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The atypical IκB protein $I\kappa B_{NS}$ is important for Toll-like receptor-induced interleukin-10 production in B cells

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Summary

Although a major function of B cells is to mediate humoral immunity by producing antigen-specific antibodies, a specific subset of B cells is important for immune suppression, which is mainly mediated by the secretion of the anti-inflammatory cytokine interleukin-10 (IL-10). However, the mechanism by which IL-10 is induced in B cells has not been fully elucidated. Here, we report that $I\kappa B_{NS}$, an inducible nuclear IκB protein, is important for Toll-like receptor (TLR)-mediated IL-10 production in B cells. Studies using $I\kappa B_{NS}$ knockout mice revealed that the number of IL-10-producing B cells is reduced in $I\kappa B_{NS}^{-/-}$ spleens and that the TLR-mediated induction of cytoplasmic IL-10-positive cells and IL-10 secretion in B cells are impaired in the absence of $I\kappa B_{NS}$. The impairment of IL-10 production by a lack of $I\kappa B_{NS}$ was not observed in TLR-triggered macrophages or T-cell-receptor-stimulated $CD4^+$ $CD25^+$ T cells. In addition, $I\kappa B_{NS}$-deficient B cells showed reduced expression of *Prdm1* and *Irf4* and failed to generate IL-10^+ $CD138^+$ plasmablasts. These results suggest that $I\kappa B_{NS}$ is selectively required for IL-10 production in B cells responding to TLR signals, so defining an additional role for $I\kappa B_{NS}$ in the control of the B-cell-mediated immune responses.

Keywords: interleukin-10-producing B cells; $I\kappa B_{NS}$; Toll-like receptors.

Introduction

Although B lymphocytes play a central role in humoral immunity through antibody production, they also contribute to immune regulation, such as antigen presentation and cytokine production. Consequently, B cells express B-cell receptor (BCR), MHC and a variety of pathogen recognition receptors including Toll-like receptors (TLRs). Both human and mouse B cells have been shown to express several TLRs, and certain TLR agonists induce B-cell proliferation and the differentiation of plasma cells.[1,2] TLRs also provide a signal for the optimal proliferation and differentiation of human naive B cells, and cooperate with BCR triggering and T-cell help.[3,4] These observations indicate that TLRs control both the innate and adaptive B-cell responses.

The TLR signals trigger downstream signalling cascades, leading to activation of the transcription factor nuclear factor-κB (NF-κB).[5,6] The activation of NF-κB is regulated by several IκB proteins, a family that consists of classical and atypical nuclear IκBs. Classical IκB proteins, such as prototypic IκBα, are ubiquitously expressed and associated with NF-κB in the cytoplasm to regulate the nuclear translocation of NF-κB.[7] Conversely, the expression of nuclear IκB genes is induced via several surface receptors, and their products are mainly localized in the nucleus and thought to positively and negatively regulate NF-κB-dependent gene expression.[8] The family of nuclear IκB proteins contains Bcl-3, IκBζ and $I\kappa B_{NS}$[9–12] as well as the recently identified IκBη and IκBL.[13,14] Although nuclear IκB proteins have been shown to play an important role in the regulation of inflammatory responses by

Abbreviations: BCR, B-cell receptor; BM, bone marrow; IL-10, interleukin-10; IRF-4, interferon regulatory factor 4; LPS, lipopolysaccharide; MZ, marginal zone; NF-κB, nuclear factor-κB; PE, phycoerythrin; T2-MZP, transitional 2-MZ precursor; TCR, T-cell receptor; TLR, Toll-like receptor

TLRs,[8] accumulating reports suggest that nuclear IκBs also play a significant role in the regulation of both innate and adaptive immunity.[15]

A nuclear protein, $I\kappa B_{NS}$, was originally identified in thymocytes undergoing negative selection. However, $I\kappa B_{NS}$-deficient mice ($I\kappa B_{NS}^{-/-}$) exhibit only a slight difference in the development of T cells.[16] Instead, Kuwata *et al.*[17] showed that $I\kappa B_{NS}$ plays a role in the control of innate immune responses: $I\kappa B_{NS}$ suppresses the TLR-induced expression of inflammatory cytokines, such as interleukin-6 (IL-6) and IL-12, in macrophages and dendritic cells. In addition, we have shown that $I\kappa B_{NS}$ significantly impacts the control of antigen-specific immune responses: $I\kappa B_{NS}$ positively regulates proliferation and IL-2 production in T cells upon T-cell receptor (TCR) stimulation,[16] and $I\kappa B_{NS}^{-/-}$ mice failed to produce antigen-specific antibodies, exhibiting a developmental defect of B-1 B and plasma cells.[18] Arnold *et al.*[19] demonstrated a critical role for *Nfkbid*, which encodes $I\kappa B_{NS}$, in the regulation of B-1 B-cell development and the extrafollicular antibody response using forward genetic screens. In this study, we further investigated the impact of $I\kappa B_{NS}$-deficiency in B cells, and we found that the number of IL-10-producing B cells is reduced in $I\kappa B_{NS}^{-/-}$ spleens and that the TLR-stimulated induction of IL-10 secretion is largely impaired in the absence of $I\kappa B_{NS}$.

Recent studies have established that B cells regulate inflammatory responses by producing anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β; B cells with suppressive function are called regulatory B cells.[20–25] The secretion of IL-10 in B cells is controlled by signals from BCRs, CD40 and TLRs.[20,22,24] Various phenotypes of IL-10-producing B cells have been reported, such as peritoneal B-1a cells,[26] less mature transitional 2-marginal zone precursor (T2-MZP) cells,[22] splenic $CD138^+$ plasma cells,[27] and a small subset of murine splenic B cells expressing a $CD1d^{hi}$ $CD5^+$ phenotype that is more enriched for IL-10-producing B (B10) cells.[28] These B-cell subsets share various surface markers, such as CD1d, CD5 and CD21. Although the therapeutic effects of IL-10-producing B cells in inflammatory and autoimmune diseases have been demonstrated in several animal models,[20–23] the developmental pathway of B10 cells and mechanisms underlying IL-10 expression in B cells remain poorly understood.

Here, we report a significant role for $I\kappa B_{NS}$ in TLR-induced IL-10 production in B cells. We also found that two IL-10-producing B-cell populations increase upon TLR-triggering: $CD138^+$ and $CD138^-$. Furthermore, $I\kappa B_{NS}^{-/-}$ B cells are unable to generate IL-10-producing $CD138^+$ $CD44^{hi}$ plasmablasts. Our findings indicate that $I\kappa B_{NS}$ is important for the regulation of B-cell function in the T-independent early phase of the immune response.

Materials and methods

Mice

C57BL/6 (B6) background $I\kappa B_{NS}^{-/-}$ mice were established as described previously[16] and were kindly provided by Dr E.L. Reinherz from the Dana-Farber Cancer Institute. B-cell-deficient B6.μMT mice[29] were kindly provided by Dr D. Kitamura from the Tokyo University of Science. B6 wild-type animals were purchased from Japan SLC Inc. (Shizuoka, Japan). Four- to eight-month-old mice were used to compare the B-cell subsets between wild-type and $I\kappa B_{NS}^{-/-}$ mice. All animal experiments and bleeding procedures were performed in accordance with the guidelines for animal experiments at Niigata University.

Mixed bone marrow chimeric mice

Bone marrow (BM) cells were prepared from the femurs and tibias of sex-matched donor mice (B6.μMT, B6 wild-type, or B6.$I\kappa B_{NS}^{-/-}$) and were depleted of red blood cells using ACK lysing buffer. BM cells from B6.μMT mice were mixed with wild-type or $I\kappa B_{NS}$-deficient BM cells at a ratio of 4 : 1, and they (1×10^7) were intravenously injected into sublethally irradiated (8 Gy) recipient B6.μMT mice. Eight weeks later, recipient mice were used to analyse splenic IL-10-producing B cells by flow cytometry. For the endotoxin-challenge assay, BM chimeric μMT mice received daily doses of 10 μg of lipopolysaccharide (LPS) intraperitoneally (*Escherichia coli* serotype 055;B5; Sigma-Aldrich, St Louis, MO) in 100 μl of PBS for 3 days. Control mice received PBS alone. Two days after the last inoculum, all mice were killed and analysed for IL-10-producing B cells in the spleens by flow cytometry.

Isolation of splenic B cells, T cells and peritoneal macrophages

Resting B cells from B6 wild-type or $I\kappa B_{NS}^{-/-}$ spleens were purified by negative selection with anti-CD43 conjugated microbeads and the MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany). Subpopulations of splenic B and lymph node T cells were isolated using a FACSAria cell sorter (BD Biosciences, San Jose, CA) with appropriate monoclonal antibodies. To isolate naive macrophages, the peritoneal cavity was washed with 6 ml of sterile PBS to retrieve resident leucocytes, and macrophages were separated by adherence to a tissue culture dish after 2 hr of incubation.

Cell stimulation

Isolated splenic B cells were resuspended in complete RPMI-1640 media at a concentration of 2×10^6 cells/ml

and stimulated with 5 μg/ml or the indicated amounts of LPS. Figure 4 shows experiments in which LPS (5 μg/ml), anti-CD40 (5 μg/ml; BioLegend, San Diego, CA), anti-IgM $F(ab')_2$ antibody (10 μg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and IL-4 (10 ng/ml; ProSpec-Tany TechnoGene Ltd., Rehovot, Israel) were used for B-cell stimulation. Except for LPS, the following TLR agonists were used in this study: Pam3CSF4 (1 μg/ml) for TLR1/2, FSC-1 (1 μg/ml) for TLR6/2, Imiquimod-R837 (2 μg/ml) for TLR7, and ODN1826 (0·2 μg/ml) for TLR9 (InvivoGen, San Diego, CA). To detect intracellular cytokines, the cells were stimulated *in vitro* for the indicated time and further treated with GolgiPlug (1/1000 dilution; BD Biosciences) for 5 hr. In some cases, PMA (40 ng/ml; Sigma-Aldrich) and calcium ionophore A23187 (400 ng/ml; Sigma-Aldrich) were added for the last 5 hr of incubation. FACS-sorted T cells (2×10^5) were resuspended in 100 μl of complete RPMI-1640 medium and stimulated with 2 μg/ml plate-bound anti-CD3ε (145-2C11; BioLegend) and 4 μg/ml soluble anti-CD28 (eBioscience) in a 96-well plate for 3 days. Peritoneal macrophages were resuspended in 100 μl of complete Dulbecco's modified Eagle's medium and stimulated with the indicated amount of LPS for 2 days. The proliferative activity was assessed using a Cell Counting Kit-8 (Dojijdo Molecular Technology, Inc., Kumamoto, Japan), and the expression of cytoplasmic IL-10 was analysed by flow cytometry. Cell culture supernatants were collected and used to measure the levels of secreted cytokines by ELISA.

Flow cytometric analysis

The following monoclonal antibodies were used: FITC-anti-CD5 (clone; 53-7.3), FITC-anti-CD44 (IM7), PerCP-Cy5.5-anti-CD1d (1B1), phycoerythrin (PE) -Cy7-anti-IgM (RMM-1), Alexa647-anti-CD19 (6D5), FITC-anti-interferon-γ (XMG1.2), PE-anti-IL-10 (JES5-16E3) and PE-Cy-7-anti-IL-10 were purchased from BioLegend. PE-anti-CD138 (Syndecan-1, 281-2) was purchased from BD Biosciences. The intracellular staining of cytokines was performed after cell fixation and permeabilization with Cytofix/Cytoperm solution (BD Biosciences). Background staining for intracellular cytokines was assessed with suitable isotype control antibodies from BioLegend or BD Biosciences. The FACSAria (BD Biosciences) and FACS Diva software were used. Dead cells were excluded from the analysis by forward and side scatter gating and propidium iodide dye exclusion.

RT-PCR

RNA isolated from purified wild-type or $I\kappa B_{NS}^{-/-}$ B cells using the TriPure isolation reagent (Roche Diagnostics GmbH, Mannheim, Germany) was used for both semi-quantitative and quantitative real-time PCR analysis. One microgram of total RNA was used to synthesize single-strand cDNA by reverse transcriptase (Transcriptor, Roche). Real-time PCR was performed using the Light Cycler (Roche Diagnostics GmbH) and SYBR Premix *Ex Taq* (TAKARA, Tokyo, Japan). The expression level of β-actin was used to normalize the template input. Assays were performed in triplicates.

ELISAs

Cytokine production was induced as described above, and the culture supernatant fluid was stored at −80° until use. Cytokines secreted in the culture supernatants were measured using a mouse ELISA MAX Standard from BioLegend.

Western blot analysis

Cytoplasmic and nuclear extracts were prepared from purified wild-type and $I\kappa B_{NS}^{-/-}$ B cells as previously described.[17] The protein concentration was assessed using a Pierce BCA kit (Thermo Fisher Scientific, Waltham, MA). To separate proteins, 30 μg of cytoplasmic extracts or 10 μg of nuclear extracts was loaded onto a 10% polyacrylamide gel. The proteins were blotted onto a polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK), and target proteins were detected using the following antibodies: NF-κB p50 (KAP-TF112; Stressgen Biotechnologies, Victoria, BC, Canada), NF-κB p65 (C-20; Santa Cruz Biotechnology, Dallas, TX), NFATc1 (7A6; BioLegend), and Lamin B (M-20; Santa Cruz). Anti-$I\kappa B_{NS}$ monoclonal antibody was purified from the culture supernatant of a hybridoma (mouse IgG2b).[12]

DNA pull-down assay

DNA pull-down assays were performed as previously described.[17] Splenic B cells were stimulated with 5 μg/ml LPS for the indicated time and lysed in a lysis buffer solution.[30] The biotinylated DNA probes of two NF-κB binding sites in the *Il10* promoter were obtained from Greiner Bio-One (Tokyo, Japan). The Origo DNA pairs were 5′-Biotin-TTTGCCAGGAAGGCCCCACTGAGC-3′ with 5′-GCTCAGTGGGGCCTTCCTGGC for NF-κB site at –50/–39[31] and 5′-Biotin-GAGGTAGTAGGAGAAGTC CCTACTGAA-3′ with 5′-TTCAGTAGGGACTTCTCCT ACTAC for the NF-κB site at –861/–851.[32] The 30 μg of nuclear extracts was pre-cleared with streptavidin Mag sepharose (GE Healthcare). A total of 1 μg of a biotinylated dsDNA probe and 10 μg of poly(dI-dC) were added, and DNA-bound proteins were collected with streptavidin Mag sepharose, washed three times with HKMG buffer (10 mM HEPES, pH 7·9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0·5%

Nonidet P-40), separated on SDS–PAGE, and identified by Western blotting using a chemiluminescence reagent (Immunostar LD; Wako, Tokyo, Japan). Protein signals were detected using anti-p50, anti-p65, or anti-IκB$_{NS}$ antibody.

Statistical analysis

The results are shown as the mean ± SD of values obtained from two or three separate experiments. For ELISAs, the results of a single representative experiment are provided. The data were analysed by unpaired two-tailed Student's *t*-test to assess the significance of differences between the wild-type and IκB$_{NS}^{-/-}$ groups. A p value of $< 0{\cdot}05$ was considered significant.

Results

IκB$_{NS}$ deficiency leads to a significant reduction in IL-10-producing B cells in the spleen

Mice deficient in IκB$_{NS}$ lack peritoneal B-1 B cells and exhibit delayed marginal zone (MZ) B-cell development.[18,19] Both cell populations are known to produce large amounts of IL-10 in response to infectious stimuli and share surface markers, such as CD1d and CD5; a small subset of splenic B10 cells potently express IL-10.[28] Based on these observations, we hypothesized that IκB$_{NS}$ plays a role in the generation of IL-10-producing B cells. We first examined the IL-10-competent CD1d^{hi} CD5^{+} B10 cells in IκB$_{NS}^{-/-}$ spleens. Because the number of MZ B cells in young IκB$_{NS}^{-/-}$ mice was previously reported to be smaller than that in wild-type mice of the same age,[18] we used mice older than 4 months, in which MZ B cells are almost fully developed, even in the absence of IκB$_{NS}$ (see Supplementary material, Fig. S1). As shown in Fig. 1(a, b), the frequency and cell number of the CD1d^{hi} CD5^{+} population in CD19^{+} splenocytes from IκB$_{NS}^{-/-}$ mice were less than half those in wild-type mice. Although the frequency of the CD1d^{hi} regulatory B cells was reduced in IκB$_{NS}^{-/-}$ spleens, the frequencies of the T2-MZP B cells with a CD19^{+} CD21^{hi} CD23^{hi} CD24^{hi} phenotype and the Tim-1-expressing B cells in IκB$_{NS}^{-/-}$ spleens were comparable with those in wild-type spleens (see Supplementary materials, Fig. S1).

To examine the IL-10-producing B cells in IκB$_{NS}^{-/-}$ mice, splenic B cells isolated from native mice were stimulated with LPS and PMA plus Ca^{2+}-ionophore for 5 hr to induce the expression of cytoplasmic IL-10 as previously described.[28] The frequencies and the number of cytoplasmic IL-10-positive B cells in IκB$_{NS}^{-/-}$ spleens were markedly reduced compared with wild-type spleens (Fig. 1c, d). These results indicate that the development of IL-10-competent CD1d^{hi} CD5^{+} B cells and IL-10-producing splenic B cells is impaired in the absence of IκB$_{NS}$.

Impairment of IL-10-producing B cells in IκB$_{NS}^{-/-}$ mice is a B-cell intrinsic property

B-cell development is supported by many environmental factors, such as antigens, cytokines and stromal cells. To

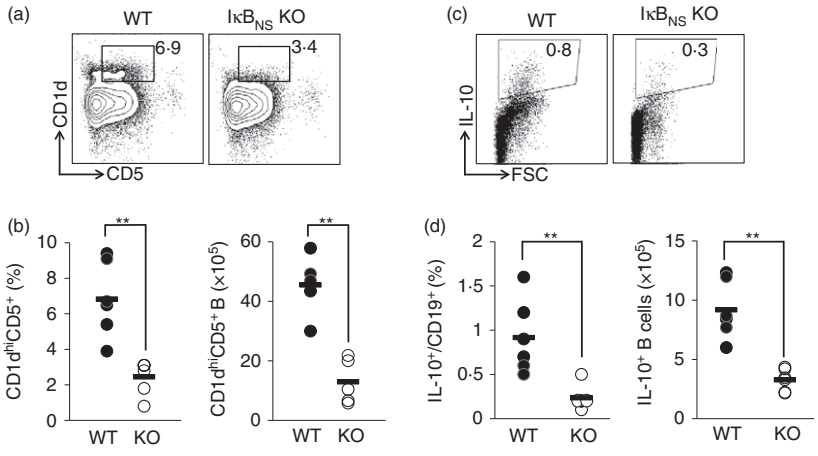

Figure 1. IκB$_{NS}^{-/-}$ mice contain reduced numbers of interleukin-10 (IL-10)-producing B cells in the spleen. (a) Flow cytometric analysis of CD1d^{hi} CD5^{+} B cells in spleen from wild-type (IκB$_{NS}^{+/+}$, WT) and IκB$_{NS}$-deficient (IκB$_{NS}^{-/-}$, KO) mice. Representative FACS profiles of CD19^{+} spleen B cells for CD1d and CD5 are shown. Numbers represent the percentage of cells in indicated gates. (b) The frequency of CD1d^{hi} CD5^{+} cells in CD19^{+} splenic B cells (left) and the numbers of CD1d^{hi} CD5^{+} B cells in wild-type and IκB$_{NS}^{-/-}$ spleens (right) are shown. (c) Flow cytometric analysis of IL-10-producing B cells in spleen. Splenocytes from wild-type and IκB$_{NS}^{-/-}$ mice were incubated with lipopolysaccharide (LPS) plus PMA, A23187 and GolgiPlug for 5 hr. The cells were then stained with anti-CD19 and anti-IL-10. Representative dot plots show the frequencies of cytoplasmic IL-10^{+} cells in CD19^{+} B cells. (d) The frequency of cytoplasmic IL-10^{+} cells in CD19^{+} splenic B cells (left) and the number of IL-10 producing B cells in the spleen (right) are shown. For (a) and (b), data are representative of at least three independent experiments. For (b) and (d), each symbol indicates an individual mouse. Horizontal bars represent the mean. $n > 5$; $^{**}P < 0{\cdot}01$.

address whether the impairment of IL-10-producing B cells in $I\kappa B_{NS}^{-/-}$ mice is a B-cell intrinsic property, we generated mixed BM chimeric mice by transferring $I\kappa B_{NS}^{-/-}$ or control B6 BM cells mixed with quadruple the number of μMT BM cells into irradiated recipient μMT mice. When donor-derived B cells were reconstituted, and the IL-10-producing B cells in recipient spleen were analysed. The B-cell compartments with MZ were observed in the spleens receiving BM cells at 2 months post transfer. Whereas full reconstitution of the B-cell compartments was observed in the spleens of wild-type BM chimeras, the frequencies of $CD1d^{hi}$ $CD5^+$ B cells and MZ B cells were lower in the spleens of $I\kappa B_{NS}^{-/-}$ BM chimeras (see Supplementary material, Fig. S2). The frequencies of IL-10-producing B cells were lower in spleens receiving $I\kappa B_{NS}^{-/-}$ BM cells, although the difference between the wild-type and $I\kappa B_{NS}^{-/-}$ groups was not significant: 0·9 ± 0·8% and 0·2 ±0·1%, respectively (Fig. 2a, b). To assess the induction of IL-10-producing B cells *in vivo*, mixed BM chimeric mice were inoculated with low-dose LPS, and IL-10-producing B cells were analysed by flow cytometry. Reconstituted wild-type B cells generated a significant level of IL-10-producing cells in response to LPS (12·0 ± 4·4%), whereas $I\kappa B_{NS}^{-/-}$ B cells were incapable of generating IL-10-producing cells (0·4 ± 0·4%, and Fig. 2). Hence, the development of IL-10-competent B cells and the endotoxin-induced generation of IL-10-producing B cells *in vivo* require B-cell intrinsic $I\kappa B_{NS}$.

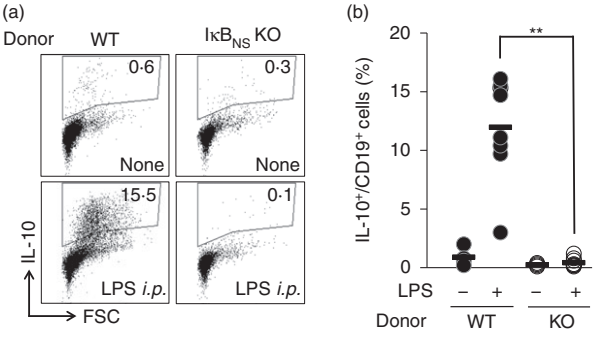

Figure 2. B-cell-intrinsic deficiency in $I\kappa B_{NS}$ results in the impaired generation of interleukin-10 (IL-10) -producing B cells *in vivo*. bone marrow (BM) cells from B6.μMT mice were mixed with BM cells from wild-type or $I\kappa B_{NS}^{-/-}$ mice at a ratio of 4:1, and 1×10^7 BM cells were transferred intravenously into irradiated B6.μMT mice. Lipopolysaccharide (LPS) -induced IL-10 production in donor-derived B cells was examined 8 weeks post transfer by the daily administration of LPS (10 μg, intraperitoneally) for 3 days. Controls received PBS alone. Two days after the last inoculum, splenocytes were stained with surface CD19 and intracellular IL-10. (a) Representative FACS profiles of $CD19^+$ splenocytes for the expression of cytoplasmic IL-10 with the percentages of cells in gates. (b) The frequency of IL-10-producing cells in $CD19^+$ splenic B cells. Data from four control and eight LPS-treated mice are shown. Each symbol shows an individual mouse, and horizontal bars represent the mean. Two independent experiments were performed, and similar results were obtained. $^{**}P < 0{\cdot}01$.

$I\kappa B_{NS}^{-/-}$ B cells fail to secrete LPS-induced IL-10

To determine the competency of IL-10 production in $I\kappa B_{NS}$-deficient B cells, purified splenic B cells from $I\kappa B_{NS}^{-/-}$ or control B6 mice were stimulated with LPS. The expression of cytoplasmic IL-10 and the amount of secreted IL-10 in the culture supernatants were examined. As shown in Fig. 3(a, b), cytoplasmic IL-10-positive cells and IL-10 secretion were almost undetectable in $I\kappa B_{NS}^{-/-}$ B cells, even in the presence of higher concentrations of LPS. The addition of PMA plus Ca^{2+}-ionophore to LPS emphasized IL-10 secretion in wild-type B cells, whereas only a slight increase in IL-10 was detected in the $I\kappa B_{NS}^{-/-}$ B-cell culture even in the presence of PMA plus Ca^{2+}-ionophore. Note that increased amounts of LPS did not enhance the frequencies of IL-10-producing cells, whereas a higher dose of LPS increased IL-10 secretion in wild-type B cells. A low dose of LPS is probably sufficient for B cells to commit to IL-10-producing cells, whereas a higher dose of LPS effectively induces B-cell proliferation. The impairment of LPS-induced IL-10 secretion in $I\kappa B_{NS}$-deficient B cells was confirmed using BALB/c background $I\kappa B_{NS}^{-/-}$ mice (data not shown).

The suppressive function of LPS-activated B cells was also examined. Although the IL-10-mediated inhibitory function of B cells participates in the suppression of antigen-presenting cells, such as dendritic cells,[24] the decrease in interferon-γ production in T cells was attenuated in the presence of LPS-stimulated $I\kappa B_{NS}^{-/-}$ B cells compared with in the presence of LPS-stimulated wild-type B cells (Fig. 3c). In addition, IL-10-producing T cells were induced only in the presence of wild-type B cells (Fig. 3c). These IL-10-producing T cells were found in $CD4^+$ cells (see Supplementary material, Fig. S3). Therefore, the $I\kappa B_{NS}$-deficiency in B cells may affect the generation of regulatory T cells as well as regulatory B cells.

Finally, we evaluated the capability of $I\kappa B_{NS}$-deficient splenic B10 cells to secrete IL-10. As shown in Fig. 3(d), $CD1d^{hi}$ $CD5^+$ B cells isolated from $I\kappa B_{NS}^{-/-}$ spleens produced significantly lower levels of IL-10 than wild-type cells, and similar results were obtained with MZ B cells from $I\kappa B_{NS}^{-/-}$ mice (Fig. 3e). Collectively, $I\kappa B_{NS}$-deficient B cells exhibit a severe defect in LPS-induced IL-10 production.

$I\kappa B_{NS}$ is required for TLR-mediated IL-10 production in B cells

We then examined IL-10 production induced by anti-CD40 and anti-IgM because the expression of $I\kappa B_{NS}$ is transiently induced by LPS, anti-CD40 and anti-IgM.[18] As

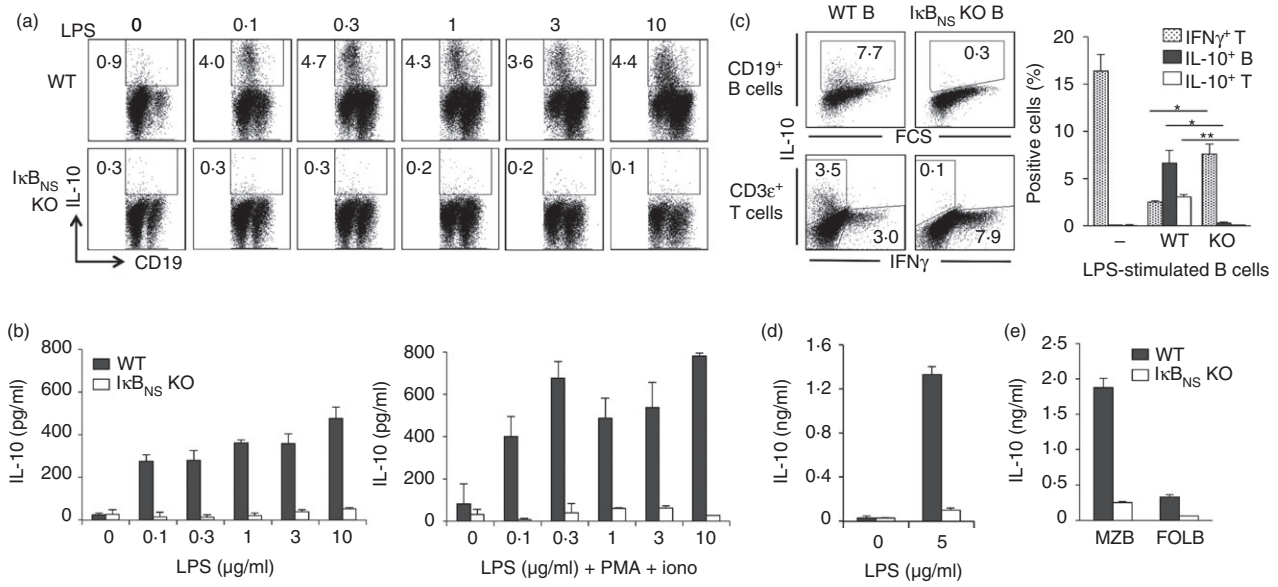

Figure 3. IκB$_{NS}$-deficient B cells fail to produce lipopolysaccharide (LPS) -induced interleukin-10 (IL-10). (a) Splenic B cells from wild-type and IκB$_{NS}^{-/-}$ mice were cultured with various concentrations of LPS for 3 days. The cells were then FACS stained with anti-CD19 and anti-IL-10. Representative FACS profiles of CD19$^+$ B cells for cytoplasmic IL-10 with the frequencies of cells in indicated gates are shown. (b) The amount of IL-10 in the culture supernatant of LPS-stimulated B cells. Splenic B cells were stimulated with LPS alone or LPS plus PMA, A23187 for 3 days. (c) Splenic B cells from wild-type and IκB$_{NS}^{-/-}$ mice were activated with LPS (5 μg/ml) for 1 day, and they were co-cultured with wild-type T cells under stimulation with anti-CD3ε and anti-CD28 for 2 days. The expression level of IL-10 in B cells and the expression levels of IL-10 and interferon-γ (IFN-γ) in CD3ε$^+$ T cells were analysed by flow cytometry. Representative FACS plots with the frequencies of cells in indicated gates are shown. The bar graph shows the frequencies of IFN-γ$^+$ cells in CD3ε$^+$ T cells (dot columns), IL-10$^+$ cells in CD19$^+$ B cells (filled columns), and IL-10$^+$ cells in CD3ε$^+$ T cells (open columns) in the co-culture. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$. (d) Purified CD1d^{hi} CD5$^+$ B cells from wild-type and IκB$_{NS}^{-/-}$ spleens were incubated with or without LPS for 2 days, and the amount of IL-10 in the culture supernatant was measured by ELISA. (e) Purified CD21^{hi} CD23^{lo} marginal zone (MZ) B cells and CD21$^+$ CD23^{hi} follicular B cells from wild-type and IκB$_{NS}^{-/-}$ spleens were incubated with LPS (5 μg/ml) for 4 days, and the amount of IL-10 in the culture supernatant was measured by ELISA. The data shown were obtained from triplicate assay and represent at least two independent experiments. Error bars represent the standard deviation.

shown in Fig. 4(a; top), these agents all induced B-cell proliferation, irrespective of the presence of IL-4. IκB$_{NS}$ deficiency modestly affected B-cell proliferation in response to anti-CD40 and anti-IgM, whereas the proliferative response to LPS was reduced in IκB$_{NS}^{-/-}$ B cells. In our *ex vivo* assay, IL-10 production in wild-type B cells was predominantly induced by LPS alone or LPS plus IL-4 but not by anti-CD40 or anti-IgM (Fig. 4a, middle). Although a defect in IL-6 due to a lack of IκB$_{NS}$ was much less severe than that in IL-10, the amount of IL-6 secreted by IκB$_{NS}^{-/-}$ B cells was also significantly reduced. This reduction probably resulted in the weak proliferative response to LPS in the absence of IκB$_{NS}$ (Fig. 4a, top and bottom).

In mice, TLR1, -2, -4, -6, -7 and -9 are expressed in most B-cell subsets.[33] Because TLR agonists other than LPS also induce the expression of *Nfkbid*, which encodes the IκB$_{NS}$ protein in B cells (Fig. 4b), we further examined the impact of IκB$_{NS}$ deficiency in TLR-mediated IL-10 production in B cells. IκB$_{NS}^{-/-}$ B cells failed to secrete IL-10 in response to TLR agonists of TLR1/2 (Pam3CSK4), TLR2/6 (FSL-1), TLR7 (Imiquimod), TLR9 (ODN1826) and TLR4 (LPS) (Fig. 4b).

Remarkably, IκB$_{NS}$-dependent IL-10 production in response to TLR appears to be B-cell-specific because the levels of IL-10 secretion in LPS-stimulated IκB$_{NS}^{-/-}$ macrophages were comparable to those in wild-type macrophages (Fig. 4c). This finding is consistent with the previously observed expression level of IL-10 in LPS-stimulated IκB$_{NS}^{-/-}$ macrophages, which was comparable to that in control macrophages.[17] In addition, a lack of IκB$_{NS}$ enhanced TCR-mediated IL-10 production in CD25$^+$ CD4$^+$ T cells (Fig. 4d), whereas the number of regulatory T cells was reduced in IκB$_{NS}^{-/-}$ mice, as previously reported by Schuster *et al.*[34] These results indicate that the regulatory mechanism of IL-10 production might depend both on the cell type and on signals via surface receptors.

A lack of IκB$_{NS}$ reduces *Il10* expression in B cells but does not alter the association of NF-κB with κB sites in the *Il10* promoter

We next examined the expression of the *Il10* gene in B cells from IκB$_{NS}^{-/-}$ mice. LPS transiently induced the expression of *Nfkbid* in wild-type B cells, as shown in

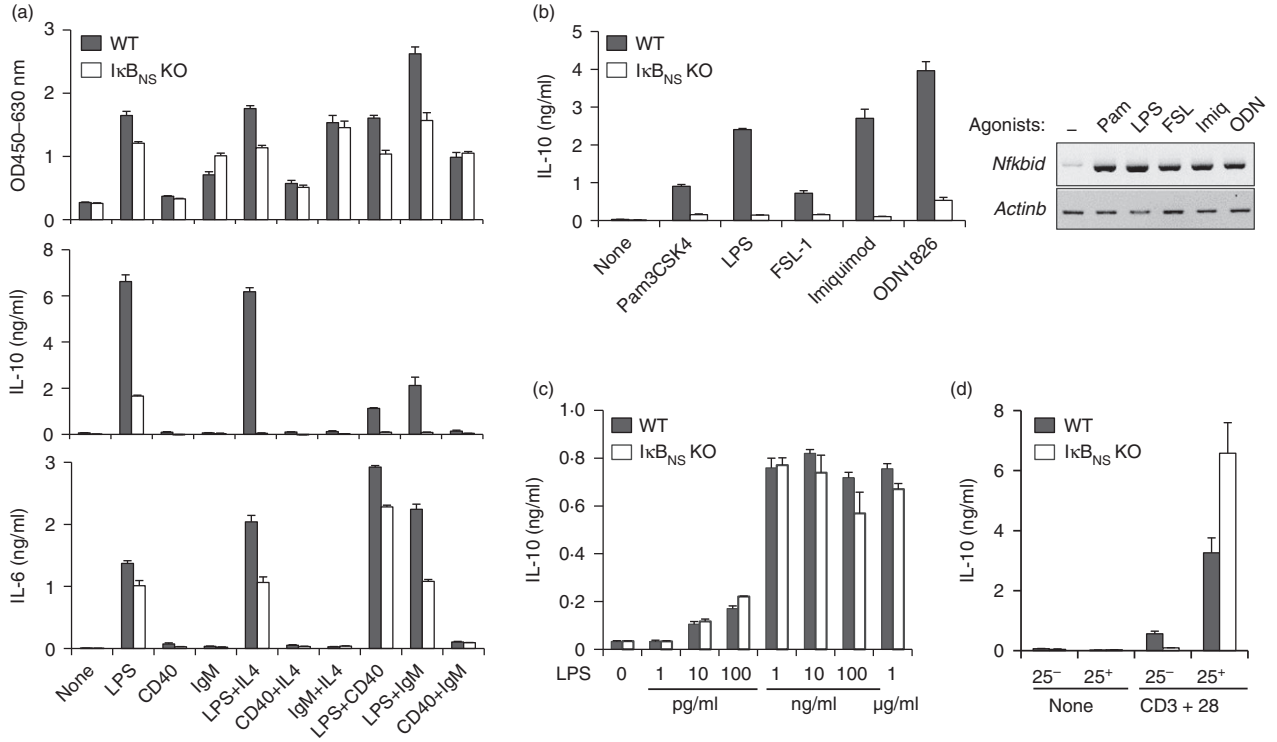

Figure 4. Toll-like receptor (TLR) -induced interleukin-10 (IL-10) production is impaired in IκB$_{NS}$-deficient B cells. (a) Splenic B cells isolated from wild-type and IκB$_{NS}^{-/-}$ spleens were incubated for 2 days in the presence of the indicated reagents [5 μg/ml lipopolysaccharide (LPS), 5 μg/ml anti-CD40, 10 μg/ml anti-IgM, 10 ng/ml rIL-4]. The proliferative activity of B cells was measured using a cell counting kit-8 (top). Culture supernatant was used for ELISA to determine the amounts of IL-10 (middle) and IL-6 (bottom). (b) Purified splenic B cells were cultured with various TLR agonists for 2 days to collect supernatants or for 2 hr for the RT-PCR analysis. The semi-quantitative RT-PCR analysis shows the TLR-induced expression of *Nfkbid* in wild-type B cells. β-Actin was used as a loading control. The concentration of each TLR agonist is described in the Materials and methods. (c) Naive macrophages in the peritoneal cavity were collected from wild-type and IκB$_{NS}^{-/-}$ mice and stimulated with the indicated amounts of LPS for 2 days. (d) CD4$^+$ CD25$^-$ conventional T cells (25$^-$) and CD4$^+$ CD25$^+$ regulatory T cells (25$^+$) from wild-type and IκB$_{NS}^{-/-}$ lymph nodes were FACS sorted and stimulated with or without anti-CD3ε and anti-CD28 for 3 days. The amount of cytokines in the culture supernatants was determined by a triplicate ELISA. Data represent at least two independent experiments.

Fig. 5(a) and as previously described.[18] The LPS-induced expression of *Il10* was observed within 2 hr of induction and continued for several days in wild-type B cells. Compared with the wild-type, the expression level of *Il10* in IκB$_{NS}^{-/-}$ B cells was lower during the first 24 hr of induction, and it increased to a level comparable to that in wild-type B cells after 48 hr of induction (Fig. 5a, b). Hence, a lack of IκB$_{NS}$ affects the early phase of *Il10* gene expression. The expression of other cytokine genes, i.e. IL-6, tumour necrosis factor-α and transforming growth factor-β, was not altered in the absence of IκB$_{NS}$.

Because TLR signalling pathways stimulate the activation of NF-κB and IκB$_{NS}$ can directly associate with NF-κB proteins in the cell nucleus,[12,17] we next examined the LPS-induced translocation of NF-κB to the nucleus and the association of NF-κB and the *Il10* promoter in the absence of IκB$_{NS}$. A Western blot analysis revealed that the levels of nuclear RelA (p65) and NF-κB1 (p50) similarly increased in LPS-stimulated wild-type and IκB$_{NS}^{-/-}$ B cells (Fig. 5c). The transient expression of IκB$_{NS}$ protein was detected only in nuclear extracts prepared from wild-type B cells, as expected.

The effect of a lack of IκB$_{NS}$ on NF-κB binding to the *Il10* promoter was examined using a DNA pull-down assay. The mouse *Il10* promoter contains two NF-κB binding sites that enhance *Il10* promoter activity in macrophages.[31,32] DNA probes containing NF-κB *cis* elements on the *Il10* proximal and distal promoters located at −55 to −46 and −861 to −851, respectively, were mixed with nuclear proteins extracted from LPS-stimulated B cells, and DNA-bound proteins were detected by Western blotting. Both p65 and p50 bound to DNA probes containing the proximal or distal κB site on the *Il10* promoter after LPS induction, and similar levels of pulled-down complexes were detected in wild-type and IκB$_{NS}^{-/-}$ extracts (Fig. 5d). IκB$_{NS}$ was not pulled down with *Il10* promoter DNA (data not shown). These results suggested that a lack of IκB$_{NS}$ does not affect NF-κB activity, at least on the *Il10* promoter in LPS-stimulated B cells.

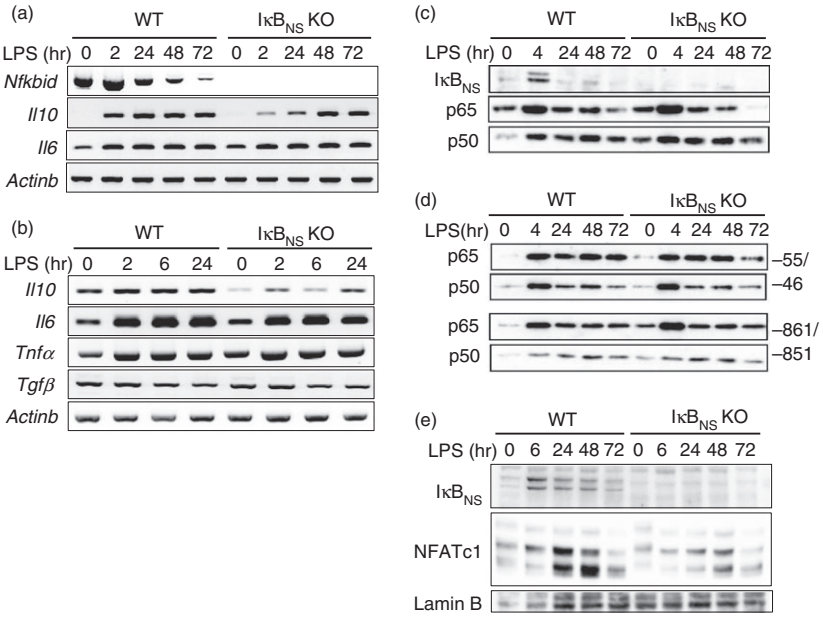

Figure 5. Expression of cytokine genes and nuclear factors in lipopolysaccharide (LPS) -activated IκB$_{NS}$-deficient B cells. (a, b) The LPS-induced transcription of *Il10* is reduced in IκB$_{NS}$-deficient B cells during the first 24 hr of induction. Total RNA was isolated from purified splenic B cells stimulated with 5 μg/ml of LPS for the indicated periods, and the semi-quantitative RT-PCR assessment of cytokine gene transcripts was performed. The LPS-induced transcription kinetics of *Nfkbid*, *Il10* and *Il6* genes (a) and cytokine expression profiles within 24 hr (b) are shown. (c) The expression and nuclear localization of IκB$_{NS}$, NF-κBp65, and p50 were analysed based on the immunoblotting of nuclear extracts prepared from LPS-stimulated wild-type and IκB$_{NS}^{-/-}$ B cells. The nuclear extracts were also used for DNA pull down with biotinylated probes for two interleukin-10 (IL-10) promoters containing a putative nuclear factor-κB (NF-κB)-binding site. (d) Western blot analysis of DNA pull down for the nuclear lysates and IL-10-promoter probes containing proximal (−55/−46) or distal (−861/−851) NF-κB motif. (e) The expression of NFATc1 was analysed based on the immunoblotting of nuclear extracts prepared from LPS-stimulated wild-type and IκB$_{NS}^{-/-}$ B cells. IκB$_{NS}$ shows two and NFATc1 shows several bands depending on their phosphorylation status. Lamin B was used as a nuclear loading control. All data represent at least two independent experiments.

A further investigation of signalling pathways in TLR-stimulated B cells revealed that the nuclear translocation and dephosphorylation of NFATc1 induced by LPS was reduced in IκB$_{NS}^{-/-}$ B cells (Fig. 5e). NFAT proteins control the Ca^{2+}-dependent signalling network and have been described to be required for IL-10 production.[35] Therefore, IκB$_{NS}$ may contribute to the Ca^{2+}-dependent signalling pathway, which positively regulates IL-10 production. Nevertheless, a direct interaction between NFATc1 and IκB$_{NS}$ was not detected by immunoprecipitation (data not shown).

Impaired differentiation of IL-10-producing plasmablasts in the absence of IκB$_{NS}$

Recent studies have shown that the expressions of Prdm1/Blimp-1 and Irf4, known as key transcription factors for the differentiation of plasma cells, are induced during IL-10 production in T and B cells.[36–38] Recently, plasmablasts and plasma cells have been identified as the main B-cell population in mice that produce cytokines, such as IL-10, IL-35, tumour necrosis factor-α, and granulocyte–macrophage colony-stimulating factor, under various conditions.[39] Because IκB$_{NS}^{-/-}$ splenic B cells exhibit a defect in plasma cell differentiation,[18] we examined the expression of *Prdm1* and *Irf4* in IL-10-competent B10 cells isolated from IκB$_{NS}^{-/-}$ mice. The expression levels of *Prdm1* and *Irf4* in CD1d^{hi} CD5^{+} B cells were significantly diminished by a lack of IκB$_{NS}$ (Fig. 6a), and similar results were obtained with MZ B cells (Fig. 6b).

Because splenic B10 cells can differentiate into antibody-secreting plasma cells[40] and splenic CD138^{+} plasma cells express IL-10,[27] we suspected that TLR-induced IL-10-producing B cells may be plasma cells. As shown in Fig. 6(c), IL-10-producing B cells could be detected by flow cytometry within 48 hr of LPS induction in control B cells, and most IL-10^{+} B cells were negative for CD138 on day 2. CD138^{+} IL-10^{+} B cells were then also differentiated from wild-type B cells on day 3. Conversely, only a small number of CD138-negative IL-10^{+} B cells were generated from IκB$_{NS}^{-/-}$ B cells, even after 3 days of induction. Notably, CD138^{+} IL-10^{+} cells observed in wild-type B cells express higher levels of CD44, and CD138^{-} IL-10^{+} cells consist of both CD44^{hi} and CD44^{med} cells. Hence, LPS induced the development of both CD138^{-} IL-10^{+} cells and CD138^{+} CD44^{hi} IL-10^{+} plasmablasts in

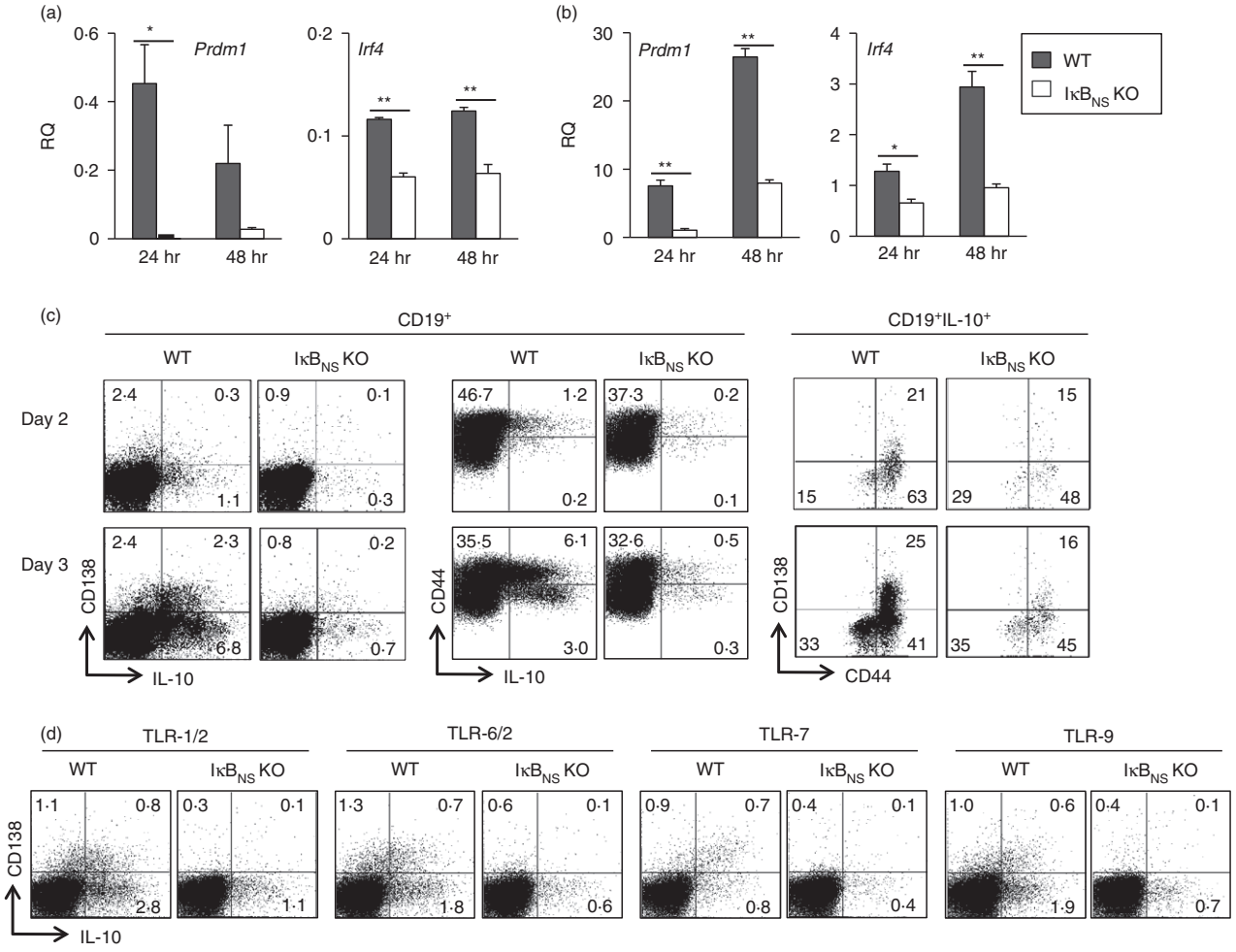

Figure 6. Toll-like receptor (TLR) -induced differentiation of IL-10$^+$ CD138$^+$ plasmablasts is impaired in the absence of IκB$_{NS}$. (a, b) Real-time PCR analysis of the expression of *Prdm1* and *Irf4*. FACS-sorted CD1d^{hi} CD5$^+$ B cells (a) and CD21^{hi} CD23^{lo} MZ B cells (b) from wild-type and IκB$_{NS}^{-/-}$ spleens were cultured for the indicated periods in the presence of 5 μg/ml lipopolysaccharide (LPS), and total RNA was isolated and subjected to quantitative PCR analysis. All expression data were normalized to β-actin expression. The data are representative of two independent experiments. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$. (c) Splenic B cells isolated from wild-type and IκB$_{NS}^{-/-}$ mice were stimulated with LPS (1·0 μg/ml) for 2 or 3 days, and the expression levels of surface CD138, CD44, and cytoplasmic interleukin 10 (IL-10) on CD19$^+$ B cells were analysed by flow cytometry. (d) Splenic B cells from wild-type and IκB$_{NS}^{-/-}$ mice were incubated with various TLR agonists for 3 days, and the expression of CD138 and cytoplasmic IL-10 were analysed by flow cytometry. Representative FACS profiles with the percentages of cells in quadrant gates are shown. The data represent three independent experiments.

wild-type B cells, whereas the development of CD138$^+$ CD44^{hi} IL-10$^+$ cells was impaired in the absence of IκB$_{NS}$. Similarly, a severe defect of the differentiation of CD138$^+$ IL-10$^+$ cells in IκB$_{NS}^{-/-}$ B-cell cultures was observed, even when we used TLR agonists other than LPS (Fig. 6d). Collectively, the TLR-induced development of IL-10-producing plasmablasts is impaired in the absence of IκB$_{NS}$.

Discussion

We have previously shown that IκB$_{NS}$ deficiency results in a defective TI antigen response and a developmental defect in peritoneal B-1 B cells, which are known to produce large amounts of IL-10 via TLR engagements.[41,42] Accordingly, we speculated that IκB$_{NS}$ plays a role in the development of IL-10-producing B cells responding to innate signals. As previously reported, splenic B cells with the CD1d^{hi} CD5$^+$ phenotype predominantly produce IL-10. Consequently, we examined the development and function of CD1d^{hi} CD5$^+$ B10 cells in IκB$_{NS}^{-/-}$ mice. Our data shown here suggest that IκB$_{NS}$ plays significant roles in the development of IL-10-competent B cells and TLR-induced IL-10 production in B cells. Although BCR-dependent IL-10 production has been demonstrated in several autoimmune models, TLR signalling has also been

shown to be required for BCR-mediated IL-10 expression.[35] Therefore, in addition to many studies of regulatory B-cell induction through the BCR signals, the current study addressed the role of innate B-cell responses in IL-10 secretion.

In addition to splenic B10 and T2-MZP populations, which can provide IL-10-dependent regulatory function in recipient mice upon adoptive transfer,[22] more recent studies identified plasmablasts/plasma cells as major B-cell populations producing immunosuppressive IL-10.[39] Recently, Matsumoto *et al.* demonstrated that IL-10-producing plasmablasts developed in the draining lymph node could suppress experimental autoimmune encephalomyelitis.[38] These findings indicate that the mechanism regulating IL-10 is, at least partially, shared with a mechanism regulating antibody production in B cells. In this context, we examined the expression of *Prdm1*, *Irf4* and CD138 in splenic B cells and found that the expression of these factors was significantly reduced by a lack of IκB$_{NS}$. This is consistent with the report that CD138^{+} plasmablasts develop under the control of Blimp1 and interferon regulatory factor 4 (IRF4) and that IRF4 regulates *Il10* expression in B cells[38] and in T cells.[36,37]

In this study, we showed that B cells stimulated with TLR agonists differentiate into two IL-10-producing populations: CD138^{-} and CD138^{+}. Although both IL-10^{+} populations were diminished in IκB$_{NS}{}^{-/-}$ B-cell cultures, a severe defect of IL-10-producing CD138^{+} CD44^{+} plasmablasts was observed in the absence of IκB$_{NS}$. The intensity of cytoplasmic IL-10 in response to TLR stimulation was higher in CD138^{-} cells than in CD138^{+} cells, although CD138^{+} plasmablasts were found to predominantly produce IL-10 during experimental autoimmune encephalomyelitis.[38] This difference might be caused by differences in the signalling components: the former consists of only TLR signalling, whereas the latter is a complex of signals by BCR, TLRs and cytokines *in vivo*.

We have examined the mechanisms by which IκB$_{NS}$ regulates IL-10 production. Because IκB$_{NS}$ lacks a DNA-binding domain, it may affect the activation of transcription factors associated with the *Il10* promoter. We first speculated that the absence of IκB$_{NS}$ results in the aberrant expression and/or activation of NF-κB. However, the LPS-induced expression and nuclear translocation of NF-κB were similar between wild-type and IκB$_{NS}{}^{-/-}$ B cells. However, we found that IκB$_{NS}{}^{-/-}$ B cells express reduced levels of NFATc1. This observation may provide a clue to understand the molecular mechanism underlying IκB$_{NS}$-dependent IL-10 production because the Ca^{2+}-dependent signalling pathway has been shown to be important in IL-10 production. Specifically, B-cell-mediated IL-10 secretion after BCR stimulation occurred in a Ca^{2+} influx-dependent fashion and consequently required NFAT activation.[35]

The involvement of IκB$_{NS}$ in BCR-mediated IL-10 expression remains to be explored. As we have previously reported, mature B cells in IκB$_{NS}{}^{-/-}$ mice express slightly higher levels of surface IgM than B cells in wild-type mice. As a consequence, the proliferative response induced by the cross-linking of IgM is slightly higher in IκB$_{NS}{}^{-/-}$ B cells. Therefore, even if IκB$_{NS}$-deficiency affects BCR-mediated signals, the increased number of IgM molecules on a cell may mask a potential response to BCR signalling in IκB$_{NS}{}^{-/-}$ B cells. However, this relationship may not definitively implicate IκB$_{NS}$ in BCR-mediated responses because IκB$_{NS}$ deficiency results in defective TI-2 antigen responses, which require extensive BCR cross-linking. Indeed, impaired NFAT expression was observed in BCR-stimulated IκB$_{NS}{}^{-/-}$ B cells (unpublished data). The recently identified function of IκB$_{NS}$ in TCR-mediated T-cell development[34,43,44] implies a role for IκB$_{NS}$ in the regulation of antigen-specific immune responses.

The phenotype of IκB$_{NS}{}^{-/-}$ B cells partly overlaps with that of IκB-ζ-deficient B cells. This overlap is noteworthy because IκB$_{NS}$ and IκB-ζ are homologous members of the nuclear IκB family, and both factors have been demonstrated to interact with p50.[11,12] IκB-ζ-deficient B cells exhibit impaired plasma cell differentiation in response to LPS.[45] Interestingly, IκB$_{NS}$ and IκB-ζ have an inverse function in the regulation of the TLR-induced expression of inflammatory cytokines, such as IL-6.[8] The elucidation of the relationship and cooperation within these IκB proteins may allow us to understand the regulatory mechanisms of NF-κB in the context of TLR signalling.

In summary, our study proposes additional roles for IκB$_{NS}$ in the development of IL-10-competent B cells and the modulation of B-cell functions induced by TLRs. B-cell-intrinsic TLR signalling is also crucial for the generation and activation of autoreactive B cells.[46] In this context, this unique signalling process could affect the pathogenesis of autoimmune diseases. Hence, further studies will be required to elucidate the exact mechanism underlying TLR ligation-triggered B-cell activation and develop new strategies for immune disorders.

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Disclosures

The authors declare no financial or commercial conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Flow cytometry analysis of B-cell subsets in $I\kappa B_{NS}^{-/-}$ spleens.

Figure S2. Reconstitution of B-cell compartments in bone marrow (BM) chimeric spleens.

Figure S3. Impaired regulatory B cell functions in $I\kappa B_{NS}$-deficient B cells (related to Fig. 3c).