FcγRIIb attenuates TLR4-mediated NF-κB signaling in B cells

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Abstract. Toll-like receptors (TLRs) serve a vital role in activating the innate immune system by sensing conserved microbial products. Fc γ receptor IIb (FcγRIIb), the inhibitory Fc receptor, exerts its immune regulatory functions by binding to the immunoglobulin G Fc domain. Although the individual roles of TLRs and FcγRIIb have been studied intensively, the cross-talk between FcγRIIb and TLR4 on B cells remains unknown. The present study demonstrated that FcγRIIb ligation by the immune complex (IC) attenuated the TLR4-triggered nuclear factor (NF)-κB activation, and decreased the release of interleukin (IL)-6 from B cells, via enhancing LYN proto-oncogene (Lyn) phosphorylation. In addition, IC treatment protected mice from lethal endotoxic shock. Accordingly, IC decreased the LPS-induced serum levels of IL-6, as well as intracellular IL-6 production in B cells *in vivo*. However, these protective and inhibitory effects of IC were not observed in FcγRIIb$^{-/-}$ mice. In conclusion, the present data demonstrated that FcγRIIb inhibited TLR4 signaling in B cells by activating Lyn phosphorylation and by inhibiting NF-κB signaling. The present study elucidated the mechanism associated with the TLR4 and FcγRIIb cross-talk in B cells.

Introduction

Upon recognition of microbial conserved structures, such as lipopolysaccharide (LPS) and viral RNA, toll-like receptors (TLRs) are triggered to induce pro-inflammatory cytokines production, thus serving important roles in the initiation of innate immunity (1). The TLR intracellular signaling pathway has been studied intensively. Binding of TLR4 agonist, LPS, to TLR4 activates myeloid differentiation protein 88 (MyD88)-dependent signaling, leading to the activation of mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB pathways, and contributing to the release of pro-inflammatory cytokines (2,3). Increased production of pro-inflammatory cytokines causes damage to the host and induces pathological inflammation. Dampening TLR signaling has therefore attracted much attention as a potential therapeutic for immune-related diseases (1,2). There are numerous reports of TLR4 signaling pathway relying on a series of phosphorylation events. For example, LPS stimulation results in myeloid differentiation factor-2 (MD-2) phosphorylation which is required for NF-κB activation (4). MD-2 phosphorylation is dependent on the Src kinase, LYN proto-oncogene (Lyn) (4). In addition, another report has suggested that Lyn positively controls TLR4-induced NF-κB activation, via regulating the activity of tumor necrosis factor (TNF) receptor-associated factor 6/tumor growth factor-β-activated kinase complex (5). Thus, regulation of phosphorylation events via kinases may be used as a potential target to dampen TLR4 signaling.

The Fc portion of immunoglobulin (Ig) G is recognized by Fc γ receptors (FcγRs). FcγRs exist in two functional subtypes: Immunoreceptor tyrosine-based activation motif (ITAM)-containing FcγRs (such as FcγRI and FcγRIIa) and immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing FcγRs (FcγRIIb). The inhibitory FcγRIIb counters the actions of activating FcγRs (6,7). As an example, co-ligation of FcγRIIa by aggregated IgG induces activation of ITAM, whereas cross-linking of FcγRIIb by immune complex (IC) initiates activation of ITIM and subsequent recruitment of SH2-domain-containing inositol phosphatase 1 (SHIP-1), ultimately resulting in the inhibition of ITAM activation pathways (8,9). Most immunocytes, such as macrophages and dendritic cells (DCs), co-express activating and inhibitory FcγRs, but B cells are reported to only express the inhibitory receptor FcγRIIb, which functions to downregulate B-cell receptor-mediated activation pathways by IC (6,7).

The individual functions of TLRs and FcγRIIb in immune responses have been investigated intensively. However, the

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Abbreviations: DC, dendritic cell; IC, immune complex; IL-6, interleukin-6; LPS, lipopolysaccharide; Ab, antibody; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation protein 88; NF-κB, nuclear factor-κB; TLR, toll-like receptor; TNF, tumor necrosis factor; WT, wild-type

Key words: B cells, Fc γ receptor IIb, immune complex, toll-like receptor

effect of the combined activation of TLRs and FcγRIIb remains unclear. Previous studies indicate that IC attenuates TLR4-induced TNF-α secretion from DCs with a high expression of FcγRIIb (10). In addition, IC inhibits TLR4-induced interleukin (IL)-6 and TNF-α production from macrophages via FcγRIIb (11). It is well known that B cells secret antibodies and mediate the humoral immune response. Furthermore, B cells function as antigen-presenting cells, inducing the activation of $CD4^+$ T cells (12). Previously, B cells have been identified to exert non-classical immune functions, such as responding to TLR ligands and secreting different types of cytokines (13-15). For example, B cells stimulated with TLR4 agonist or TLR2 agonist produce high levels of IL-6 (13). However, whether FcγRIIb regulates TLR4-triggered innate immune responses in B cells has not been elucidated to date.

The present study demonstrated that FcγRIIb ligation by IC negatively regulated TLR4-triggered secretion of IL-6 in B cells, by enhancing activation of Lyn and inhibiting activation of NF-κB signaling. IC treatment protected mice from lethal endotoxic shock in an FcγRIIb-dependent manner. Accordingly, IC treatment decreased LPS-induced serum and intracellular levels of IL-6 in B cells via FcγRIIb *in vivo*. The present results enrich the understanding of the cross-talk between TLR4 and FcγRIIb during immune response.

Materials and methods

Mice. A total of 135 male C57BL/6J mice (5-6 weeks of age, 17-21 g) were purchased from the Animal Center of Yangzhou University (Yangzhou, China). A total of 45 male $Fc\gamma IIb^{-/-}$ mice on a C57BL/6J background (5-6 weeks of age, 17-21 g) were purchased from Jackson Laboratory (Ben Harbor, ME, USA). All mice were maintained in an animal facility at 22-24˚C with 50-60% humidity, a 12-h light/dark cycle and with free access to food and water. Animal experiments were conducted with approval from the Institutional Animal Care and Use Committee of Yangzhou University.

IC preparation. IC was generated as described previously (11). Briefly, anti-ovalbumin (OVA) antibody (Ab; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was incubated with OVA (Sigma-Aldrich; Merck KGaA) at a 10:1 mass ratio at 37˚C for 1 h. For *in vitro* experiments, IC was used at a concentration of 10 μg/ml OVA + 100 μg/ml anti-OVA. For *in vivo* experiments, IC was used at 100 μg OVA + 1 mg anti-OVA per mouse. IC was tested for LPS contamination by limulus amebocyte lysate test (Zhanjiang A&C Biological Ltd., Zhanjiang, China).

B cells stimulation. Splenic B cells were prepared using CD19 microbeads (Miltenyi Biotec, Inc., Cambridge, MA, USA) according to the manufacturer's protocols. Cells were plated at $2x10^5$ cells per well and stimulated with IC, 200 ng/ml LPS (from *Escherichia coli* O111:B4; Merck KGaA) or IC plus LPS for 24 h. Where indicated, 100 nM of the NF-κB inhibitor ammonium pyrrolidinedithiocarbamate (PDTC; Merck KGaA), or 1 nM of the Lyn inhibitor radicicol (Merck KGaA), was used to pretreat B cells 1 h prior to stimulation with LPS.

Western blot assay. Protein was extracted from B cells as described previously (16). The protein concentrations were measured using the bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Equal amounts of protein (30 μg) were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membranes. Membranes were then incubated with primary Abs targeting Lyn (cat. no. 2796), phosphorylated (p)-Lyn (cat. no. 2731), Bruton tyrosine kinase (Btk; cat. no. 3533), p-Btk (cat. no. 5082), spleen associated tyrosine kinase (Syk; cat. no. 2712), p-Syk (cat. no. 2710), NF-κB inhibitor a (IκBa; cat. no. 4812), p-IκBa (cat. no. 2859), p65 (official name, RELA proto-oncogene; cat. no. 8242), p-p65 (cat. no. 3033), inhibitor of NF-κB kinase subunit β (IKKβ; cat. no. 8943), p-IKKβ (cat. no. 2697), 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 1:1,000 (v/v) dilution overnight at 4˚C. β-actin (cat. no. 4970; 1:1,000; Cell Signaling Technology, Inc.) was used as a loading control. Following washing, membranes were incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated secondary Abs (cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. Finally, relevant bands were detected by enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.).

IC injection and endotoxic shock. IC was injected intraperitoneally into mice for 24 h and then 20 mg/kg of LPS was injected intraperitoneally into the IC-pretreated mice. Survival rate was monitored for 4 days. In some experiments, the mice were sacrificed 2 h following LPS injection. Blood samples were acquired for IL-6 detection in sera. Splenic single-cell suspensions were prepared as reported (17) for intracellular IL-6 detection in B cells.

IL-6 measurement. For detecting IL-6 in sera and culture supernatants, a CBA Flex Set kit (BD Biosciences, San Jose, CA, USA) was used following the manufacturer's protocol. For measurement of intracellular IL-6 in B cells, single cell suspensions were incubated with brefeldin A (Sigma-Aldrich; Merck KGaA) at 10 μg/ml for 6 h, and labeled with anti-CD19 (cat. no. 553785; BD Biosciences) and anti-IL-6 (cat. no. 554401; BD Biosciences) or isotype-matched Ig control Abs (cat. no. 554685; BD Biosciences) at 1:20 dilution using a Cytofix/Cytoperm kit (BD Biosciences) following the manufacturer's protocol. Results from both assays were analyzed by flow cytometry. Flow cytometry was done with a FACSCalibur (BD Biosciences) and data were analyzed with FlowJo version 10.0 software (FlowJo, LLC, Ashland, OR, USA).

Statistical analysis. For comparisons between two groups, data were analyzed using Student's t-test. For comparisons among multiple groups, data were analyzed using one-way analysis of variance followed by the Tukey post hoc test. GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for data analysis. Survival curves were drawn using GraphPad Prism 5.0 (GraphPad Software, Inc.), and survival analysis was performed using the log-rank (Mantel-Cox) test.

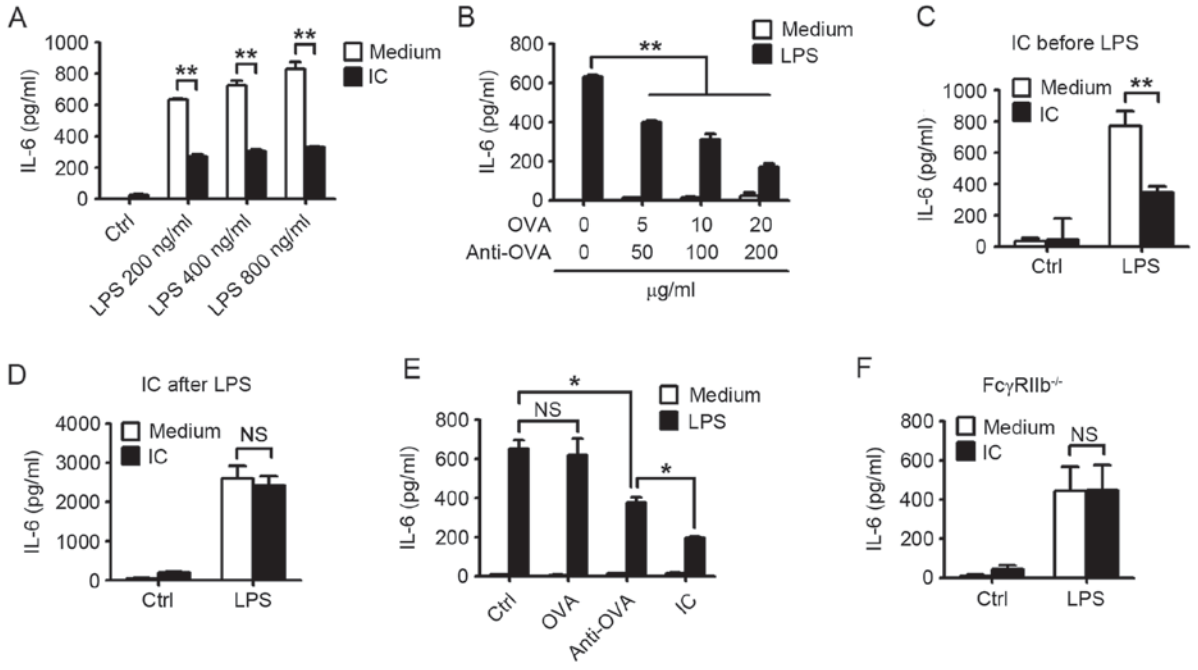

Figure 1. IC inhibits LPS-triggered IL-6 secretion in splenic B cells via FcγIIb. (A) Splenic B cells were stimulated with IC (containing 10 μg/ml OVA + 100 μg/ml anti-OVA antibody), LPS at indicated concentrations, or LPS plus IC for 24 h. (B) Splenic B cells were stimulated with IC at indicated concentrations, LPS (200 ng/ml), or LPS plus IC for 24 h. (C) Splenic B cells were pretreated with either medium control or IC (containing 10 μg/ml OVA + 100 μg/ml anti-OVA) for 24 h and then stimulated with 0 (Ctrl) or 200 ng/ml LPS for another 24 h. (D) Splenic B cells were stimulated with 0 (Ctrl) or 200 ng/ml LPS for 24 h and then treated with either medium control or IC (containing 10 μg/ml OVA + 100 μg/ml anti-OVA) for another 24 h. (E) Splenic B cells were cultured with 10 μg/ml OVA, 100 μg/ml anti-OVA or IC (containing 10 μg/ml OVA + 100 μg/ml anti-OVA) in the presence or absence of LPS for 24 h. (F) FcγIIb$^{-/-}$ splenic B cells were stimulated with IC (containing 10 μg/ml OVA + 100 μg/ml anti-OVA), 200 ng/ml LPS, or LPS plus IC for 24 h. Data represent the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, with comparisons indicated by lines. IC, immune complex; LPS, lipopolysaccharide; IL, interleukin; FcγIIb, Fc γ receptor IIb; OVA, ovalbumin; Ctrl, control; NS, not significant.

Results

IC inhibits LPS-triggered IL-6 release from B cells via FcγRIIb. B cells were stimulated with IC and LPS for 24 h and then cytokine production was examined in the supernatants. IC treatment did not affect TNF-α, IL-1β, interferon (IFN) γ and IFNγ-induced protein 10 production (data not shown). However, IL-6 secretion was significantly inhibited in B cells stimulated with 200-800 ng/ml LPS, compared with unstimulated B cells (Fig. 1A). As illustrated in Fig. 1B, IC treatment inhibited IL-6 production in LPS-activated B cells in a dose-dependent manner. Furthermore, LPS-activated B cells exhibited attenuated IL-6 secretion when pretreated with IC for 24 h (Fig. 1C). However, IC treatment could not inhibit LPS-induced IL-6 secretion in B cells, if LPS stimulation had already occurred prior to IC treatment (Fig. 1D). Considering that IC is formed from free OVA and anti-OVA Ab, the effect of free OVA or anti-OVA Ab treatment alone on LPS-induced IL-6 production was tested as a control. As illustrated in Fig. 1E, free OVA did not reduce LPS-triggered IL-6 secretion, while the ability of the anti-OVA Ab alone to inhibit IL-6 production was significantly decreased compared with IC treatment. To further confirm that FcγRIIb mediated the inhibitory action of IC on IL-6 release in LPS-activated B cells, B cells from FcγRIIb$^{-/-}$ mice were activated with IC and LPS. As demonstrated in Fig. 1F, the inhibitory action of IC on LPS-triggered IL-6 secretion was lost in FcγRIIb$^{-/-}$ B cells. These results indicated that FcγRIIb mediated the inhibitory effect of IC on B cell IL-6 production.

IC inhibits LPS-triggered NF-κB activation via FcγRIIb. It is reported that TLR4 ligation activates the MAPK and NF-κB pathways, resulting in the production of pro-inflammatory cytokines (18). As illustrated in Fig. 2A, LPS-induced NF-κB activation (demonstrated by the phosphorylation of IKKα/β, IκBα and p65) was inhibited in IC-treated B cells, compared with B cells treated with LPS alone. By contrast, phosphorylation of ERK, JNK and p38 was not affected by IC treatment (Fig. 2B), indicating that the MAPK pathway is not involved in the IC-mediated inhibition of IL-6 production in B cells. Pretreatment with the NF-κB inhibitor PDTC significantly blocked IL-6 production in LPS-stimulated B cells (Fig. 2C), suggesting that NF-κB activation is necessary for B cells to produce high levels of IL-6. Furthermore, LPS activated-B cells deficient in FcγRIIb displayed similar levels of p-IκBα and p-p65 with or without IC treatment (Fig. 2D), suggesting that FcγRIIb mediates the inhibitory action of IC on LPS-triggered NF-κB activation.

Lyn downregulates LPS-triggered IL-6 secretion by inhibiting NF-κB pathway activation. In order to explore which signal molecule may mediate the function of IC in inhibiting TLR4-mediated NF-κB activation, the activation status of various tyrosine kinases (Lyn, Btk and Syk) was examined in IC-stimulated B cells. Btk and Syk exhibited a similar activation status in B cells with or without IC stimulation, while phosphorylation of Lyn, one of the Src family members, was markedly increased in wild-type B cells following IC stimulation (Fig. 3A). However, phosphorylation of Lyn was not altered

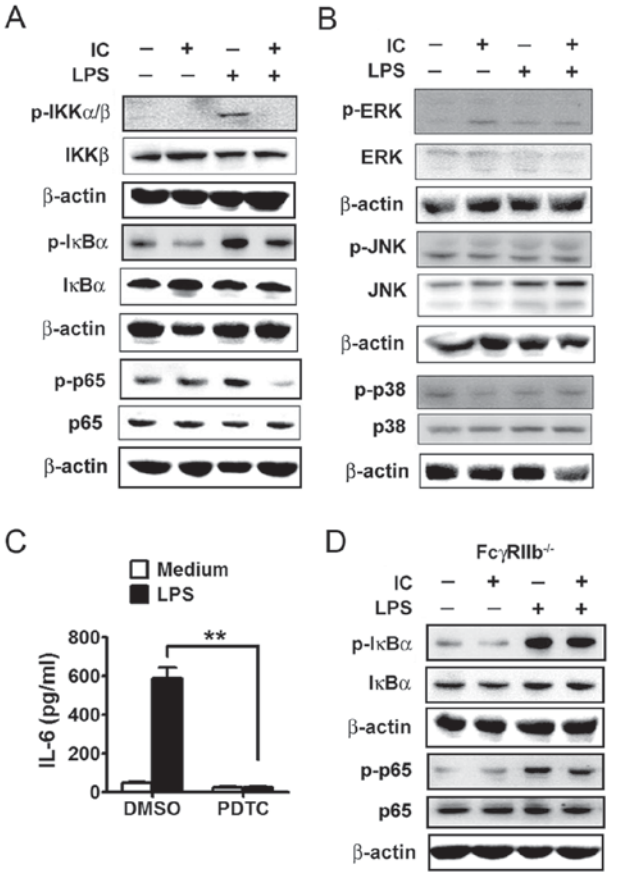

Figure 2. IC attenuates NF-κB activation in LPS-stimulated B cells in an FcγRIIb-dependent manner. (A) Western blot analysis of NF-κB pathway molecules and (B) MAPK pathway molecules in lysates of splenic B cells stimulated for 30 min with IC, LPS or LPS plus IC. (C) IL-6 production was measured in the supernatants of splenic B cells pretreated with 100 nM NF-κB inhibitor PDTC or DMSO vehicle control for 1 h, then stimulated with LPS for 24 h. (D) Western blot analysis of IκBα and p65 phosphorylation in lysates of FcγIIb$^{-/-}$ splenic B cells stimulated for 30 min with IC, LPS or LPS plus IC. β-actin was used as a loading control. Data represent the mean ± standard deviation of three independent experiments. **P<0.01, with comparisons indicated by lines. IC, immune complex; NF, nuclear factor; LPS, lipopolysaccharide; FcγIIb, Fc γ receptor IIb; MAPK, mitogen-activated protein kinase; IL, interleukin; PDTC, ammonium pyrrolidinedithiocarbamate; DMSO, dimethyl sulfoxide; IκB, NF-κB inhibitor; p65, RELA proto-oncogene; p, phosphorylated; IKK, inhibitor of NF-κB kinase subunit; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p38, MAPK14.

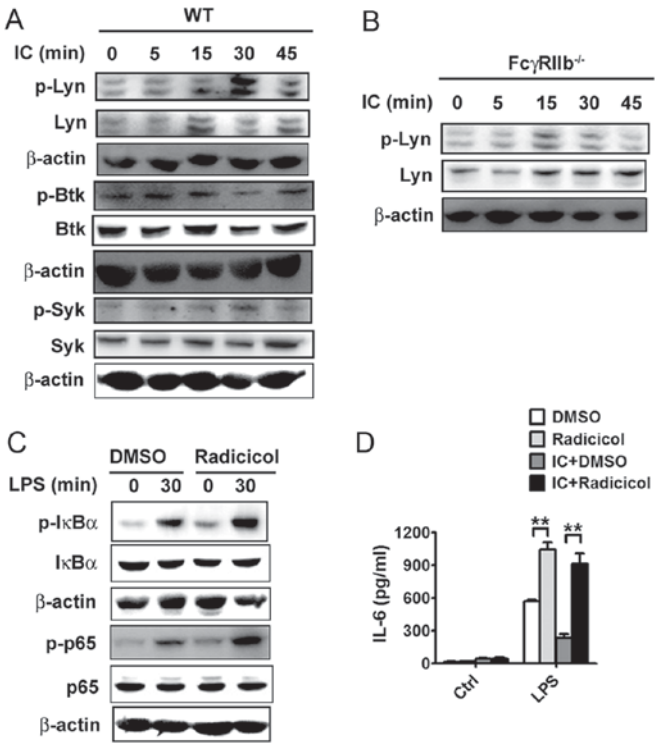

Figure 3. IC negatively regulates LPS-induced IL-6 production by enhancing activation of Lyn. (A) Western blot analysis of phosphorylated molecules in lysates of WT splenic B cells stimulated for the indicated times with IC. (B) Western blot analysis of Lyn phosphorylation in lysates of FcγIIb$^{-/-}$ splenic B cells stimulated for the indicated times with IC. (C) Western blot analysis of IκBα and p65 phosphorylation in lysates of splenic B cells pretreated for 1 h with 1 nM radicicol (an inhibitor of p-Lyn) or DMSO vehicle control, followed by LPS stimulation. β-actin was used as a loading control. (D) IL-6 production was measured in the supernatants of splenic B cells pretreated with 1 nM radicicol for 1 h, then stimulated with LPS or/and IC for 24 h. Data represent the mean ± standard deviation of three independent experiments. **P<0.01, with comparisons indicated by lines. IC, immune complex; LPS, lipopolysaccharide; IL, interleukin; Lyn, LYN proto-oncogene; WT, wild-type; FcγIIb, Fc γ receptor IIb; IκB, NF-κB inhibitor; p65, RELA proto-oncogene; DMSO, dimethyl sulfoxide; p, phosphorylated; Btk, Bruton tyrosine kinase; Syk, spleen associated tyrosine kinase.

by IC stimulation in B cells derived from FcγRIIb$^{-/-}$ mice (Fig. 3B), suggesting that IC-induced Lyn activation is FcγRIIb dependent. To explore the role of Lyn in the LPS-induced activation of the NF-κB pathway, B cells were pretreated with radicicol, an inhibitor of p-Lyn, and then stimulated with LPS. As illustrated in Fig. 3C, radicicol enhanced the LPS-induced NF-κB activation, as measured by phosphorylation of IκBα and p65. In addition, the inhibitory effect of IC on IL-6 production in LPS-activated B cells was abrogated following radicicol treatment (Fig. 3D). These results demonstrated that IC inhibits LPS-triggered IL-6 production in B cells by enhancing Lyn activation and subsequently suppressing the NF-κB pathway.

IC protects mice from endotoxic shock in an FcγRIIb-dependent manner. As demonstrated in Fig. 1, IC suppressed IL-6 secretion in LPS-activated B cells *in vitro*. Therefore, the hypothesis that IC may exhibit a protective effect on LPS-induced endotoxic shock was tested *in vivo*. As illustrated in Fig. 4A, WT mice died within 2 days following LPS intraperitoneal administration. However, only ~30% of IC-pretreated WT mice died within 3 days, suggesting that pretreatment of IC protected the mice from endotoxic shock (Fig. 4A). When FcγIIb$^{-/-}$ mice were tested, IC pretreatment did not protect the mice from endotoxic shock and the mice died within 3 days following administration of LPS (Fig. 4A). Similar to results obtained with B cells *in vitro*, IC-pretreatment resulted in decreased IL-6 expression levels in the sera (Fig. 4B) and the splenic B cells (Fig. 4C) of the LPS-treated mice, compared with mice treated with LPS alone. By contrast, IL-6 expression levels in the sera and the splenic B cells of LPS-treated FcγIIb$^{-/-}$ mice remained unchanged by IC pretreatment (Fig. 4B and C). Taken together, these results suggested that IC exhibited a protective effect on LPS-induced endotoxic shock via FcγIIb *in vivo*.

Discussion

TLRs serve a crucial role in allowing immune cells, such as DCs and macrophages, to recognize microbial pathogens and

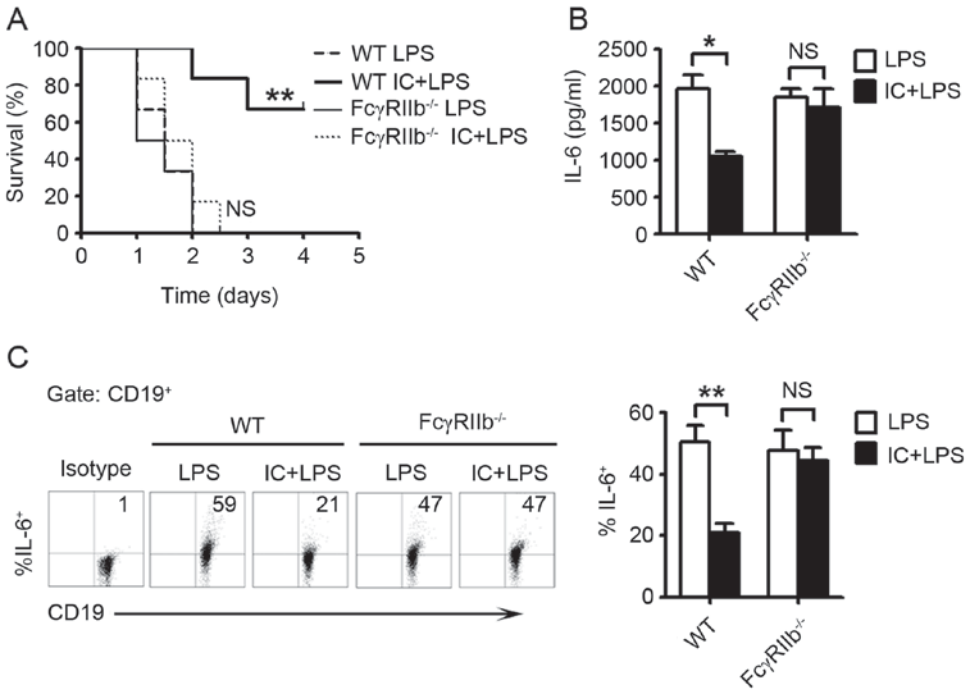

Figure 4. Protection of mice from lethal endotoxic shock by IC pretreatment is mediated by FcγRIIb. WT and FcγIIb$^{-/-}$ mice were injected intraperitoneally with IC 24 h prior to administration with 20 mg/kg of LPS. (A) Survival of the treated mice was observed for the subsequent 4 days (n=6 per group). **P<0.01 vs. WT LPS group; NS, not significant vs. FcγIIb$^{-/-}$ LPS group. (B) Sera were collected from mice at 2 h following LPS administration and IL-6 levels were measured. (C) Single cell suspension from spleens were prepared at 2 h following LPS administration and IL-6 levels were measured by flow cytometry. Representative plots and quantification for the % of CD19^{+}/IL-6^{+} B cells from total CD19^{+} B cells. Data are presented as the mean ± standard deviation (n=3 mice per group). *P<0.05 and **P<0.01, with comparisons indicated by lines. IC, immune complex; FcγIIb, Fc γ receptor IIb; WT, wild-type; LPS, lipopolysaccharide; IL, interleukin; NS, not significant.

initiate protective responses. However, TLRs have also been reported to take part in the development of several inflammatory and autoimmune diseases (1). FcγRIIb has been reported to be important in regulating Abs generation. A recent study has highlighted new functions for FcγRIIb, such as inhibiting the innate immune system via downregulating TLR signaling (19).

Antigen-presenting cells, including DCs, B cells and macrophages, co-express TLRs and FcγRIIb (7,13). Previous studies indicate that DCs from patients with rheumatoid arthritis express high levels of FcγRIIb and that these FcγRIIb-expressing DCs inhibit TLR4-triggered production of TNF-α and IL-12p70 following IC stimulation (10). Blocking FcγRIIb with specific Abs fully abrogated the inhibitory role of IC on TLR4-triggered TNF-α production, suggesting that FcγRIIb mediates the inhibitory role of IC on TLR4 signaling in DCs (10). IC/Ig has also been reported to negatively regulate TLR4-triggered IL-6 and TNF-α production through FcγRIIb in macrophages (11). In the present study, IC/Ig was demonstrated to suppress TLR4-triggered IL-6 secretion, but not TNF-α production, in B cells. A previous study has reported that LPS-triggered IL-6 release from RAW264.7 macrophages is inhibited by 16α, 17α-epoxypregnenolone-20-oxime (EPREGO), but EPREGO does not affect TNF-α secretion (20). Therefore, it is possible that LPS may induce activation of different signaling pathways for different cytokine secretion and the signaling pathways responsible for IL-6 secretion, but for TNF-α secretion, are inhibited by IC. In macrophages, prostaglandin E2 (PGE2) has been reported to be responsible for the inhibitory effect of IC on TLR4 signaling (11). When testing whether PGE2 is involved in IC function in B cells, results demonstrated that IC induced PGE2 production in an FcγRIIB-independent manner and PGE2 was not responsible for the IC-mediated inhibitory effect in B cells (data not shown). Thus, different mechanisms may account for the inhibitory effect of IC on TLR4 pathway in different cell types.

The mechanisms underlying the FcγRIIb-mediated inhibition of TLR4 have not yet been fully identified (10). It has been reported that CD11b inhibits TLR4 pathway activation by activating Src and Syk tyrosine kinases (21). In the present study, various tyrosine kinases were screened and Lyn, a member of the Src family of cytoplasmic tyrosine kinases, was demonstrated to be involved. Inhibiting Lyn resulted in an increase of TLR4-mediated NF-κB activation and IL-6 production from B cells *in vitro*. Lyn has been reported to phosphorylate adaptor molecules, such as Dok proteins, which sustain SHIP-1 activation and inhibit NF-κB pathway activation (22,23). Thus, Dok proteins may be involved in the cross-talk between FcγRIIb and TLR4. It has also been reported that the tyrosine kinase Syk induces MyD88 and TIR-domain-containing adapter inducing interferon-β (TRIF) activation, resulting in their degradation by the E3 ubiquitin ligase Cbl-b, and subsequent inhibition of TLR signaling in innate immune responses (21). These findings raise the possibility that FcγRIIb may inhibit TLR4-mediated signaling via Lyn activation and MyD88 and TRIF degradation. Further studies are required to fully elucidate the exact mechanism by which Lyn activation inhibits the TLR4-triggered NF-κB pathway.

To date, inhibition of TLR4-induced pro-inflammatory cytokine production by IC has been reported *in vitro* for various immunocytes, such as DCs and macrophages (10,11). The present study demonstrated that B cells pretreated with IC exhibited decreased IL-6 production following LPS activation both *in vitro* and *in vivo*, and IC protected mice from LPS-induced endotoxic shock via FcγIIb. Because overproduction of pro-inflammatory cytokines induces acute endotoxic shock and chronic pathological inflammation (24), the present study, together with others, may provide novel therapeutic approaches for treating TLR4-mediated pathological inflammation. Furthermore, IC pretreatment decreased TNF-α expression levels in the sera, of mice but not in the splenic B cells (data not shown), suggesting that B cells are not responsible for the decreased TNF-α levels in sera. Death caused by LPS challenge in mice is more likely due to the high levels of TNF-α. Macrophages and other myeloid cells are the major source of inflammatory cytokines during sepsis. Thus, macrophages or other myeloid cells may serve more important roles than B cells in the therapeutic effect of IC.

Taken together, the present results demonstrated that IC negatively regulated TLR4 signaling in B cells via FcγIIb and Lyn activation. These findings may add insights to the mechanism by which FcγIIb and TLR4 cross-talk to prohibit damage to host tissues from exaggerated immune responses, such as in sepsis.

Acknowledgements

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