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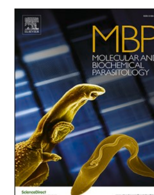

Deficiency in TLR4 impairs regulatory B cells production induced by *Schistosome* soluble egg antigen

Fang Tian [a,b,c,*], Kangwen Xian [a,e], Bin Yang [a], Qiufang Duan [a], Li Qian [a,b,c], Chanhong Shi [d,**]

[a] Department of Pathogen Biology and Immunology, Institute of Translational Medicine, Medical college, Yangzhou University, Yangzhou 225001, China
[b] Jiangsu Key Laboratory of Experimental & Translational Non-coding RNA Research, Yangzhou 225001, China
[c] Jiangsu Key Laboratory of Zoonosis, Yangzhou 225001, China
[d] Department of Neurology, Yiwu Hospital Affiliated to Wenzhou Medical University and Yiwu Central Hospital, YiWu 322000, China
[e] Department of Psychiatry, Affiliated Wu Tai Shan Hospital of Medical College of Yangzhou University, Yangzhou 225003, China

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ABSTRACT

Regulatory B cells (Bregs) producing IL-10 have negative regulatory function. Several studies have shown the important roles for Toll-like receptor 2 (TLR2), TLR4, and TLR9 ligation in the development of Bregs. We have reported that *Schistosome* soluble egg antigen (SEA) induced the production of Bregs. However, it remains unclear whether such activation is via the TLR pathway. The present study showed that IL-10 and TLR4 mRNA expression in spleen B cells of significantly increased in C57BL/10 J mice spleen B cells following SEA stimulation. The level of secreted IL-10 and IL-10$^+$ B cell proportion decreased in spleen B cells derived from TLR4-deficient C57BL/10ScNJ (TLR4$^{-/-}$) mice following SEA or LPS stimulation compared with C57BL/10 J mice. The CD1d^{hi}CD5$^+$ B cells proportion decreased in spleen B cells of TLR4$^{-/-}$ mice following SEA stimulation compared with control mice. NF-κB, ERK, p38MAPK and JNK signal transduction inhibitors significantly suppressed IL-10 secretion in CD1d^{hi}CD5$^+$ B cells induced by SEA or LPS. The phosphorylation levels of IκBα, p65, ERK, JNK and p38 were increased in CD1d^{hi}CD5$^+$ B cell of C57BL/10 J mice treated with LPS or SEA. In conclusion, this study suggests that TLR4 plays a critical role in Bregs activation induced by SEA. And the TLR4-triggered NF-κB and MAPK pathways activation in CD1d^{hi}CD5$^+$ B cells stimulated with SEA. The findings elucidated the mechanism of SEA induction of CD1d^{hi}CD5$^+$ B cells and helped us to understand the immune regulation during *Schistosoma japonicum* infection.

1. Introduction

Schistosomiasis is a typical chronic infectious disease. The immune response mechanisms of host–parasite interaction are complex during *Schistosoma* infection. *Schistosome* soluble egg antigen (SEA) is an important source of antigens and mainly responsible for the immunopathology during *S. japonicum* infection [1–3]. The interaction of SEA with immune cells elicit a switch in host immune response [4].

The regulatory B cells (Bregs) have immunosupressive function via secreting IL-10, IL-35, and TGF-β or cytokine-independent mechanisms. At present, Bregs have diversified phenotypes, but no specific transcription factors to distinguish them from other B cells [5–7]. The capacity of human and mouse regulatory B cells is central to the negative regulation of inflammation, autoimmune disease, immunodeficiency disorders, infectious disease and malignant tumor [8,9].

Much of the research has examined the Bregs could be induced during *Schistosoma* infection and the phenotype of Bregs is CD1d^{hi}CD5$^+$ [10,11]. In *vitro*, SEA stimulation could promote the IL-10 expression in splenic CD5$^+$ B cells [12]. We also have reported that SEA induced the production of Bregs secreting IL-10 in *vivo and vitro* [13]. Haeberlein S et al. found that both SEA and egg glycoprotein IPSE/alpha-1 could induce Bregs in mice and man [14]. IFN-I signaling could enhance IL-10 secretion by SEA-primed Bregs, but it wasn't necessary for Bregs development induced by *Schistosoma* [15]. Therefore, the signals required for the activation of SEA-Bregs are not well characterized.

Toll-like receptor (TLR) and/or CD40 activation is the best-

* Corresponding author at: Department of Pathogen Biology and Immunology, Institute of Translational Medicine, Medical college, Yangzhou University, Yangzhou 225001, China.
** Corresponding author.
E-mail addresses: ftian@yzu.edu.cn (F. Tian), chanhongs@163.com (C. Shi).

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characterized signal known to induce Bregs differentiation [16]. And pro-inflammatory cytokines such as IL-35, IL-21 and IFN-I can also drive the induction of Bregs [17]. Several mice studies have shown the important roles of TLR2, TLR4, TLR7 and TLR9 ligations in the development of Bregs, and highlighted their boosting potential [18,19]. Lipopolysaccharide (LPS) is a crucial structural component of the outer membrane of Gram-negative bacteria, with which regulatory B cells secrete IL-10 by TLR4 pathway [20]. The LPS/TLR4 signal transduction pathway involves transcription factors nuclear factor-κB (NF-κB), extracellular regulated kinase (ERK), p38 mitogen activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK) [21]. TLR2 and TLR4 might direct the distinct adaptive immune responses in a mouse model infected with *Schistosoma japonicum* [22]. However, it remains unclear whether these Bregs activation stimulated by SEA is via the TLR pathway. In this study, the contributions of TLR2 and TLR4 to Bregs induced with SEA were investigated, and whether the deficiency in TLR4 pathway impaired Breg cell production induced by SEA was analyzed.

2. Materials and methods

2.1. Ethics statement

The use of experimental animals was approved by the Ethics Committee of Yangzhou University Medical College, Yangzhou, China; Approval number YXYLL-2020–50.

2.2. Mice

Female mice (aged 6–8 wk) were used in all experiments. TLR4-deficient C57BL/10ScNJ mice and control C57BL/10 J mice were purchased from Model Animal Research Center, Nanjing University (Nanjing, China). TLR4-deficient ($TLR4^{-/-}$) murine strain C57BL/10ScNJ on a C57BL/10 J background does not express TLR4 due to deletion of this gene. The mice were maintained under specific pathogen-free conditions and bred in Laboratory Animal Center of Yangzhou University (Yangzhou, China).

2.3. Preparation of antigens

S. japonicum eggs were extracted from the livers of infected rabbits and enriched. SEA was then prepared from the homogenized eggs as previously described [23]. Protein concentration was determined by BCA protein Assay kit (Pierce Biotechnology, Inc., Rockford, Illinois) according to the instructions provided by the manufacturer. The concentration of endotoxin in SEA was less than 150 ng/mg protein, as assayed by Tachypleus Amoebocyte Lysate (TAL) test (Zhanjiang bokang marine biological Co., LTD, Zhanjiang, China) according to the instructions provided by the manufacturer.

2.4. Signal transduction inhibitors

PDTC for NF-κB, SB203580 for p38MAPK, SP600125 for JNK, and PD98059 for ERK were purchased from Beyotime Biotechnology (Shanghai, China).

2.5. Cell sorting and culture

In *vitro*, single cell suspensions of splenocytes were prepared by mincing normal mouse and $TLR4^{-/-}$ mouse spleens in phosphate buffered saline (PBS) containing 1% fetal bovine serum (FBS) (Gibco, Grand Island, New York) and 1% EDTA. Red blood cells were lysed using ACK lysis buffer containing ammonium chloride (Beyotime Ltd, Shanghai, China). $CD19^+$ B cells were enriched using anti-CD19 MicroBeads (Miltenyi Biotec, Teterow, Germany) and the purity of $CD19^+$B cells was 96% after sorting. The 2×10^6/ml purified B cells were stimulated with 20 μg/ml SEA or 10 μg/ml LPS or PBS for 72 h at 37 °C in complete RPMI1640 medium (Gibco) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Culture supernatants were collected for enzyme-linked immunosorbent assay (ELISA). The cells were collected for fluorescence activated cell sorting (FACS) or extraction of RNA after incubation.

2.6. Cell treatment

In *vitro*, $CD19^+$ B cells sorted using anti-CD19 MicroBeads were cultured in complete RPMI1640 medium (Gibco) containing 10% FBS, 2 mM L-glutamine. Then 2×10^6 cells per well in 1 ml of complete media were culture in 24 well plate. Subsequently, 10 μmol/L PD98059, 50 μmol/L SP600125, 40 μmol/L SB203580, 100 μmol/L PDTC and DMSO were added to the cells suspension, respectively. After 30 min, the B cells suspension were stimulated with 20 μg/ml SEA or 10 μg/ml LPS or PBS for 72 h at 37 °C. Culture supernatants were collected for ELISA.

2.7. Extraction of RNA, reverse transcription, and quantitative real-time polymerase chain reaction (RT- PCR)

Antigen-pulsed normal mouse $CD19^+$ B cells were lysed and total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, California) and reverse transcribed to cDNA using PrimeScript RT reagent kit (Takara, Otsu, Shiga, Japan). cDNA was amplified with SYBR Premix Ex Taq (Takara) in a 7500 Real-time PCR System (Applied Biosystems, Foster City, California). Primers specific for β-actin, IL-10 (*il-10*), TLR2 (*tlr2*) and TLR4 (*tlr4*) are shown in Table 1. Real-time PCR was run in triplicates in a volume of 20 μl containing 10 μl of SYBR Premix Ex Taq, 300 nM of each primer, and 50 ng cDNA. Reaction conditions were as follows: 50 °C for 2 min, 95 °C for 30 s, 40 cycles of 95 °C for 30 s, and 60 °C for 20 s. Housekeeping gene β-actin was used as an internal control. Quantitation of relative differences in expression was finally calculated using the comparative $2^{-\Delta\Delta CT}$ method [24].

2.8. Cytokine detection

The concentrations of cytokines IL-10, IFN-γ and IL-4 in the culture supernatant were measured by ELISA kits (Dakewei, Beijing, China) according to the manufacturer's protocol.

2.9. Flow cytometry

To detect Bregs, single cell suspensions of $CD19^+$ B cells from each mouse were prepared, and 1×10^6 cells from each sample were stimulated with 25 ng/ml PMA and 1 μg/ml ionomycin in complete RPMI1640 medium for 1 hr 37 °C in 5% CO_2. After 1 h, the cells were cultured for 5 h after addition of BFA. Then the cells were collected and washed, fixed, permeabilized with Cytofix/Cytoperm buffer (BD PharMingen, provide location of manufacturer), intracellularly stained with PE-conjugated antibodies against IL-10 (or isotype IgG2a control

Table 1
Primers and annealing temperatures used for the amplification of each target gene.

Gene	Primer (5′−3′)	Annealing temperature (°C)	Product size (bp)
β-actin	F:CCTCTATGCCAACACAGTGC R:GTACTCCTGCTTGCTGATCC	59	216
il-10	F: CAACATACTGCTAACCGACTC R: CATTCATGGCCTTGTAGACAC	59	276
tlr4	F:TGTCCTTGAGAAGGTTGAGA R:TCCTCTGCTGTTTGCTCAGG	59	180
tlr2	F:GAAACCTCAGACAAAGCGTC R:GCTTTTCATGGCTGCTGTGA	59	200

antibody) (eBioscience) to detect IL-10$^+$ B cells, and analyzed with FACS Calibur flow cytometer.

The spleen CD19$^+$ B cells stimulated by SEA from two groups were stained with antibodies against CD1d -Alexa Fluor488 and CD5- PerCP-Cy 5.5 according to the manufacturer's protocol, and were analyzed or sorted with FACS Calibur flow cytometer.

2.10. Western blot assay

The protein was extracted from the CD1d^{hi}CD5$^+$ B cell after stimulating with SEA and LPS. Protein extraction reagent with protease inhibitor to lysates on ice, and centrifugation at 12,000 g for 10 min to collect supernatants. The concentrations of the protein were determined by bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the instructions. The same amount of proteins (30 μg) were separated by 12% SDS-PAGE, electrophoresed, and then transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary Abs targeting NF-κB inhibitor (IκBa; cat. no. 4812), p-IκBa (cat. no. 2859), p65 (RELA proto-oncogene; cat. no. 8242), p-p65 (cat. no. 3033), extracellular signal-regulated kinase (ERK; cat.no. 4695), p-ERK (cat. no. 4370), c-Jun N-terminal kinase (JNK; cat. no. 9252), p-JNK (cat. no. 4671), p38 (MAPK14; cat. no. 9212) and p-p38 (cat. no. 4511; all from Cell Signaling Technology, Inc., Danvers, CA, USA) at 1:1000 (v/v) dilution overnight, shaking at 4°C. β-actin (cat. no. 4970; 1:1000; Cell Signaling Technology, Inc.) was used as a loading control. Each membrane was washed and membranes were incubated with an HRP-conjugated secondary Abs (cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 1 h. Finally, relevant bands were detected using enhanced chemiluminescence (ECL) (Pierce; Thermo Fisher Scientific, Inc.).

2.11. Statistical analysis

Statistical analysis was performed using the SPSS version 22.0 software. The data were expressed as mean±SEM. Statistical significance was determined by one-way ANOVA. Significant differences were as follows: * $P < 0.05$; * * $P < 0.001$.

3. Results

3.1. TLR2, TLR4 and IL-10 mRNA expressions levels

Both SEA and LPS stimulations significantly induced the expressions of IL-10 and TLR4 mRNA in B cells derived from C57BL/10 J mice. However, there was no significant change in TLR2 mRNA expression. Therefore, the expression of TLR4 and IL-10 mRNA in B cells increased after SEA and LPS stimulation (Fig. 1).

3.2. Cytokine profile

In order to investigate the cytokines secreted by SEA- or LPS-stimulated B cells derived from TLR4$^{-/-}$ mice, splenic CD19$^+$ B cells were sorted and stimulated by SEA or LPS, and the levels of cytokines in the supernatants were detected (Fig. 2). The levels of IL-10 secreted from B cells of TLR4$^{-/-}$ mice following SEA or LPS stimulation decreased compared with that of C57BL/10 J mice, though they were still quite higher than the PBS control. However, IFN-γ and IL-4 secretions were similar.

3.3. IL-10$^+$ B cells proportions

After stimulation with SEA or LPS in *vitro*, the percentages of IL-10$^+$ B cells in CD19$^+$ B cells of TLR4$^{-/-}$ mice significantly decreased compared with those of C57BL/10 J mice (Fig. 3).

3.4. CD1d^{hi}CD5$^+$ B cells proportions

We tested the proportions of CD1d^{hi}CD5$^+$ cells in the spleen CD19$^+$ B cells. After stimulation with SEA in *vitro*, the proportion of CD1d^{hi}CD5$^+$ B cells of TLR4$^{-/-}$ mice decreased compared with that of C57BL/10 J mice (Fig. 4).

3.5. IL-10 levels produced by CD1d^{hi}CD5$^+$B cells treated with signal transduction inhibitors

SB203580, SP600125, PD98059 and PDTC significantly inhibited IL-10 secretion in CD1d^{hi}CD5$^+$ B cells induced by SEA or LPS. These data suggested that IL-10 secretion of CD1d^{hi}CD5$^+$ B cells partly depended on transcription factors NF-κB, ERK, p38MAPK and JNK involved in the TLR4 signal transduction pathway (Fig. 5).

3.6. SEA-induced CD1d^{hi}CD5$^+$B cells activated by NF-κB and MAPK pathways

It has been reported that TLR4 ligand can activate the NF-κB and MAPK pathways to produce IL-10. The phosphorylation levels of IκBα and p65 were increased in CD1d^{hi}CD5$^+$ B cell of C57BL/10 J mice treated with LPS and SEA. The extents of ERK, JNK and p38 phosphorylation were also significantly increased in CD1d^{hi}CD5$^+$ B cell of C57BL/10 J mice after LPS and SEA treatment. The results suggested that the NF-κB and MAPK pathways are involved in the CD1d^{hi}CD5$^+$ B cells induced by SEA (Fig. 6).

4. Discussion

The gold standard for Bregs identification are endogenous or transferred B cells with suppression function [25]. We and others have recently reported that SEA managed to induce Bregs secreting IL-10 in vitro and in vivo [13,14]. However, it remains unclear that the pathway of SEA activating the Bregs. In this study, we analyzed the TLR2 and TLR4 expressions of Bregs induced by SEA, and evaluated the resulting production capacity of Bregs in TLR4$^{-/-}$ mice. The results show that the

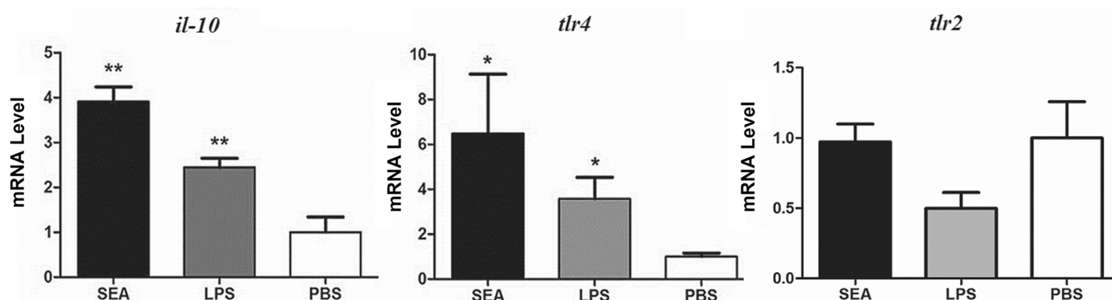

Fig. 1. SEA and LPS stimulation significantly increased *il-10* and *tlr4* mRNA expression in spleen CD19$^+$ B cells from C57BL/10 J mice. Data are expressed as mean ± SEM of 3 independent experiments with six C57BL/10 J mice per group in each experiment. * $p < 0.05$; ** $p < 0.01$, compare to control group with PBS.

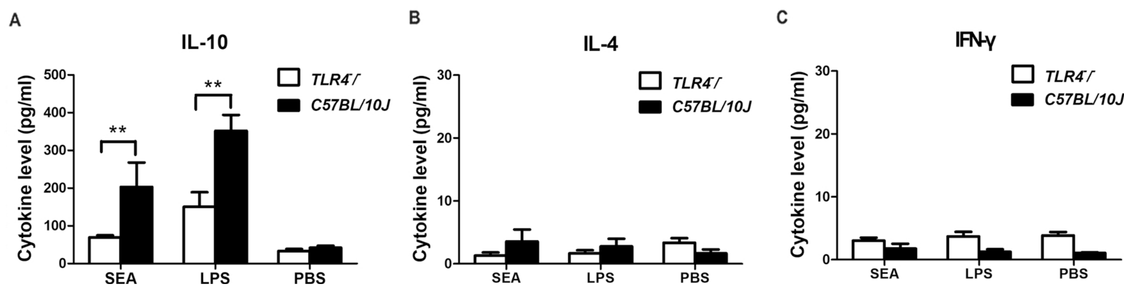

Fig. 2. IL-10 levels significantly decreased in B cells of TLR4-/- mice following SEA or LPS stimulation. Data are expressed as mean ± SEM of 3 independent experiments with six mice per group in each experiment. * $p < 0.05$; * * $p < 0.01$, compare to C57BL/10 J mice.

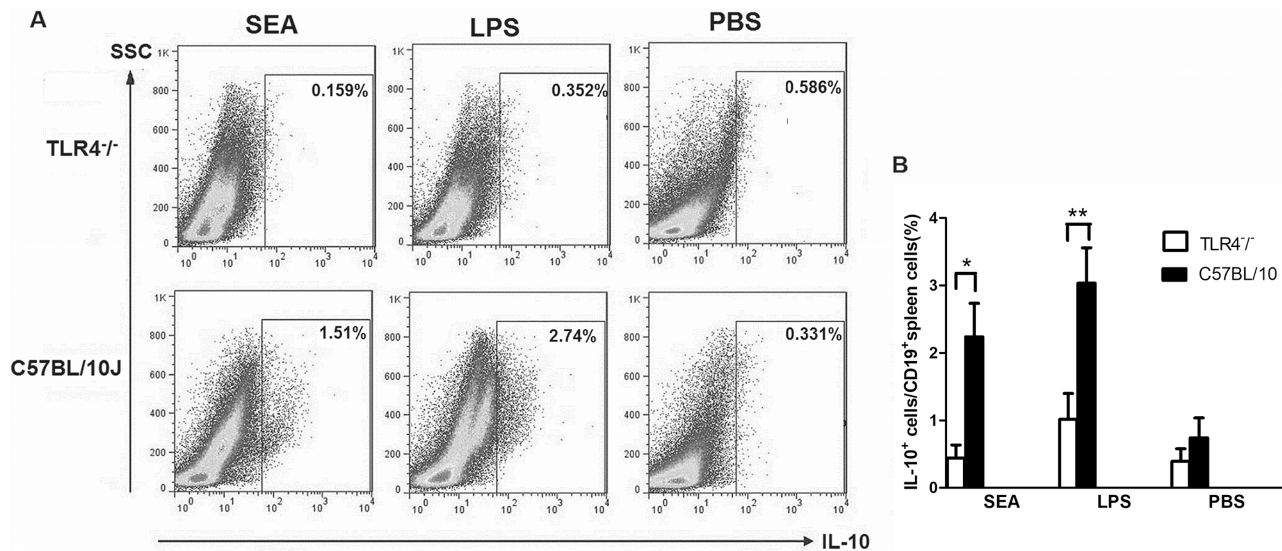

Fig. 3. Proportions of IL-10+ cells significantly decreased in CD19+ spleen cells derived from TLR4-/- mice stimulated with SEA, LPS. A. Flow cytometric analysis from one representative experiment. B. Proportion of IL-10+ cells in CD19+ B cells. Data are expressed as mean ± SEM of 3 independent experiments with six mice per group in each experiment. * $p < 0.05$; ** $p < 0.01$, compare to C57BL/10 J mice.

deficiency in TLR4 impairs Bregs production induced by SEA.

There are at least two possible pathways by which splenic B cells may differentiate into Bregs. In acquired immune-mediated diseases, Bregs may be activated through BCR ligation with self-Ag and/or CD40/ CD40L interaction. In diseases involving exposure to bacterial products, they may be activated through TLR pathways [7,26,27]. A lipid fraction from *Schistosoma mansoni* eggs containing lysophosphatidylserine, in a TLR2-dependent mechanism, can induce the activation of DCs that promote Th2 and regulatory T-cell development [28]. Lacto-N-fucopentaose III was conjugated to a 40-kDa molecule of Dex (LNFPIII-Dex), a synthetic copy of *Schistosome* egg glycan, could facilitate Th2 differentiation by DCs via a TLR4-dependent pathway [29]. Therefore, TLR4 and/or TLR2 play(s) an important role in the recognition of SEA by DCs and macrophages and in the development of Th2 responses [30]. Whether SEA induces the activation of Bregs via the TLR2 and/or TLR4 pathway is still undefined. Our results showed that SEA and LPS stimulation both significantly induced the expressions of IL-10 and TLR4 mRNA in B cells derived from C57BL/10 J mice. These suggested that TLR4 was related with SEA-induced Bregs activation.

Distinct TLR4 ligands induce either Th1 or Th2 responses. As an important structural component of the outer membrane of Gram-negative bacteria [31]. LPS is a TLR4 ligand which activates DCs to induce Th1 responses [29]. It has also been described as a strong inducer of IL-10 expression in murine B cells [32]. LNFPIII is the predominant carbohydrate component of SEA that activates B and B-1 cells to produce IL-10 [33,34]. And SEA and egg glycoprotein IPSE/alpha-1 could induce Bregs [14]. In this study, a few B cells of TLR4-/- mice could differentiate into IL-10+ B cells and secrete a certain amount of IL-10 after SEA and LPS stimulation, but the IL-10+ B cells proportion and IL-10 level significantly decreased compared with B cells derived from C57BL/10 J mice. Studies showed that the phenotype of spleen Bregs secreting IL-10 was mainly CD1d^{high}CD5+ [10,12]. So we analyzed the percentages of CD1d^{high}CD5+ B cells, and the proportion of CD1d^{high}CD5+ B cells decreased in TLR4-/- mice. These indicated that TLR4 participated in IL-10 secretion of CD1d^{high}CD5+ Bregs responding to SEA. These findings are in line with previous studies showing that TLR4 supports the expansion of CD1d^{high}CD5+ Bregs in contact hypersensitivity [35]. And TLR4 signaling plays an important role in regulating IL-10 secretion by Bregs in multiple sclerosis [36]. However, whether SEA can induce the production of Bregs with other phenotypes and its mechanism need to be further investigated. Whether TLR is involved in SEA induced the production of CD1d^{high}CD5+ Bregs in *vivo* also needs to be further confirmed.

NF-κB and MAPK kinase pathways are the key downstream signaling networks for TLR4 activated in B cells [37,38]. NF-κB predominantly controls cell cycle and survival in response to BCR or TLR4 signals [39]. In B cells, MAPK is the downstream signaling of TLRs. MAPK pathways comprise ubiquitous multi-tiered kinase cascades that activate conserved effector serine/threonine kinase, ERK, plus the stress activated protein kinases (SAP), JNK and p38 [40,41]. In our study, signal transduction inhibitors of NF-κB, p38MAPK, JNK, and ERK significantly inhibited IL-10 level secretion in CD1d^{hi}CD5+ Bregs induced by SEA or

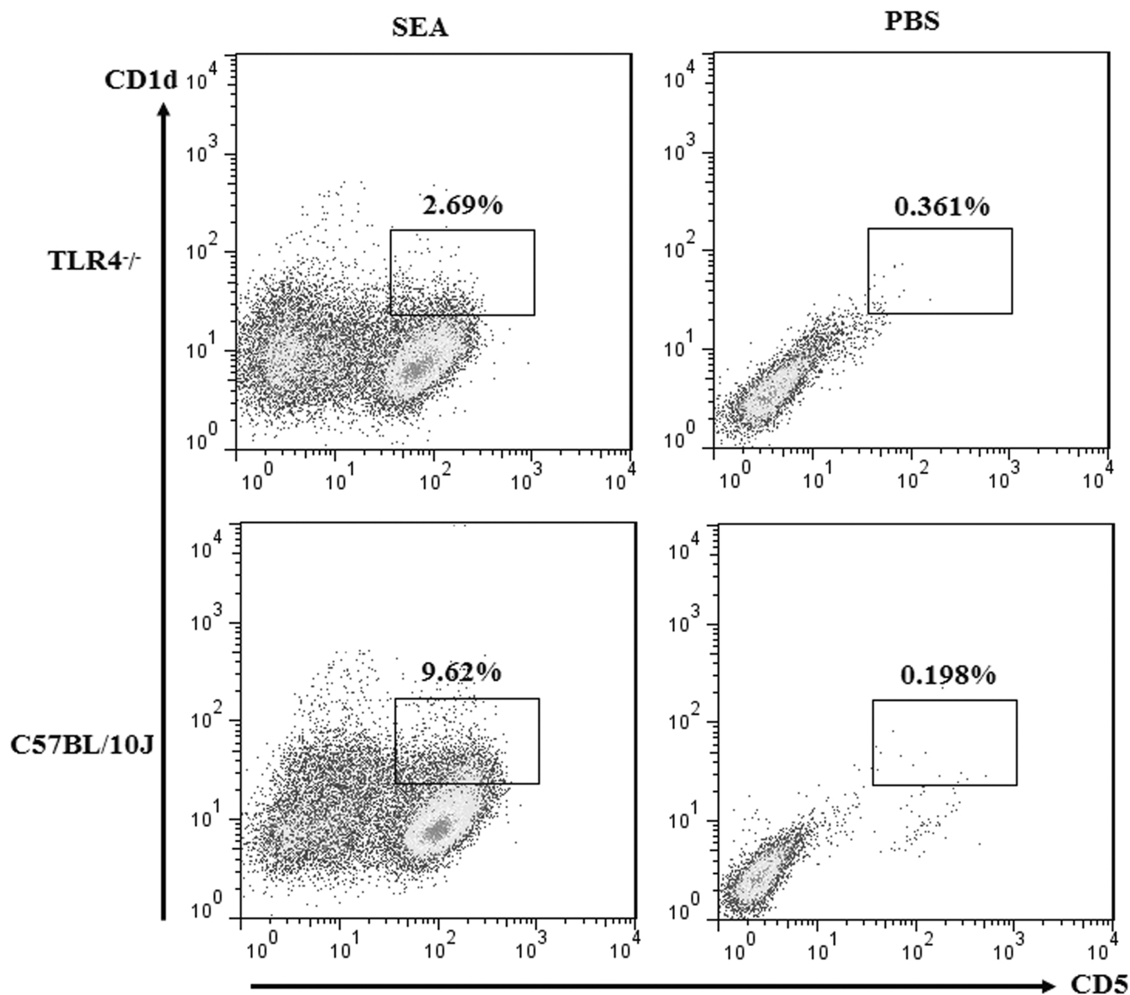

Fig. 4. Proportions of CD1d^{hi}CD5^{+} cells significantly reduced in CD19^{+} B cells derived from TLR4$^{-/-}$ mice stimulated with SEA. Data represent three independent experiments with six mice per group per experiment. *$p < 0.05$; **$p < 0.01$, compare to C57BL/10 J mice.

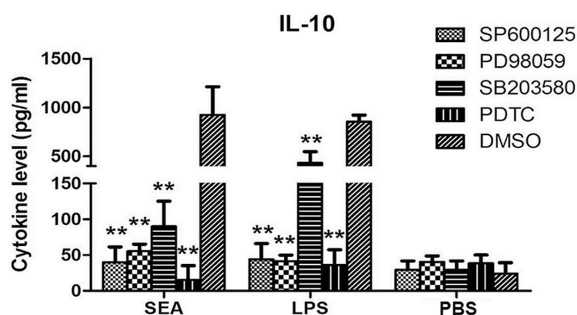

Fig. 5. IL-10 levels significantly decreased in supernatants produced by CD1d^{hi}CD5^{+} B cells treated with NF-κB (PDTC), ERK (PD98059), JNK (SP600125) and p38MAPK (SB203580) pathway inhibitors and stimulated with SEA and LPS. Data are expressed as mean ± SEM of 3 independent experiments with six C57BL/10 J mice per group in each experiment. *$p < 0.05$; **$p < 0.01$, compare to control group with DMSO.

LPS. And SEA-induced CD1d^{hi}CD5^{+} Bregs activated by NF-κB and MAPK Pathways. so the production of CD1d^{hi}CD5^{+} Bregs is partly dependent on these transcription factors involved in the TLR4 signal transduction pathway.

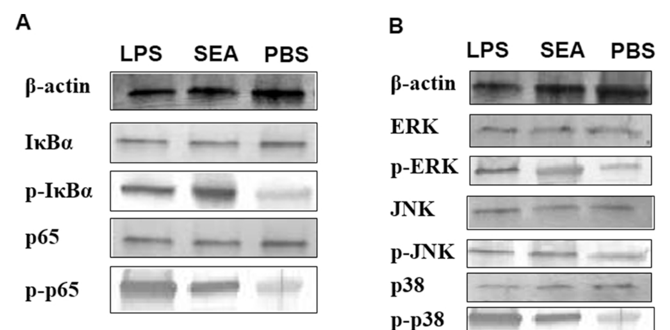

Fig. 6. The phosphorylation levels of IκBα, p65, ERK, JNK and p38 significantly increased in CD1d^{hi}CD5^{+} B cell of C57BL/10 J mice treated with LPS or SEA. (A) Western blot analysis of NF-κB pathway related molecules. (B) Western blot analysis of MAPK pathway related molecules. Data also represent three independent experiments, compare to control group with PBS.

5. Conclusions

In summary, the generation of CD1d^{hi}CD5^{+} Bregs secreting IL-10 induced by SEA partly depended on the TLR4 pathway, being related with involved NF-κB and MAPK pathways. This study suggests that TLR4 may exert critical effects on Bregs secreting IL-10 activation, and contribute to the understanding of the immune regulation during *S. japonicum* infection.

Ethical standards

Animal experiments were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Animal Welfare Association of Jiangsu province (Jiangsu, China). All animal procedures were approved by the Institutional Animal Care and Use Committee of Yangzhou University Medical College.

CRediT authorship contribution statement

Fang Tian: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **Kangwen Xian**: Data curation, Writing – original draft. **Bin Yang**: Data curation, Validation. **Qiufang Duan**: Data curation, Formal analysis. **Li Qian**: Formal analysis, Visualization. **Chanhong Shi**: Formal analysis, Writing – review & editing.

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Conflicts of interest

The authors declare no conflict of interests.

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