokines and transcription factor expression (TGF-β, Foxp3 and CD25) but reduced IL-17A and RORγt levels (Figure 3). This data showed that *L.acidophilus* PGNs were potential stimulators to improve Treg-dominant immunity, which might be responsible for inhibiting IgE-induced allergy. Next, we further investigated the relationship between *L.acidophilus* PGN-induced TLR2 pathway and Treg/Th17 immune response in β-lg-sensitized mouse. Several studies examined the effect of TLRs signaling on modulating $CD4^+$T cell development in vivo and in vitro. Loures et al [14] and Nichols et al [15] observed that TLR2 signaling played an important role in promoting predominantly Treg responses. Furthermore, Schabussova et al [42] indicated that *L.paracasei* induced TLR2 and TLR4 signalling led to promote regulatory cytokines in birch pollen allergic mouse. Rizzo et al [43]confirmed that *L.plantarum* suppressed IL-17 and IL-23 production in *S. pyogenes* infection via the TLR2/TLR4 pathway. As expected, our results showed that the gene expression of TLR2 and NF-κB were positively correlated with the mRNA levels of Foxp3, but were negatively with RORγt in high dose of *L. acidophilus* PGN group compared to allergy group. Taken together, these findings suggested that the TLR2/NF-κB triggered by *L. acidophilus* PGN might be in involved in inhibiting Th17-dominant responses.

In conclusion we observed that *L. acidophilus* PGN, the major components of probiotics cell wall, was recognized by the TLR2 signaling, and subsequently induced Treg immune responses in β-lg-sensitized mice. This preliminary study was helpful to extend the previous knowledge concerning the preventive activity of probiotics against allergic inflammatory.

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