

# Immune complex negatively regulates toll-like receptor 3-triggered tumour necrosis factor $\alpha$ production in B cells

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## Abstract

*Inappropriate activation of toll-like receptor 3 (TLR3) has been implicated in the pathogenesis of autoimmune diseases, so the negative regulation of TLR3-triggered immune response has received increasing attention. Nonpathogenic immune complex (IC) has been used as treatment for many inflammatory and autoimmune diseases. However, the role of IC in the regulation of TLR3-triggered immune responses and the underlying mechanisms need to be investigated. In this study we demonstrate that IC or intravenous immunoglobulin (Ig) stimulation of B cells attenuates polyinosinic:polycytidylic acid (poly I:C)-induced CD40 expression; IC, but not Ig, can significantly inhibit poly I:C-induced pro-inflammatory tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) production by B cells. Moreover, IC/Ig stimulation does not alter the expression of TLR3 in B cells. Further experiments suggest that receptor for the Fc portion of IgG1b (Fc $\gamma$ RIIb) is involved in the suppressive effect of IC on TLR3-mediated TNF- $\alpha$  production, but not CD40 expression. Thus, we provide a new means of negative regulation of TLR3-triggered immune responses in B cells via Fc $\gamma$ RIIb, and we provide a new mechanistic explanation of the therapeutic effect of nonpathogenic IC on inflammatory or autoimmune diseases.*

**Key words:** B cells, Fc $\gamma$ RIIb, immune complex, Toll-like receptor.

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## Introduction

Toll-like receptors (TLRs) are pivotal components of the innate immune response, which is responsible for discriminating invading microbial pathogens [1, 2]. Toll-like receptors also initiate adaptive immune responses and lead to amplified immune response against microbial pathogens by activating T and B cells [3-5]. For example, most TLR ligands, including viral double-stranded RNA (dsRNA), activate B cells. This is characterised by the up-regulation of MHC class II, induction of co-stimulatory molecules, and secretion of pro-inflammatory cytokines, which can presumably increase their potential activity for antigen presentation [6, 7].

But excessive responses to TLR agonists may induce immunopathological conditions, such as autoimmune diseases [8-11]. For example, expression of TLR3 was significantly higher in rheumatoid arthritis (RA) synovial fibroblasts compared with healthy controls or patients with non-inflammatory arthritis. Toll-like receptor 3 activation

increased disease severity in the rat pristane-induced arthritis and rat collagen-induced arthritis (CIA) models, and small interfering RNA targeting TLR3 *in vivo* reduced disease severity [12]. Treatment of pristane-induced arthritis in rats with a microRNA-26a decreased TLR3 expression and disease symptoms, confirming a role for TLR3 in this model [13]. Therefore, inhibition of TLR3 activation may have therapeutic benefit in such autoimmune diseases.

Receptors for the Fc portion of IgG (Fc $\gamma$ Rs) are important in both promoting and regulating the immune and inflammatory response to immune complex (IC) [14, 15]. Most Fc $\gamma$ Rs are activating receptors and Fc $\gamma$ RIIb is the only Fc $\gamma$ R that has an inhibitory function. Fc $\gamma$ RIIb is the only Fc $\gamma$ R expressed by B cells, and engagement of Fc $\gamma$ -RIIb can negatively regulate B cell activation [16, 17]. In accordance with its inhibitory function, Fc $\gamma$ RIIb deficiency increases disease severity in CIA and Fc $\gamma$ RIIb B cell transgenic mice show a reduction in disease severity and in collagen-specific IgG titres [18]. Interaction between

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nonpathogenic soluble IC and Fc $\gamma$ RIIb has been demonstrated to be remarkably effective in the treatment of many autoimmune and inflammatory diseases [19-22]. However, the role of soluble IC and Fc $\gamma$ RIIb in the regulation of TLR3-mediated activation of B cells and their influence on the expression of TLR3 remain to be further identified.

In this study, we demonstrated that soluble IC (composed of OVA Ag and anti-OVA mAb) can negatively regulate TLR3-triggered activation in B cells. Most notably, our experiments demonstrate that Fc $\gamma$ RIIb is responsible for the suppressive effect of IC on TLR3-mediated TNF- $\alpha$  production in B cells. Thus, we provide a new means of negative regulation of TLR3-triggered immune responses in B cells via Fc $\gamma$ RIIb.

## Material and methods

### Mice

C57BL/6J mice and Fc $\gamma$ IIb-deficient mice were obtained from the Experimental Animal Centre of Yangzhou University (Jiangsu, China) and Jackson Laboratory (Bar Harbor, ME, USA), respectively, and used at the age of 5-6 weeks. Animal experiments were performed in 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). The study was approved by the Ethics Committee of Yangzhou University.

### Preparation of immune complex

Soluble IC was prepared as previously described [22]. Briefly, OVA stock (Sigma-Aldrich, St. Louis, MO, USA) was prepared to 10 mg/ml using PBS, and then 50  $\mu$ g of OVA was incubated with 500  $\mu$ g mouse-derived anti-OVA monoclonal antibody (mAb; IgG1, Sigma-Aldrich) at 37°C for 1 h to obtain anti-OVA IC. IC (OVA/anti-OVA mAb) was used at a final concentration of 10  $\mu$ g/ml OVA plus 100  $\mu$ g/ml anti-OVA mAb. Endotoxin contamination in OVA and anti-OVA reagent was measured using Limulus amoebocyte lysate (ZhanJiang A&C Biological, Shanghai, China), and the level of endotoxin in 10  $\mu$ g of OVA plus 100  $\mu$ g of anti-OVA mAb was lower than 0.03 EU.

### Isolation of mouse CD19<sup>+</sup> B cells

Spleen-derived B cells or Fc $\gamma$ IIb<sup>-/-</sup> B cells were enriched using CD19-conjugated microbeads as recommended by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated mouse CD19 cells was > 96%, as analysed by flow cytometry. The isolated B cells were cultured in RPMI1640 medium supplemented with 10% FBS, 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Gibco RBL, Grand Island, NY, USA).

### CD19<sup>+</sup> B cells stimulation

Purified splenic B cells or Fc $\gamma$ IIb<sup>-/-</sup> B cells were plated in 96-well flat-bottom plates at a density of  $2.0 \times 10^5$  per well, and then stimulated with IC, Ig (anti-OVA mAb, 100  $\mu$ g/ml), poly I:C (10  $\mu$ g/ml), poly I:C plus Ig, or poly I:C plus IC. Poly I:C were purchased from InvivoGen (San Diego, CA, USA).

### Measurement of interleukin 6 and tumour necrosis factor $\alpha$

At 24 h, B cell culture supernatants were collected and measured for IL-6 and TNF- $\alpha$  by ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

### Flow cytometric analysis

At 24 h, B cells were washed with ice-cold PBS buffer and then stained with antibodies to CD19-APC, CD40-PE, CD86-PE, MHC II (Ia<sup>b</sup>)-PE (BD Biosciences), or TLR3-PE (BioLegend, San Diego, CA, USA) for 30 min at 4°C. For intracellular TLR3 staining, cells were stained with antibody to CD19-APC and then fixed and permeabilised with a Cytotfix/Cytoperm kit according to BD Biosciences. Permeabilised cells were incubated with PE-conjugated anti-TLR3 mAb. Data were collected using FACS Calibur (BD Biosciences) and analysed by FlowJo software.

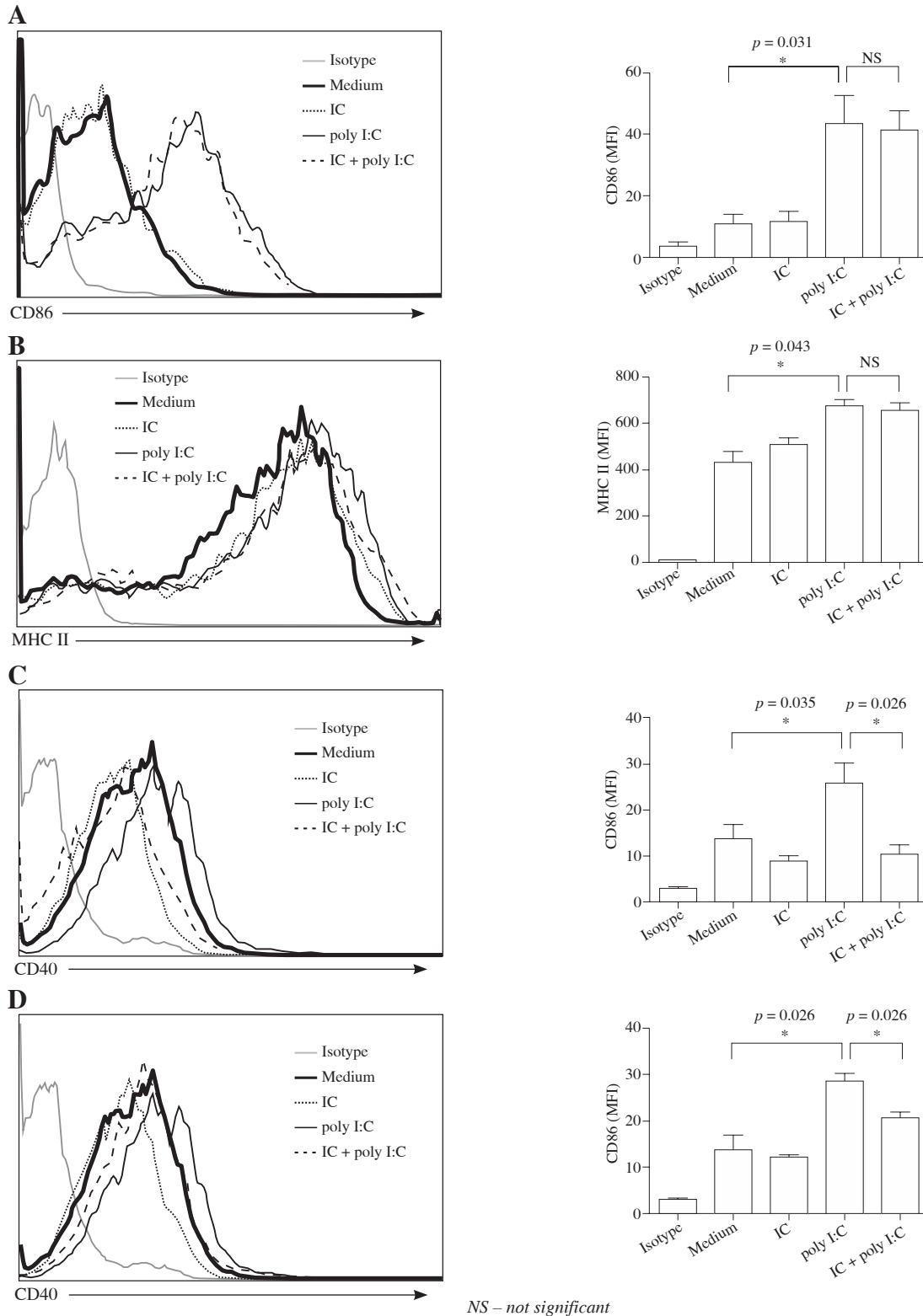
### Statistics

Data are presented as mean  $\pm$  SD. Statistical comparisons were made using GraphPad PRISM software. A two-tailed Student *t* test was applied. *P* < 0.05 was considered to indicate statistically significant differences.

## Results

### Immune complex/Ig stimulation inhibits TLR3-mediated CD40 expression on B cells

First, we observed the effect of IC on poly I:C (TLR3 agonist)-induced membrane molecule expression of B cells. We found that B cells did not display an altered expression of CD86 and MHC II upon stimulation with IC or without IC in the presence of poly I:C (Fig. 1A, B). Interestingly, the combination of IC and poly I:C resulted in a decrease in the expression of CD40 by B cells, as compared with that observed in B cells stimulated with poly I:C alone (Fig. 1C). Furthermore, we observed the effect of free intravenous immunoglobulin (Ig, anti-OVA mAb) on poly I:C-induced CD40 expression by B cells. We found that Ig could also reduce poly I:C-induced CD40 expression (Fig. 1D). Taken together, these results suggested that IC/Ig stimulation of B cells can inhibit poly I:C-induced CD40 expression.



**Fig. 1.** IC/Ig inhibits poly I:C-induced CD40 expression of splenic B cells. Freshly purified splenic B cells were stimulated for 24 h with IC, poly I:C, or poly I:C plus IC. Expression of CD86 (A), MHC II (B), and CD40 (C) on CD19<sup>+</sup> B cells was detected by flow cytometry. D) Freshly purified splenic B cells were stimulated for 24 h with Ig, poly I:C, or poly I:C plus Ig. Expression of CD40 on CD19<sup>+</sup> B cells was detected by flow cytometry. Left panels, histograms represent one of three independent experiments. Right panels, numbers indicate mean fluorescence intensity (MFI) of test samples. Data are presented as the means  $\pm$  SD, \* $p < 0.05$

### Immune complex stimulation inhibits TLR3-mediated TNF- $\alpha$ secretion of B cells

To further determine whether the poly I:C-induced production of pro-inflammatory cytokines in B cells is affected by IC, we stimulated B cells with a combination of poly I:C and IC and compared them with B cells stimulated with poly I:C alone. We found that both unstimulated and stimulated B cells failed to release any detectable IL-12p70, IP-10, IFN-g, or IL-17A (data not shown). Poly I:C alone could significantly induce the production of IL-6 and TNF- $\alpha$  from B cells (Fig. 2A, B). There was no significant difference in production of IL-6 by B cell stimulation with or without IC in the presence of poly I:C (Fig. 2A), suggesting that IC stimulation of B cells cannot inhibit poly I:C-induced IL-6 production. However, IC clearly inhibited the production of TNF- $\alpha$  from poly I:C-activated B cells (Fig. 2B). Moreover, we found that Ig could not reduce poly I:C-induced TNF- $\alpha$  production (Fig. 2C). Taken together, these results suggested that treatment of B cells with IC can significantly suppress poly I:C-induced TNF- $\alpha$  production.

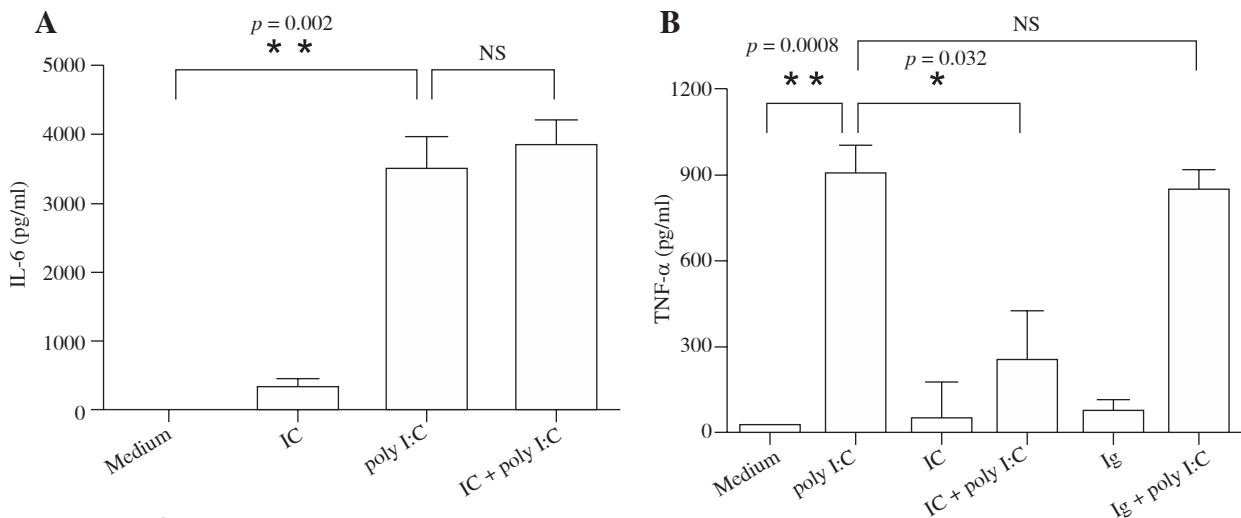
### Immune complex/Ig stimulation has no effect on TLR3 expression in B cells

Then we wondered whether IC/Ig negatively regulates TLR3-mediated immune response by down-regulation of TLR3 expression in B cells. We stimulated B cells with IC or Ig and then detected the expression of TLR3 in B cells.

As shown in Figure 3, both cell surface and intracellular TLR3 expression in B cells remained unchanged after IC or Ig stimulation. These data suggest that IC/Ig-mediated suppression of TLR3-mediated immune response in B cells is not due to down-regulation of TLR3 expression.

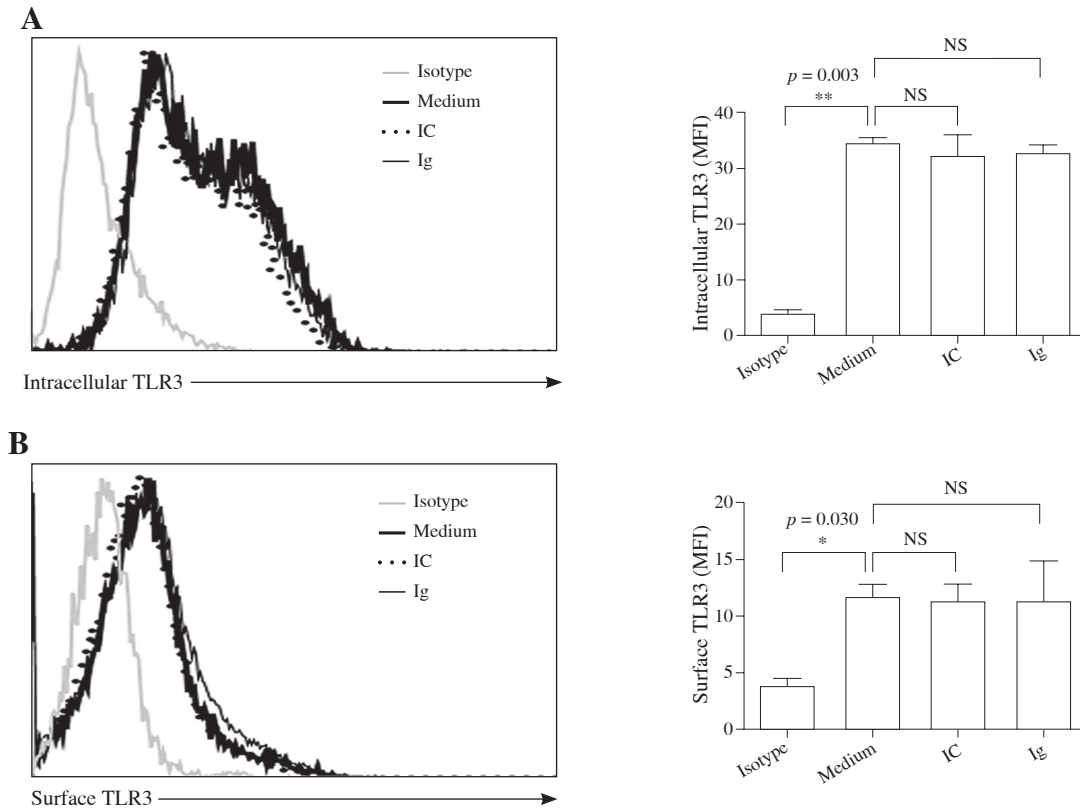
### Fc $\gamma$ RIIb is responsible for the suppressive effect of immune complex on poly I:C-triggered TNF- $\alpha$ production

It has been well-established that Fc $\gamma$ RIIb, the unique inhibitory Fc $\gamma$ R expressed on B cells, plays a critical role in negative regulation of immune responses, and IC can bind to Fc $\gamma$ RIIb to exert its biological activities [17, 22]. We then wondered whether Fc $\gamma$ RIIb was responsible for IC/Ig-mediated suppression of TLR3 signalling in B cells. So, we analysed the CD40 expression and TNF- $\alpha$  secretion by poly I:C-stimulated Fc $\gamma$ RIIb<sup>-/-</sup> B cells treated with or without IC or Ig. As shown in Fig. 4A, B, IC/Ig stimulation of Fc $\gamma$ RIIb<sup>-/-</sup> B cells significantly suppressed poly I:C-induced CD40 expression, similar to that of wild type B cells (Fig. 1C, D). These results suggest that Fc $\gamma$ RIIb does not play an important role in the IC/Ig-mediated inhibition of CD40 expression. Interestingly, IC treatment lost its inhibitory effect poly I:C-induced TNF- $\alpha$  secretion when Fc $\gamma$ RIIb<sup>-/-</sup> B cells were used (Fig. 4C), indicating that Fc $\gamma$ RIIb is responsible for the suppressive effect of IC on TLR3-mediated TNF- $\alpha$  secretion.



NS – not significant

**Fig. 2.** IC inhibits poly I:C-induced TNF- $\alpha$  secretion from splenic B cells. A) Freshly purified splenic B cells were stimulated for 24 h with IC, poly I:C, or poly I:C plus IC. IL-6 in the supernatants was measured by ELISA. B) Freshly purified splenic B cells were stimulated for 24 h with IC/Ig, poly I:C, or poly I:C plus IC/Ig. Culture supernatants were collected and assayed for TNF- $\alpha$  production by ELISA. Results are representative of three independent experiments. Data are expressed as mean  $\pm$  SD, \* $p$  < 0.05; \*\* $p$  < 0.01



NS – not significant

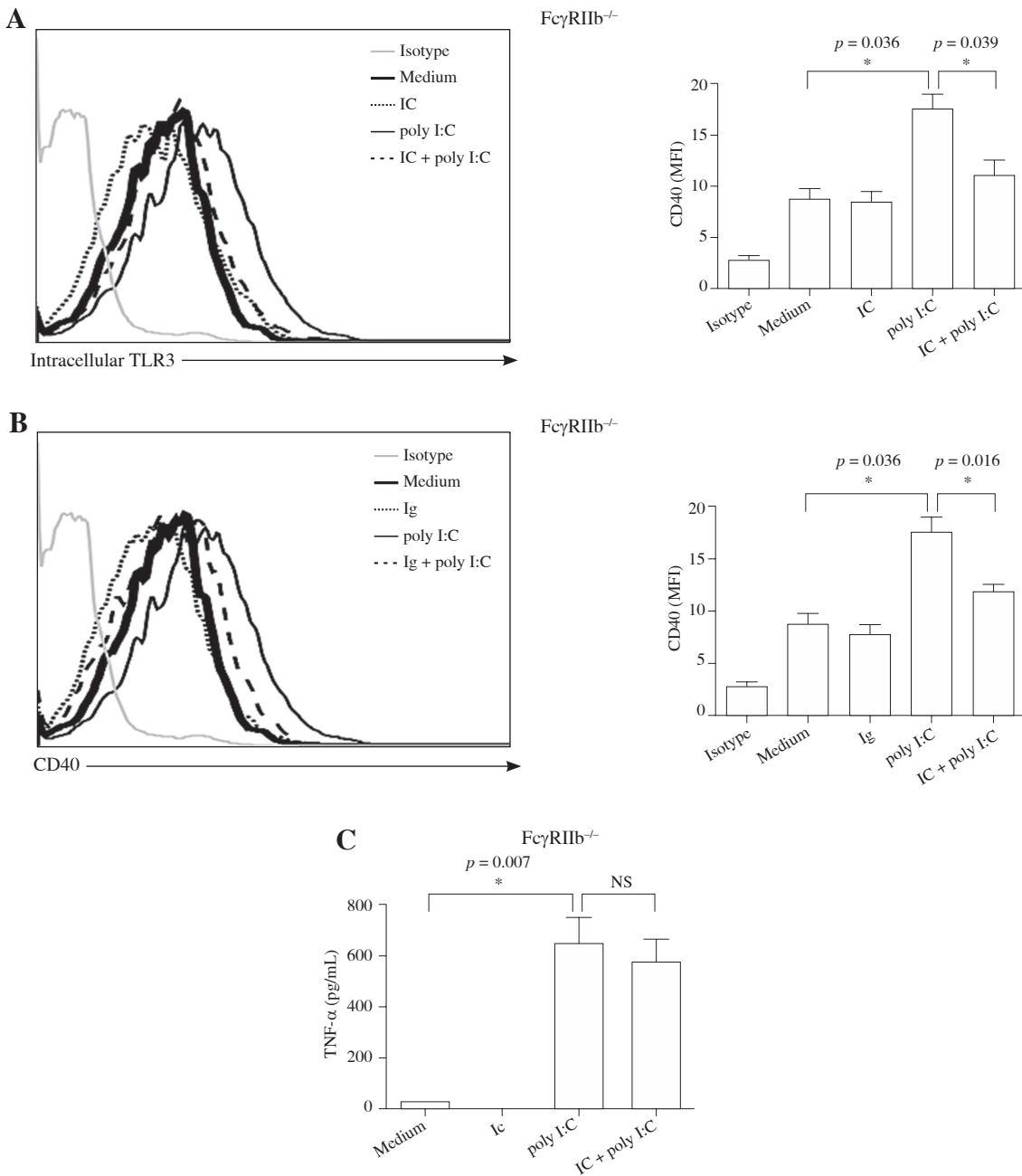
**Fig. 3.** IC/Ig has no effect on TLR3 expression in splenic B cells. Freshly purified splenic B cells were stimulated for 24 h with IC or Ig. A) TLR3 in CD19<sup>+</sup> B cells was detected by flow cytometry with intracellular staining. B) Expression of TLR3 on CD19<sup>+</sup> B cells was detected by flow cytometry. Left panels, histograms represent one of three independent experiments. Right panels, numbers indicate MFI of test samples. Data are presented as the means  $\pm$  SD, \* $p < 0.05$ ; \*\* $p < 0.01$

## Discussion

Toll-like receptor 3 has been identified as an endosomal or cell-surface receptor that recognises and mediates responses to viral dsRNA or poly I:C, an analogue of viral dsRNA [23]. It has been demonstrated that B cells express TLR3, and that they can be activated via TLR3 ligands [24]. This was confirmed by our finding that poly I:C induced up-regulation of MHC II, CD86, and CD40, and production of TNF- $\alpha$  and IL-6 from B cells.

Intravenous Ig has been used in an increasing number of immune-mediated conditions, including acute and chronic/relapsing autoimmune diseases and systemic inflammatory disorders [25, 26]. Recent data indicate that IgG reactive with soluble antigens (soluble IC) can mimic the therapeutic effects of IV Ig in treating some autoimmune diseases [19]. It was previously reported that IV Ig-induced down-regulation of MHC II and CD86 on dendritic cells resulted in the inhibition of autoreactive and

alloreactive T-cell activation and proliferation [27]. However, our results showed that the expression of MHC II and CD86 was not modified in poly I:C-activated B cells in the presence of IC. These differences could be due to differences related to the cell types involved. Interestingly, we showed that IC/Ig inhibited CD40 upregulation on poly I:C-activated B cells. Evidence from several sources supports the key role of CD40L/CD40 interactions in initiating and/or propagating chronic inflammatory autoimmune diseases such as rheumatoid arthritis (RA) [28]. The administration of antagonistic anti-CD40L mAbs significantly ameliorates collagen-induced arthritis [29], indicating that disruption of this pathway could be a beneficial treatment in RA. Furthermore, highly elevated levels of tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) and TNF- $\alpha$  polymorphisms have been associated with various disease states, such as RA [10, 30]. Biological agents targeting TNF- $\alpha$  show significant clinical benefit and halt radiographic progression, confirming the key role of TNF- $\alpha$  in RA [30, 31].



NS – not significant

**Fig. 4.** IC inhibits TNF- $\alpha$  production from poly I:C-stimulated B cells via inhibitory Fc $\gamma$ IIb. A, B) Freshly purified splenic Fc $\gamma$ IIb $^{-/-}$  B cells were stimulated with IC/Ig, poly I:C, or poly I:C plus IC/Ig. After 24 h, cells were collected and analysed by flow cytometry for CD40 expression. Left panels, histograms represent one of three independent experiments. Right panels, numbers indicate MFI of test samples. Data are presented as the means  $\pm$  SD, \* $p < 0.05$ . C) Freshly purified splenic Fc $\gamma$ IIb $^{-/-}$  B cells were stimulated with IC, poly I:C, or poly I:C plus IC. Culture supernatants were collected at 24 h and assayed for TNF- $\alpha$  by ELISA. Data are presented as the means  $\pm$  SD. \*\* $p < 0.01$

In the present study, we demonstrate that IC decreases the expression of TNF- $\alpha$  and CD40 from poly I:C-activated B cells. In consideration of the above findings, the present study may provide an additional mechanistic explanation

of the therapeutic effect of IC on autoimmune diseases such as RA.

In addition, we found that IC inhibited TLR3-mediated TNF- $\alpha$  production but did not inhibit another pro-in-

flammatory cytokine, IL-6. We speculated that different pro-inflammatory cytokines secretion, such as IL-6 and TNF- $\alpha$ , may be regulated by different signalling pathways in B cells. It is possible that IC might inhibit the signalling pathways responsible for TNF- $\alpha$  production but not the signalling pathways responsible for IL-6 production.

The precise mechanism by which IC/Ig exerts its action on B cells is not yet clear. We tried to investigate whether IC/Ig inhibits poly I:C-induced immune response by down-regulation of TLR3 expression in B cells. However, we did not observe the altered expression of TLR3 in B cells or poly I:C-activated B cells by IC/Ig (data not shown). Next, we investigated the role of Fc $\gamma$ RIIb in IC-mediated inhibition of B cell activation. As expected, we found that ICs lost their inhibitory effect on poly I:C-induced expression of TNF- $\alpha$  when Fc $\gamma$ RIIb<sup>-/-</sup> B cells were used, suggesting that IC negatively regulates poly I:C-induced TNF- $\alpha$  secretion from B cells through its interaction with Fc $\gamma$ RIIb. However, we found that Fc $\gamma$ RIIb was not responsible for the inhibitory effect of IC/Ig on CD40 expression. A recent study demonstrated that IV Ig can exert its action on B cells through binding of its sialic-acid-bearing glycans to the negative regulator CD22 [32]. So, we speculate that other receptor(s) such as CD22 might be involved in IC-induced inhibition of TLR3 signalling, which may also account for the reason why IC and Ig exert different regulatory roles in poly I:C-activated B cells. However, which receptor(s) is (are) involved needs to be further investigated.

In summary, in this study we have provided novel insights for the mechanisms underlying the negative regulation of TLR3-triggered immune responses in B cells by IC. It also contributes to a better understand of the mechanisms of clinical administration of IV Ig for prevention of serious inflammation or treatment of autoimmune diseases.

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*The authors declare that they have no conflicting interests.*

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