

TLR4 supports the expansion of FasL⁺ CD5⁺ CD1d^{hi} regulatory B cells, which decreases in contact hypersensitivity



Keng Wang^{a,b,c,1}, Lei Tao^{a,b,1}, Jianbing Su^{a,b}, Yueyang Zhang^{a,b}, Binhua Zou^{a,b}, Yiyuan Wang^{a,b}, Min Zou^b, Nana Chen^{a,b}, Linsheng Lei^{a,b}, Xiaojuan Li^{a,b,*}

^a Laboratory of Anti-inflammatory Immunomodulatory Pharmacology, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, PR China

^b Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, PR China

^c Department of Clinical Pharmacy, The Affiliated Nanhai Hospital of Southern Medical University, Foshan 528200, PR China

ARTICLE INFO

Keywords:

Regulatory B cell

FasL

TLR4

TLR9

Anti-CD40

Contact hypersensitivity

ABSTRACT

Certain B cells termed as “regulatory B cells” (Bregs) can suppress the ongoing immune responses and a splenic CD5⁺ CD1d^{hi} Breg subset identified earlier was shown to exert its regulatory functions through secretion of IL-10. Though FasL expression is an alternative mechanism of immune suppression used by B cells, little is known about the FasL expressing CD5⁺ CD1d^{hi} Bregs. In this study, we isolated splenocytes or splenic CD19⁺ B cells and compared the efficiency of toll-like receptor (TLR)4 ligand (lipopolysaccharide) with TLR9 ligand (CpG), anti-CD40 and TLR9 ligand (CpG) plus anti-CD40 on the FasL expression of splenic CD5⁺ CD1d^{hi} Bregs by flow cytometry. FasL expression in CD5⁺ CD1d^{hi} B cells was rapidly increased after TLR4 ligation. Intriguingly, anti-CD40 and CpG plus anti-CD40 combinations failed to stimulate FasL expression in CD5⁺ CD1d^{hi} B cells although the IL-10 production was up-regulated in this subset. In addition, LPS and other B10-cell inducers increased the expression of surface molecules like CD86 and CD25, which are correlated to the regulatory functions of B cells. Furthermore, NF-κB and NF-AT inhibitors decreased the TLR4-activated FasL expression in CD5⁺ CD1d^{hi} B cells. Then we sorted splenic CD5⁺ CD1d^{hi} Bregs using flow cytometry and found that TLR4-activated CD5⁺ CD1d^{hi} Bregs suppressed the proliferation of CFSE-labeled CD4⁺ T cells *in vitro*, which was partly blocked by anti-FasL antibody. In oxazolone-sensitized mice having contact hypersensitivity, FasL expression in splenic CD5⁺ CD1d^{hi} B cells was decreased compared to the control group after TLR4 ligation. Our findings suggest that the regulatory function of CD5⁺ CD1d^{hi} B cells could be partly mediated by Fas-FasL pathway and this FasL expressing CD5⁺ CD1d^{hi} Bregs might participate in the regulation of inflammatory diseases.

1. Introduction

B cells produce antibodies, which are generally considered as positive regulators of immune responses. However, a suppressive role for B cells has been identified recently (Mauri and Ehrenstein, 2008). The suppressive capacity of Breg is ascribed mainly to their secretion of IL-10 (Lykken et al., 2015). In mice, two well-characterized IL-10 producing Breg subsets are identified until now (Matsushita, 2014; Yang et al., 2013): T2-MZP (Transitional 2-marginal zone precursor) B cell subsets with the phenotype of CD19⁺ CD23⁺ CD21^{hi} CD24^{hi} CD1d^{hi} (Evans et al., 2007) and “B10 cells” with the phenotype of CD19⁺ CD5⁺ CD1d^{hi} (Yanaba et al., 2008). Both of these two Breg subsets were identified from murine splenocytes. IL-10 competent CD19⁺ CD5⁺ CD1d^{hi} Bregs from wild-type (WT) mice can suppress Th1 responses and inhibit the exaggerated contact hypersensitivity

(CHS) reactions (Yanaba et al., 2008), murine lupus (Watanabe et al., 2010), DSS-induced intestinal injury (Yanaba et al., 2011) and imiquimod-induced psoriasis-like skin inflammation (Yanaba et al., 2013) in IL-10 deficient CD19^{−/−} mice. In addition, transfer of BAFF-induced IL-10 producing CD5⁺ CD1d^{hi} B cells suppressed collagen induced arthritis (Yang et al., 2010) and the transfer of myelin oligodendrocyte glycoprotein(33-35)-sensitized CD5⁺ CD1d^{hi} B10 cells into WT mice reduced the initiation of experimental autoimmune encephalomyelitis (EAE) dramatically but not the ongoing EAE disease (Matsushita et al., 2010).

Though IL-10 is pivotal for the regulatory function of CD5⁺ CD1d^{hi} B cells, in fact only a small portion of CD5⁺ CD1d^{hi} Breg subset secretes IL-10 (Yanaba et al., 2008). Interestingly, Bregs can also suppress immune responses in IL-10 independent manner (Floudas et al., 2016). One important alternative regulatory mechanism used by B cells is FasL

* Corresponding author at: Southern Medical University, 1838 Guangzhou Avenue North, Guangzhou 510515, PR China.

E-mail address: lixiaoj@smu.edu.cn (X. Li).

¹ Keng Wang and Lei Tao contributed equally to this work.

(CD178) (Lundy, 2009). After binding to Fas receptor (CD95), FasL could induce apoptosis in target cells such as CD4⁺ T cells (Lundy and Fox, 2009). Splenic B cells activated by lipopolysaccharide (LPS) expressed FasL and delayed the onset of diabetes (Tian et al., 2001). Infection with *Schistosomas* induced FasL⁺ B cells, which were enriched in CD5⁺ B cell population (Lundy and Boros, 2002). The FasL⁺CD5⁺ B cells reduced in severe collagen-induced arthritis (Lundy and Fox, 2009). Moreover, B cells from FasL deficient mice could not transfer tolerance in a skin grafting model (Minagawa et al., 2004). In summary, FasL⁺ B cells potentially played a role in down-regulating the immune responses and inflammatory immune diseases.

FasL expression was enriched in naïve CD5⁺CD1d^{hi} B cells but not CD23⁺CD21^{hi} T2-MZP B cells in murine splenocytes (Klinker et al., 2013). As previously described, CD5⁺CD1d^{hi} Bregs usually exert suppressive function after their activation. For example, adoptive transfer of CD5⁺CD1d^{hi} B cells purified from DNFB-sensitized mice altered DNFB but not oxazolone-induced CHS responses in CD19^{-/-} recipients (Yanaba et al., 2008). TLR4 ligation (by LPS) is an innate signal required for the development and function of CD5⁺CD1d^{hi} Bregs producing IL-10 (Yanaba et al., 2009), while the effects of TLR4 ligand on FasL expressing CD5⁺CD1d^{hi} Bregs are not explored until now.

At present, we firstly observed the effects of LPS (TLR4 ligand) and other B10-cell inducers on FasL expression along with IL-10, CD86 and CD25 expressions in CD5⁺CD1d^{hi} Breg population. It is interesting to find that LPS can rapidly and potently up-regulate FasL expression in CD5⁺CD1d^{hi} Breg subset, while some other B10-cell inducers such as anti-CD40, CpG plus anti-CD40 signals fail to increase the FasL expression though the IL-10 and CD86 expressions are strongly increased in this subset. FasL expression in CD5⁺CD1d^{hi} Bregs activated by TLR4 ligation could be reduced by both NF-κB and NF-AT inhibitors. Furthermore, the regulatory function of CD5⁺CD1d^{hi} Bregs activated by TLR4 ligation was down-regulated by anti-FasL treatment *in vitro* and FasL expressing CD5⁺CD1d^{hi} Bregs were decreased in mice having contact hypersensitivity. Thus this study shows that FasL could partly mediate the regulatory function of CD5⁺CD1d^{hi} Bregs and TLR4 signals are required to generate splenic FasL expressing CD5⁺CD1d^{hi} Bregs, which indicates a possible role for these IL-10-independent Bregs in inflammatory diseases.

2. Materials and methods

2.1. Mice

Female C57BL/6 WT mice were purchased from Guangdong Medical Laboratory Animal Center. All the mice were bred in a specific pathogen-free barrier facility. All the animals were used between the ages of 8 and 12 weeks. Care, use and treatment of mice were in strict accordance with the Guide for the Care and Use of Laboratory Animals. Ethical approval was given by Southern Medical University Experimental Animal Ethics Committee.

2.2. Isolation of splenic lymphocytes

Spleens were dissected and mashed through nylon cell strainer under sterile conditions. Splenic lymphocytes were collected by mouse lymphocyte separation medium (DKW33-R0100, Dakewe Biotech Company). The trapped cells mainly consist of B cells and T cells. When necessary, the residual erythrocytes were eliminated using red cell lysis buffer (420301, Biolegend). CD19⁺ B cells were purified through negative selection using a B cell isolation kit (19854, StemCell) according to the manufacturer's instructions with purity of > 95%.

2.3. Cell culture and stimulation

The splenocytes or isolated CD19⁺ B cells were cultured with LPS (10 µg/ml, *Escherichia coli* serotype O111:B4, L4391, Sigma-Aldrich),

CpG-ODN 1826 (50 nM, tlr1-1826, InvivoGen), anti-CD40 (2 µg/ml, 553721, BD Biosciences) or CpG plus anti-CD40 separately in 200 µl of complete medium in a 96-well flat bottom plate for 12 h, 24 h or 48 h for measuring surface FasL, CD86, CD25 and intracellular IL-10. For detection of intracellular cytokine IL-10 of B cell subsets, phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, P8139, Sigma-Aldrich), ionomycin (500 ng/ml, I0634, Sigma-Aldrich) and monensin (2 µM, 00-4505-51, eBioscience) were added to the culture for the terminal 5 h in addition to LPS or other B10-cell inducers. When treated with LPS in purified CD19⁺ B cells, BAY11-7082 (2 µM; 1 µM, B5556, Sigma-Aldrich) and CsA (1 µM; 0.5 µM, Sigma-Aldrich) were used as NF-κB and NF-AT inhibitor respectively and added simultaneously with LPS for 24 h to detect IL-10 and FasL expression. For the detection of intracellular cytokine IFN-γ in CD4⁺ T splenocytes in animal models, splenocytes were isolated from CHS and control mice, and stimulated with anti-CD3 and PIM (PMA, ionomycin and monensin) for 5 h.

2.4. Flow cytometric analysis

Cells were collected and washed with PBS supplemented with 0.5% (wt/vol) BSA. For the detection of cell-surface markers, cells were stained with different combinations of the following antibodies: PerCP-conjugated CD19 (551001), FITC-conjugated CD5 (553021), PE-conjugated CD1d (553846), APC-conjugated CD86 (558703), APC-conjugated CD25 (561048) and APC-conjugated CD178. For intracellular staining of IL-10 or IFN-γ, cells were fixed and permeabilized using a Cytofix/Cytoperm kit (54714, BD Biosciences) according to the manufacturer's instructions. Permeabilized cells were stained with APC-conjugated IL-10 (554468) or IFN-γ (562018). All the anti-mouse monoclonal antibodies were purchased from BD Biosciences except APC-conjugated CD178 (17-5911, eBioscience). The stained cells were acquired with a BD Canto II flow cytometer (BD Biosciences) and analyzed by FlowJo software (version 7.6, LLC, Ashland, OR, USA).

2.5. Sorting of CD5⁺CD1d^{hi} B cells

A B cell isolation kit was used to purify CD19⁺ B cells as described in 2.2. The CD19⁺ B cells were activated with LPS (10 µg/ml) in a 24 well-plate for 24 h. PE-Cy7-conjugated CD19 (552854, BD Biosciences), FITC-conjugated CD5 and PE-conjugated CD1d were used to stain the cell surface molecules, CD5⁺CD1d^{hi} and CD5⁺CD1d^{low} B cells were then sorted with a BD FACS Aria III flow cytometer (BD Biosciences) with purity of 90%–95%.

2.6. T cell proliferation assay

CD4⁺ T cells were isolated from the splenocytes of WT mice by negative selection (19812A, StemCell) according to the instructions with purity of > 90%. The purified cells were stained with 4 µM CFSE (C34554, Invitrogen) for 10 min at 37 °C. CFSE-labeled CD4⁺ T cells (1.5 × 10⁵ cells/well) were cultured in a 96 well-plate precoated with anti-CD3 (2 µg/ml, 16-0031-82, eBioscience) and anti-CD28 (2 µg/ml, 16-0281-82, eBioscience) antibodies. Sorted CD5⁺CD1d^{hi} or CD5⁺CD1d^{low} B cells after LPS stimulation were co-cultured with CFSE-labeled CD4⁺ T cells at a ratio of 1:1 in the presence or absence of anti-FasL (5 µg/ml, 16-5911-81, eBioscience) (Montandon et al., 2013) for 72 h and then stained with APC-conjugated CD4. CD4⁺ T cells were gated to analyze CFSE by flow cytometry.

2.7. Induction of contact hypersensitivity (CHS) reactions

WT mice were divided into CHS model and control groups, with 5 mice in each group. In CHS model group, mice were sensitized with 25 µl of oxazolone (100 mg/ml, E0753, Sigma-Aldrich) in 4:1 (vol/vol) acetone/olive oil applied to their shaved abdomens on days –6 and –5. Oxazolone (10 mg/ml) in 4:1 acetone/olive oil was painted on

both sides of the right ears (5 μ l on the dorsal side and 5 μ l on the ventral side) on day 0 as a challenge dose. An identical amount of the solvent was applied to the left ears. Ear thickness was measured after oxazolone challenge for 24 h, 48 h and 72 h. To detect the changes of IFN- γ produced by splenic CD4⁺ T cells and FasL expressed on CD5⁺CD1d^{hi} B cells in CHS models, after oxazolone challenge for 48 h (Day 2), splenic lymphocytes of CHS and control mice were isolated and pooled to plate in 96-well plates. IFN- γ production of CD4⁺ T cells in splenocytes of CHS and control groups after stimulation with anti-CD3 plus PIM for 5 h was measured by flow cytometry. FasL expression in CD5⁺CD1d^{hi} B cells was also detected by flow cytometry after stimulation with LPS for 24 h.

2.8. Statistical analyses

All the data were expressed as mean $\pm$ standard error of the mean (SEM). Data analysis was performed with GraphPad Prism software (version 5.0, San Diego, CA). One-way ANOVA test followed by a Tukey multiple comparison was used to compare the individual groups. Differences between the ear thickness of mice were assessed using the Student's test. $P < 0.05$ was considered statistically significant; ns, not significant. All the experiments were repeated at least twice to ensure reproducibility.

3. Results

3.1. TLR4 ligand but not all other B10-cell inducers up-regulated FasL expression in splenic CD5⁺CD1d^{hi} Bregs

TLRs are key components for immune cell activation, including TLR4 and TLR9 (Meyer-Bahlburg and Rawlings, 2008). Anti-CD40 is an activator of CD40/CD40L pathway. TLR4, TLR9 ligands and anti-CD40 are widely known as B10-cell inducers (Kalampokis et al., 2013). To evaluate the inducers on FasL expression, LPS, CpG, anti-CD40 and CpG plus anti-CD40 were used as the activators of TLR4, TLR9, CD40/CD40L, TLR9 plus CD40/CD40L pathways respectively (Blair et al., 2009; Lampropoulou et al., 2010; Lampropoulou et al., 2008; Lenert et al., 2005). We found that FasL expression was up-regulated significantly when stimulated with LPS in splenocytes. After stimulation with LPS for 12 h, the frequency of FasL⁺ in CD5⁺CD1d^{hi} Bregs was about 20% (Fig. 1A). This value increased constantly and reached about 40% at 24 h (Fig. 1B). CpG also up-regulated FasL expression in CD5⁺CD1d^{hi} Bregs, however the expression level was lower than LPS stimulation (Fig. 1A–B). On the other hand, anti-CD40 and CpG plus anti-CD40 stimulations failed to up-regulate FasL expression in CD5⁺CD1d^{hi} Bregs (Fig. 1A–B). FasL expression in CD5⁺CD1d^{hi} Bregs after LPS or CpG plus anti-CD40 stimulation in the purified CD19⁺ B cells were similar to the results observed with splenocytes (Fig. 2A–C).

3.2. TLR4 ligand and other B10-cell inducers increased the IL-10 production in CD5⁺CD1d^{hi} Bregs

IL-10 was one of major cytokines for B cells to exert their regulatory function (van der Vlugt et al., 2014). We used LPS, CpG, anti-CD40 and CpG plus anti-CD40 as B10-cell inducers to stimulate splenocytes and found that they all succeeded in inducing IL-10 production in CD5⁺CD1d^{hi} Bregs (Fig. 3A). Based on previous results, LPS and CpG plus anti-CD40 showed different effects on FasL expression, we added LPS or CpG plus anti-CD40 to detect the secretion of IL-10 in purified CD19⁺ B cells (Fig. 3B). Though CpG plus anti-CD40 failed to up-regulate FasL expression in CD5⁺CD1d^{hi} Bregs, IL-10 secretion was induced significantly (Fig. 3B). It is plausible that though both FasL and IL-10 exert regulatory functions, they may employ different signaling pathways, and could be mutually exclusive population of cells within the CD5⁺CD1d^{hi} Breg subset.

3.3. TLR4 ligand and other B10-cell inducers increased the CD86 expression in CD5⁺CD1d^{hi} Bregs

CD86 was a co-regulator along with CD80 in the induction and regulation of immune responses. Specifically, CD86 was known as a signaling pathway for activation and hence used as an activation marker for B cells (Cheng et al., 2016). CD86 was also related to the regulatory function of Bregs: B cells could induce CD4⁺CD25⁺ Tregs (regulatory T cells) via CD86-dependent mechanism and the blocking of CD86 could exacerbate EAE (Mann et al., 2007; Racke et al., 1995). In our study, we found that CD86 molecules were highly expressed in LPS-activated CD5⁺CD1d^{hi} B cells (Fig. 4A). CpG and CpG plus anti-CD40 also up-regulated CD86 expression in CD5⁺CD1d^{hi} Bregs (Fig. 4A). Individual use of anti-CD40 also increased the expression of CD86 in CD5⁺CD1d^{hi} Bregs, however the expression levels were much weaker compared to other activators ($P < 0.001$ compared to LPS group, $P < 0.001$ compared to CpG group, $P < 0.001$ compared to CpG plus anti-CD40 group).

3.4. TLR4 ligand and other B10-cell inducers activated CD25 expression in CD5⁺CD1d^{hi} Bregs

CD25 is a cell surface glycoprotein, alpha chain of interleukin-2 receptor. Similar to CD86, CD25 is related to B cell activation and highly expressed in activated B cells (Meyer-Bahlburg and Rawlings, 2008). Also, CD25 is used as one of the Breg cell surface markers. CD19⁺CD25⁺ B cells are recognized as one of the Breg subsets, which could suppress the proliferation of CD4⁺ T cells but enhance Foxp3 and CTLA-4 expressions in Tregs (de Andres et al., 2014; Kessel et al., 2012). In our studies demonstrated that LPS, CpG and CpG plus anti-CD40 except CD40 highly increased the expression of CD25 (Fig. 4B), similar to CD86 expression levels. Hence, it is clear that the expression of CD25 and CD86 molecules did not correlate with FasL expression in CD5⁺CD1d^{hi} B cells.

3.5. NF- κ B and NF-AT inhibitors reduced the TLR4 ligand expanded FasL expressing CD5⁺CD1d^{hi} Bregs

NF- κ B and NF-AT signaling pathways are known to be involved in IL-10 production (Saraiva and O'Garra, 2010). Since we proved that FasL expression in CD5⁺CD1d^{hi} Bregs was increased after TLR4 ligation, as a next step, we intended to confirm whether FasL expression is related to NF- κ B and NF-AT pathways in CD5⁺CD1d^{hi} Bregs. For this purpose, we used BAY11-7082 and CsA which are known as specific inhibitors of NF- κ B and NF-AT respectively and observed their effects on FasL expression in CD19⁺ B cells. We found that BAY11-7082 at a concentration of 2 μ M and CsA at 2 μ M and 1 μ M inhibited both the IL-10 production and FasL expression in LPS activated CD5⁺CD1d^{hi} Bregs (Fig. 5A–B). When the concentration of BAY11-7082 was reduced to 1 μ M, the suppression on FasL expression was disappeared, though IL-10 production was still at low levels (Fig. 5A–B). These results suggest that the expression of FasL in CD5⁺CD1d^{hi} Bregs might be functioning via both NF- κ B and NF-AT signaling pathways.

3.6. Anti-FasL treatment reduced the regulatory function of LPS activated CD5⁺CD1d^{hi} Bregs in vitro

Earlier it was shown that CD5⁺CD1d^{hi} Bregs exerted their regulatory function by suppressing the proliferation of CD4⁺ T cells (Sheng et al., 2014). In our study, LPS-induced CD5⁺CD1d^{hi} Bregs suppressed the proliferation of anti-CD3/CD28 activated CD4⁺ T cells compared to CD5⁺CD1d^{low} B cell subset. The purities of isolated CD5⁺CD1d^{hi}, CD5⁺CD1d^{low} B cell subsets and CD4⁺ T cells were well above 90% (Fig. 6A–B). To verify whether this function was mediated by FasL, we used anti-FasL antibodies to block the effects of FasL expression on CD5⁺CD1d^{hi} Bregs. We found that proliferation of CD4⁺ T cells was

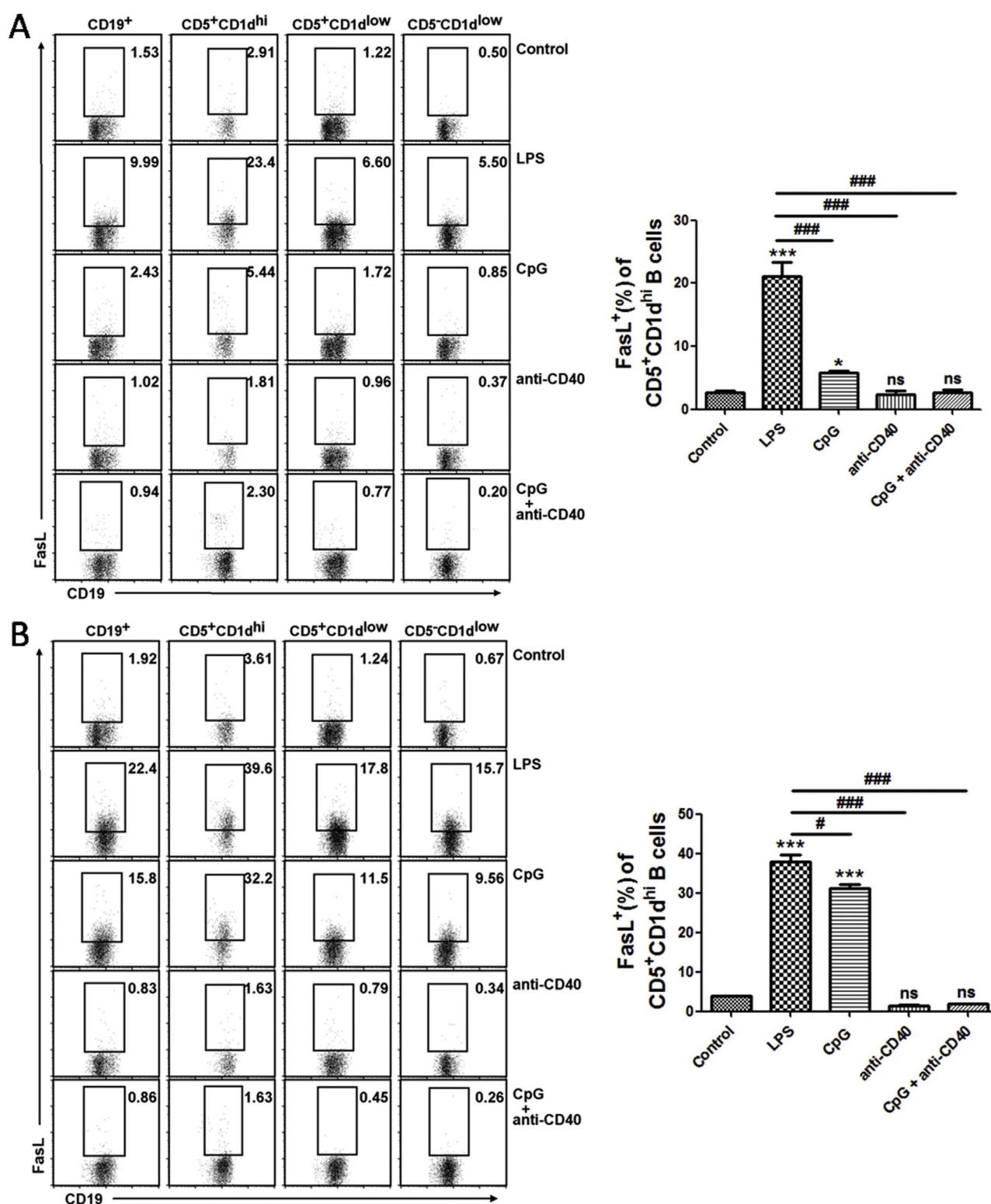


Fig. 1. TLR4 ligation up-regulated FasL expression of CD5⁺CD1d^{hi} Bregs in splenocytes. Splenocytes from mice were treated with LPS, CpG, anti-CD40 or CpG plus anti-CD40 for 12 h (A) or 24 h (B) and stained with CD19, CD5, CD1d and FasL specific mAbs. Representative scatter plots demonstrate FasL⁺ B cell frequency in different B cell subsets. Bar graphs represent the mean $\pm$ SEM of FasL⁺ B cell frequency in CD5⁺CD1d^{hi} B cell subset. Data represent three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs control group. #P < 0.05, ##P < 0.01, ###P < 0.001 vs LPS group.

inhibited by co-culturing with CD5⁺CD1d^{hi} Bregs but not CD5⁺CD1d^{low} B cells as evidenced by reduced cell division after CFSE staining (Fig. 6C). Interestingly, the proliferation of CD4⁺ T cells was enhanced after the addition of anti-FasL antibody when co-cultured with CD5⁺CD1d^{hi} Bregs, which suggested that the regulatory function of

CD5⁺CD1d^{hi} Bregs was modulated by anti-FasL treatment (Fig. 6C). These results suggest that the TLR4-ligated CD5⁺CD1d^{hi} Bregs can lead to the suppression of CD4⁺ T cell proliferation, and such suppression is mediated partly by FasL expression.

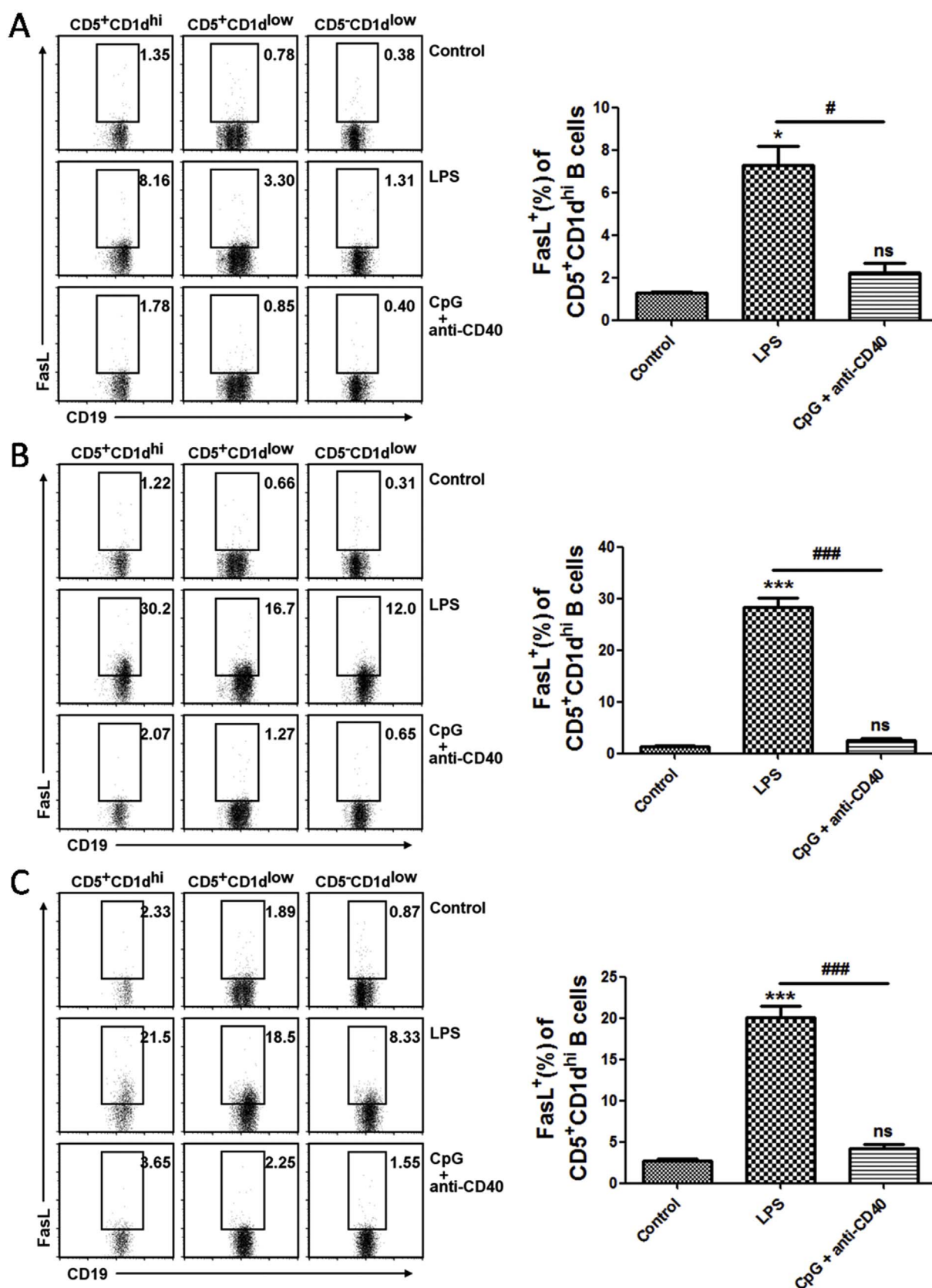


Fig. 2. TLR4 ligation up-regulated FasL expression of CD5⁺CD1d^{hi} Bregs in purified CD19⁺ B cells. Splenic CD19⁺ B cells were isolated from C57BL/6 mice and stimulated with LPS or CpG plus anti-CD40 for 12 h (A), 24 h (B) or 48 h (C) and stained with CD19, CD5, CD1d and FasL specific mAbs. Representative scatter plots demonstrate the frequency of FasL⁺ B cells in different B cell subsets. Bar graphs indicate the mean $\pm$ SEM of FasL⁺ B cell frequency in CD5⁺CD1d^{hi} B cell subset. Data represent three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs control group. #P < 0.05, ##P < 0.01, ###P < 0.001 vs LPS group.

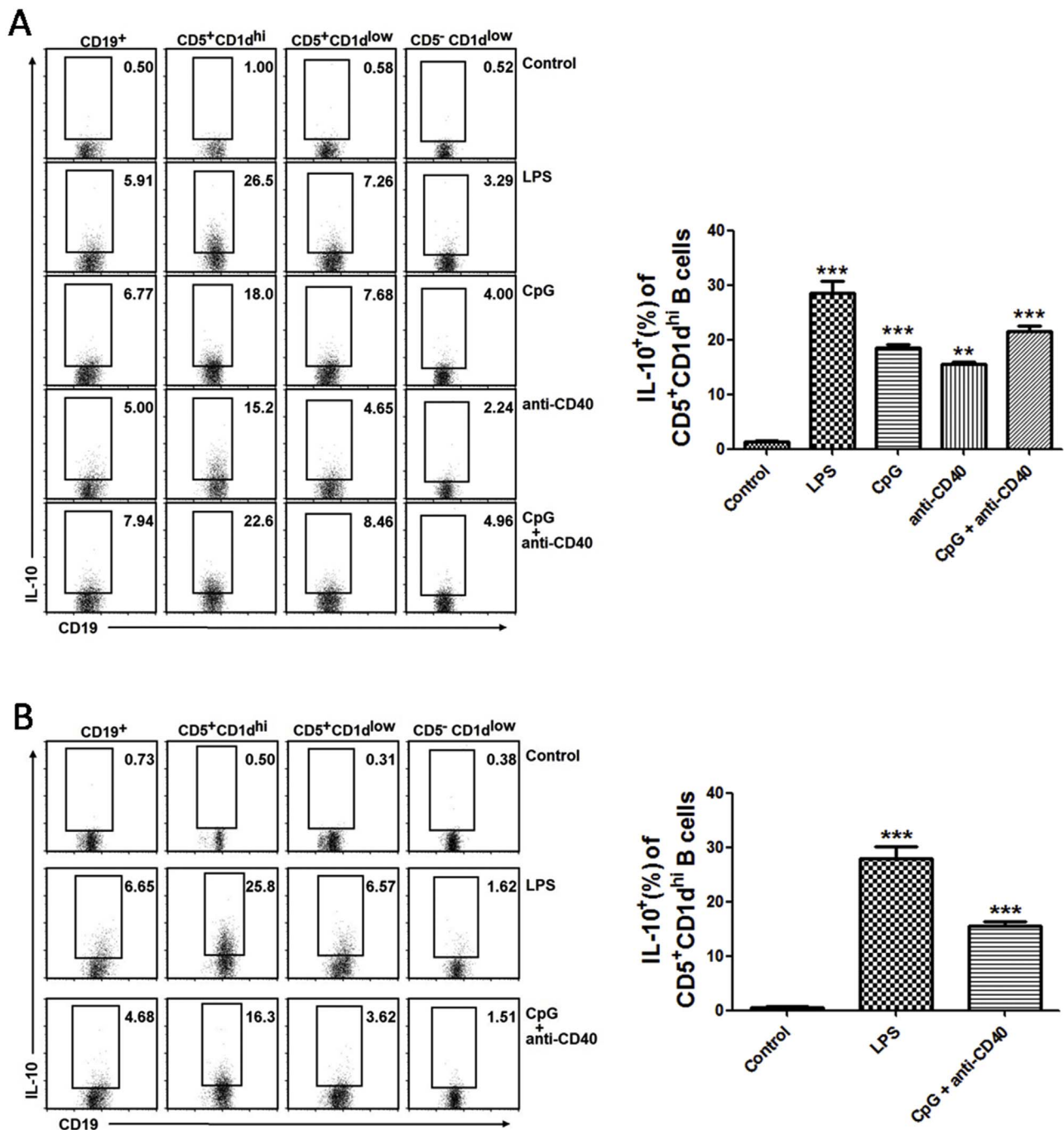


Fig. 3. TLR4 ligand and other B10-cell inducers increased IL-10 expression in CD5⁺CD1d^{hi} Bregs. Splenocytes were treated with LPS, CpG, anti-CD40 or CpG plus anti-CD40 for 24 h (A), or splenic CD19⁺ B cells were treated with LPS, CpG plus anti-CD40 for 24 h (B). PMA, ionomycin and monensin (PIM) were added for the last 5 h and cells were stained with CD19, CD5, CD1d, IL-10 specific mAbs. Representative scatter plots of flow cytometry indicate IL-10⁺ cell frequencies in different B cell subsets. Bar graphs indicate the mean $\pm$ SEM of IL-10⁺ B cell frequency in CD5⁺CD1d^{hi} B cell subset. Data represent three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs control group.

3.7. TLR4 ligand expanded FasL⁺CD5⁺CD1d^{hi} Bregs decreased in mice having contact hypersensitivity

To further study the role of FasL⁺CD5⁺CD1d^{hi} Bregs during inflammation, we used CHS inflammation model. After being sensitized with oxazolone, ears of WT mice swelled significantly (Fig. 7A) and IFN- γ secreted by splenic CD4⁺ T cells rose dramatically (Fig. 7B). On the other hand, LPS-expanded FasL⁺CD5⁺CD1d^{hi} Bregs significantly declined in these CHS mice after oxazolone challenged for 48 h (Fig. 7C). This decline in FasL⁺CD5⁺CD1d^{hi} Bregs was accompanied by an increased ear thickness and IFN- γ production. Our results suggest

that FasL⁺CD5⁺CD1d^{hi} Bregs might very well be participating in the regulation of inflammatory diseases *in vivo*.

4. Discussion

Murine splenic CD5⁺CD1d^{hi} B cell subset is one of the well-characterized functional Breg subsets, which were shown to inhibit Th1 responses, proliferation of CD4⁺CD25⁺ T effector cells and inflammation (Bashi et al., 2016; Bodhankar et al., 2011; Yanaba et al., 2008; Yang et al., 2010). CD5⁺CD1d^{hi} Bregs can suppress immune responses by secreting IL-10. However, only around 10, 30%

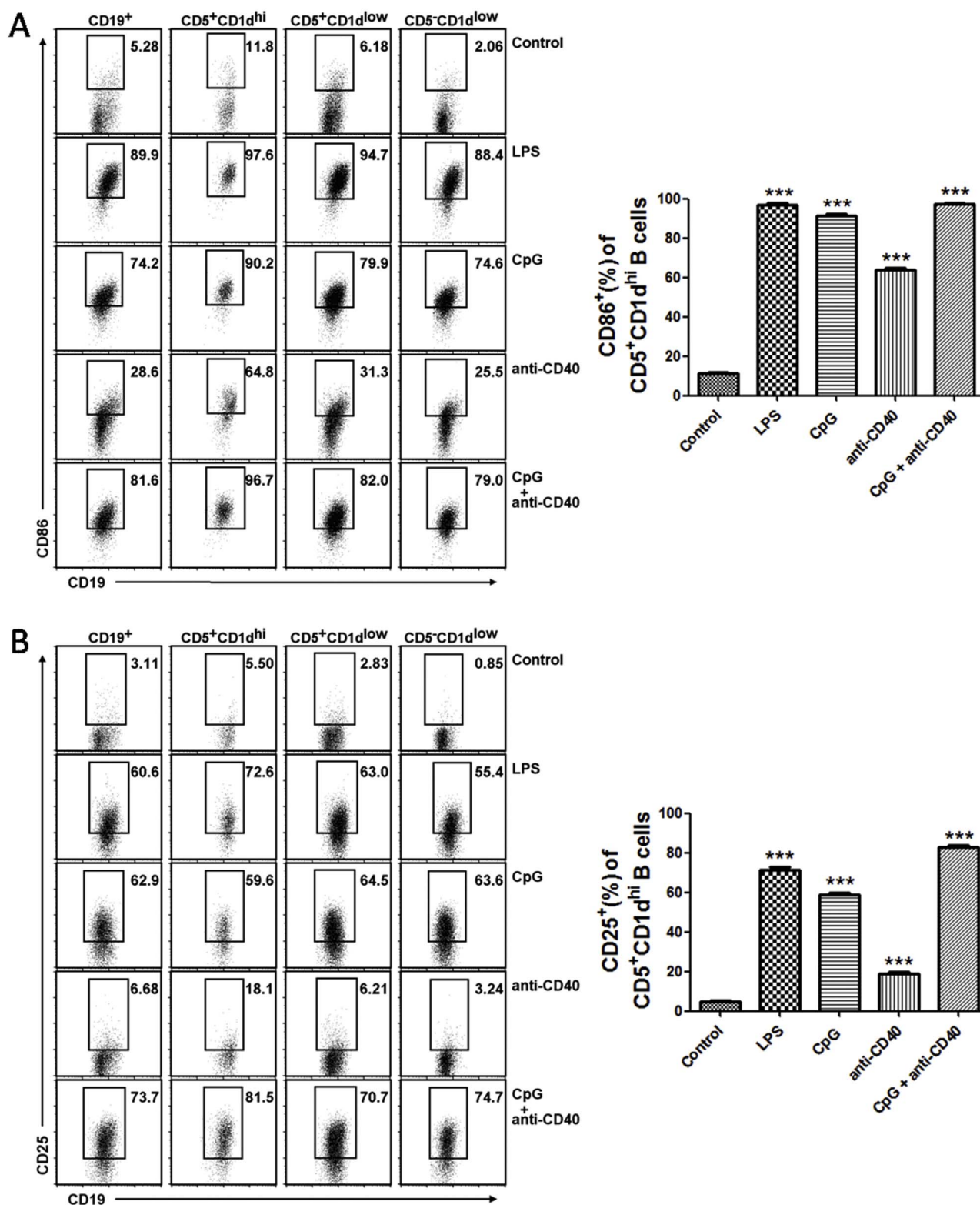


Fig. 4. TLR4 ligand and other B10-cell inducers stimulated CD86 and CD25 expression in CD5⁺CD1d^{hi} Bregs. Splenocytes were treated with LPS, CpG, anti-CD40 or CpG plus anti-CD40 for 24 h and then stained with CD19, CD5, CD1d and CD86 or CD25 specific mAbs. Representative scatter plots demonstrate the frequency of CD86⁺ (A) or CD25⁺ (B) B cells in different B cell subsets. Bar graphs present the mean $\pm$ SEM percentages of CD86⁺ or CD25⁺ B cell proportion in CD5⁺CD1d^{hi} B cell subset. Data represent three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs control group.

of these CD5⁺CD1d^{hi} B cells are IL-10 producing cells after LPS plus PIM stimulation for 5, 48 h respectively *in vitro* (Tedder, 2015; Yanaba et al., 2009). Hence, it is possible that IL-10 independent regulatory pathways could contribute to this functional property of Bregs. FasL is an important suppressive mechanism employed by B cells, which

induces apoptosis in Fas expressing immune cells (Lundy and Klinker, 2014). As previously described, naïve splenic CD19⁺FasL⁺ B cells showed a significant enhancement of CD5, CD1d expression but not CD21 and CD23 expression compared to CD19⁺FasL⁻ B cells (Klinker et al., 2013). Hence, this study was undertaken to understand FasL

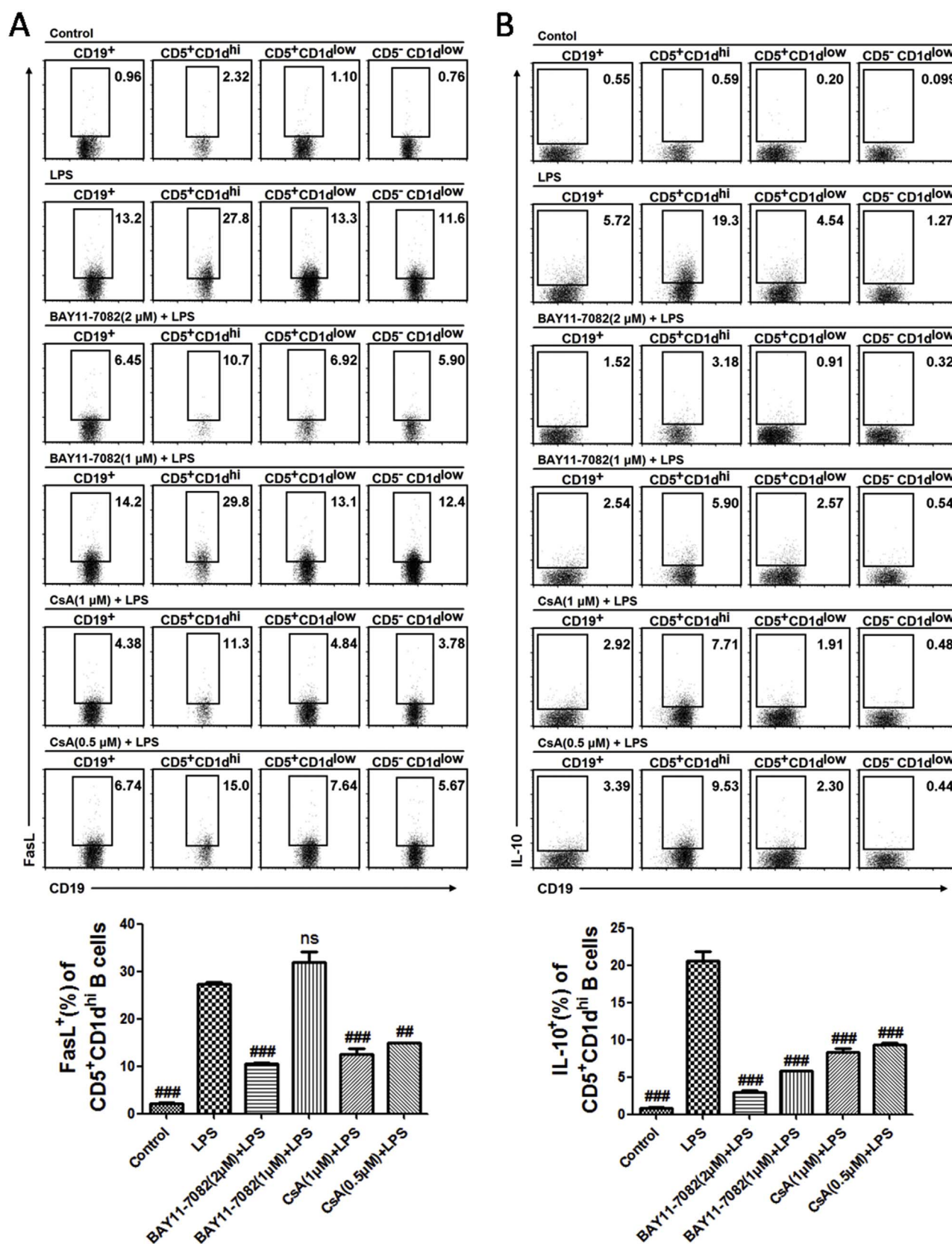


Fig. 5. NF- κ B and NF-AT inhibitors reduced TLR4 ligand stimulated FasL expression in CD5⁺CD1d^{hi} Bregs. Splenic CD19⁺ B cells purified from C57BL/6 mice were treated with LPS, LPS plus BAY11-7082 (2 μM; 1 μM) or CsA (1 μM; 0.5 μM) for 24 h and stained with CD19, CD5 and CD1d and FasL specific mAbs (A). For IL-10 analysis, PMA, ionomycin and monensin (PIM) were added to the cultures for the last 5 h. After fixation and permeabilization, B cells were stained with IL-10 or isotype mAb (B). Representative scatter plots demonstrate the frequency of FasL⁺ or IL-10⁺ B cells in different B cell subsets. Bar graphs indicate mean $\pm$ SEM of FasL⁺ or IL-10⁺ B cell frequency in CD5⁺CD1d^{hi} B cell subset. Data represent three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 vs LPS group.

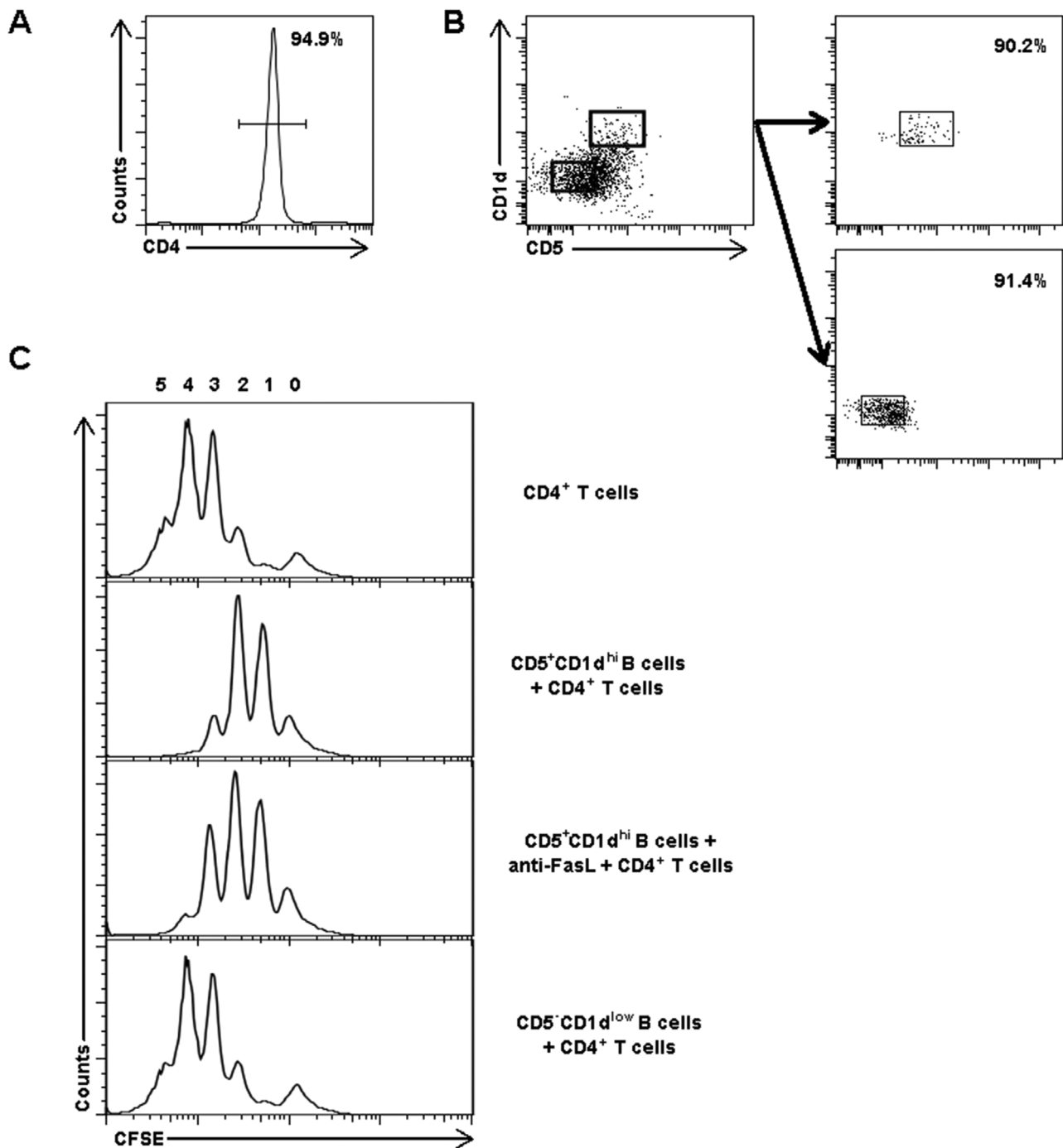


Fig. 6. Anti-FasL antibody weakened TLR4 ligand induced regulatory function of $CD5^+CD1d^{hi}$ Bregs *in vitro*. $CD4^+$ T cells were purified by negative selection (A) and labeled with CFSE. $CD19^+$ B cells were administrated with LPS for 24 h and $CD5^+CD1d^{hi}$ or $CD5^+CD1d^{lo}$ Bregs were sorted with a flow cytometer (B). Isolated $CD5^+CD1d^{hi}$ or $CD5^+CD1d^{lo}$ B cell subsets were co-cultured with CFSE-labeled $CD4^+$ T cells at a ratio of 1:1 in a 96 well-plate precoated with anti-CD3 and anti-CD28 antibodies for 72 h in the presence or absence of anti-FasL antibody. T cell proliferation was evaluated by flow cytometry after staining with CD4 mAb (C). Results represent three independent experiments.

expression in $CD5^+CD1d^{hi}$ Bregs after TLR4 activation and the functional relevance of this FasL $^+$ $CD5^+CD1d^{hi}$ Bregs in inflammatory CHS.

At first, we found that TLR4 ligation induced a strong FasL expression in $CD5^+CD1d^{hi}$ Bregs rapidly, around 20, 40% of $CD5^+CD1d^{hi}$ Bregs expressed FasL after LPS stimulation for 12, 24 h respectively. Normally, regulatory B cells acquire regulatory function after stimulation (Matsushita et al., 2010). TLR, CD40-CD40L, BAFF and BCR signals have been shown to initiate IL-10 production in naïve B cells or $CD5^+CD1d^{hi}$ Bregs (Fillatreau et al., 2002; Hussain and Delovitch, 2007; Poe et al., 2011; Yehudai et al., 2012). LPS (TLR4 ligand) and anti-CD40 could strongly induce IL-10 producing splenic

$CD5^+CD1d^{hi}$ Bregs in mice, while the effect of anti-IgM (BCR signal) was much milder (only 1.3% B cells produce IL-10 after 48 h stimulation) (Yanaba et al., 2009). TLR9 ligand is also a potent inducer of IL-10 production especially in human B cells which express TLR9 (Li et al., 2012). Human B cells or Breg subsets activated by TLR9 plus CD40L produced IL-10 and acquired the regulatory functions *in vitro* (Iwata et al., 2011; Li et al., 2012; Khoder et al., 2014). In our previous study, we found that murine $CD5^+CD1d^{hi}$ B cells could also potentially produce IL-10 after CpG plus anti-CD40 stimulation. So we studied the effects of TLR4 compared to TLR9, anti-CD40 and TLR9 plus anti-CD40 stimulations on FasL and IL-10 expression in $CD5^+CD1d^{hi}$ Bregs. As expected,

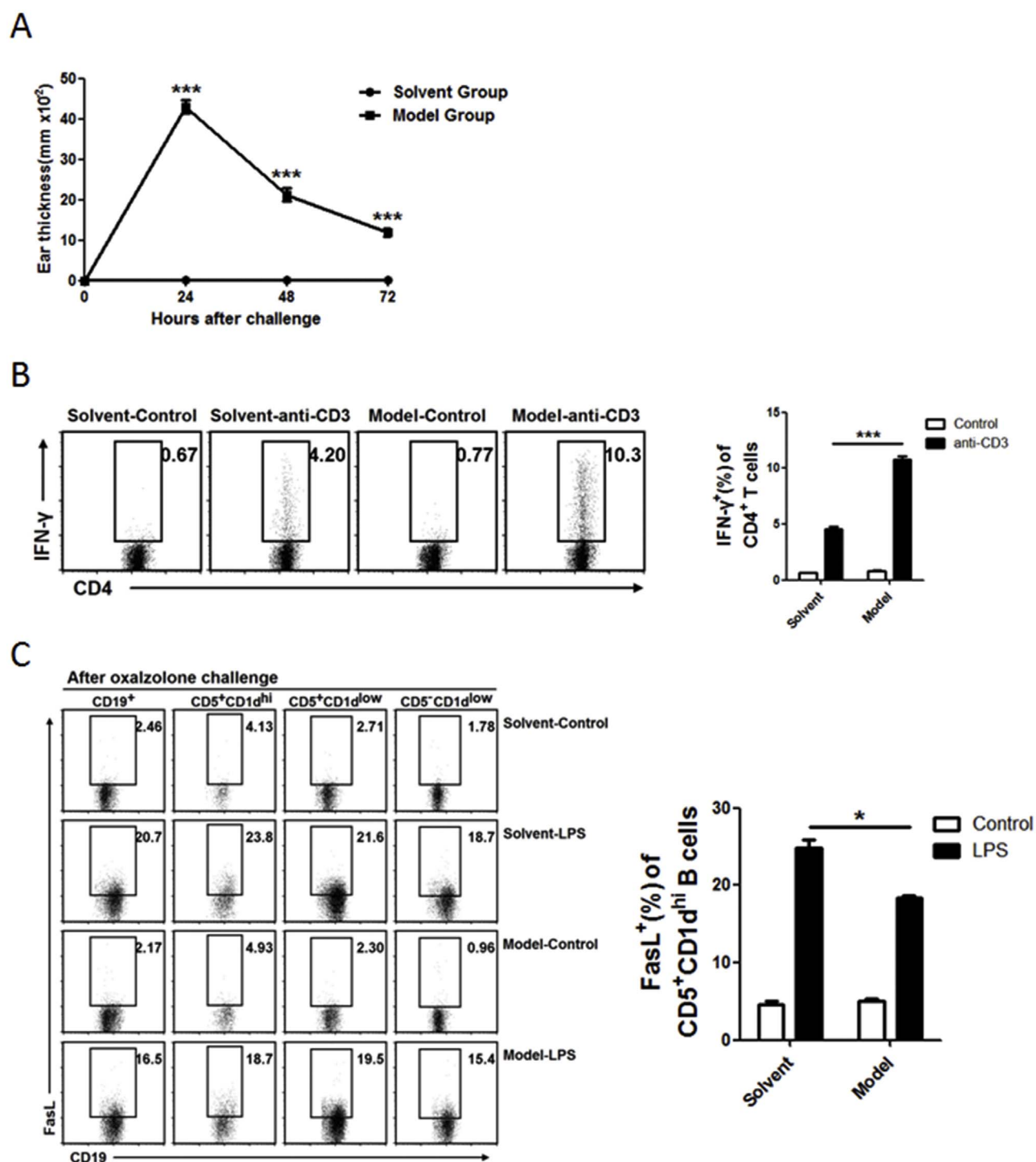


Fig. 7. TLR4 ligand expanded FasL⁺CD5⁺CD1d^{hi} Bregs decreased in mice having contact hypersensitivity. Ear thickness of WT mice was measured after oxazolone sensitization for 24 h, 48 h and 72 h (A). After oxazolone challenge for 48 h, IFN- γ production of splenic CD4⁺ T cells from WT mice during CHS responses was measured (B). FasL expression of CD5⁺CD1d^{hi} Bregs after 24 h LPS stimulation in oxazolone sensitized mice (C). Representative results are obtained from five mice in each group and repeated twice. *P < 0.05, **P < 0.01, ***P < 0.001 compared between CHS and solvent groups.

LPS, CpG, anti-CD40 and CpG plus anti-CD40 stimulants induced IL-10 production in CD5⁺CD1d^{hi} Bregs. However, it is interesting to note that anti-CD40 and CpG plus anti-CD40 stimulants failed to up-regulate FasL expression. This suggested that even though IL-10 producing and FasL expressing cells could very well be elevated and enriched in the same subset, they might be distinct populations existing in activated CD5⁺CD1d^{hi} Bregs. This is in accordance with the previous report that IL-10 production is not enriched in FasL⁺ B cells since naïve FasL⁺ B cells and FasL⁺ B cells showed no difference in IL-10 production (Klinker

et al., 2013).

We further investigated the expression of CD86 and CD25 molecules which correlated with Bregs and found that both of them could be induced by LPS and other B10-cell inducers. LPS, CpG, anti-CD40 as well as CpG plus anti-CD40 increased the CD86 expression strongly (about 60–98%) *in vitro*; whereas only LPS, CpG, CpG plus anti-CD40 highly increased the CD25 expression (about 60–80%). On the other hand, anti-CD40 alone increased CD25 expression was less than 20% in CD5⁺CD1d^{hi} Bregs. Based on earlier published studies and our current

results of FasL and IL-10 expression, it is now clear that the surface molecular markers CD25 and CD86 could not be used to distinguish FasL and IL-10 expressing populations within the activated CD5⁺CD1d^{hi} Breg populations. CD25 and especially CD86 might be present in both FasL expressing and IL-10 producing CD5⁺CD1d^{hi} Bregs. Previous research indicated that FasL expression could be increased in parallel with B cell activation status (Bonardelle et al., 2005; Tang et al., 2016). As CD25 and CD86 also serve as activation makers of lymphocytes, our data suggest that FasL expression in B cells or CD5⁺CD1d^{hi} Breg subsets might not be correlating with B cell activation, nonetheless affected by the different signaling pathways.

NF- κ B activation is required for IL-10 production in B cells and CD5⁺CD1d^{hi} Bregs (Jin et al., 2013). NF-AT is dephosphorylated by Ca²⁺ activated calcineurin and the calcineurin inhibitor cyclosporine A (CsA) was found to block IL-10 production, which suggested the involvement of NF-AT in IL-10 producing Bregs (Baba et al., 2015). Here, we observed that the NF- κ B specific inhibitor BAY11-7082 at a higher concentration (2 μ M) and NF-AT inhibitor CsA (at 1 μ M and 2 μ M) could suppress both the IL-10 and FasL expression in CD5⁺CD1d^{hi} Bregs. However, BAY11-7082 at a lower concentration (1 μ M) inhibited only IL-10 synthesis but not FasL expression in CD5⁺CD1d^{hi} Bregs. These results suggest FasL and IL-10 expressing CD5⁺CD1d^{hi} Bregs might share NF- κ B and NF-AT signaling pathways after TLR4 activation.

In mice having contact hypersensitivity (CHS), we observed that TLR4 ligand activated FasL⁺CD5⁺CD1d^{hi} Bregs were reduced compared to control mice. In addition, TLR4 ligand activated CD5⁺CD1d^{hi} Bregs from WT mice suppressed the proliferation of CD4⁺ T cells and this regulatory effect was partly mitigated by anti-FasL antibody treatment. Hence, Fas-FasL pathway could be one of possible mechanisms for CD5⁺CD1d^{hi} Bregs to exert their regulatory function. Furthermore, FasL induced apoptosis in target cells such as CD4⁺ T cells after binding to Fas receptor (CD95) (Lettau et al., 2011); FasL expressing B cells suppressed the onset of diabetes and inhibited the proliferation of CD4⁺ T cells, which could be reduced by anti-FasL antibody treatment (Montandon et al., 2013); B cells from FasL deficient mice did not transfer tolerance in a skin grafting model (Klinker and Lundy, 2012). These observations are in accordance with our results. Thus, it is plausible that FasL mediated regulatory function of CD5⁺CD1d^{hi} Bregs might be reduced in CHS which directly correlates with the enhancement of the inflammation observed in these mice.

In summary, we found that TLR4 ligation induced FasL expression in CD5⁺CD1d^{hi} Bregs in an IL-10 independent manner; TLR4 ligand activated CD5⁺CD1d^{hi} Bregs suppressed the proliferation of CD4⁺ T cells partly via FasL pathway; FasL⁺CD5⁺CD1d^{hi} Bregs might participate in CHS reactions. These results also suggest that CD86 and CD25 molecules should not be used to distinguish FasL⁺CD5⁺CD1d^{hi} Bregs and IL-10⁺CD5⁺CD1d^{hi} Bregs. It was earlier reported that IL-5R expression was higher in FasL⁺ B cells than FasL⁻ B cells (Klinker et al., 2013), while other publications indicted up-regulation of IL-5 genes in IL-10⁺ B cells (Luckey et al., 2012; Matsushita et al., 2016; Szodoray et al., 2006). Hence, search for additional markers to distinguish between FasL⁺CD5⁺CD1d^{hi} Bregs and IL-10⁺CD5⁺CD1d^{hi} Bregs are needed for further analysis of these regulatory B cell subsets.

Conflict of interest

The authors have no conflict interests.

Acknowledgements

Xiao-juan Li received project grants describing this work from the National Natural Science Foundation of China (81073119, 81611130078).

We thank Professor Kutty Selva Nandakumar for critical reading and

correction of this manuscript.

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