

B-cell linker protein expression contributes to controlling allergic and autoimmune diseases by mediating IL-10 production in regulatory B cells

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Background: Regulatory B cells that exhibit the cell-surface CD1d^{hi}CD5⁺ phenotype and produce IL-10 are termed B10 cells. Although B10 cells exert potent suppressive functions in patients with various allergic and autoimmunity disorders, the precise signaling mechanisms required for B10 cell functions remain unknown. B-cell linker protein (BLNK) is an essential component of the B-cell antigen receptor signaling pathway and is required for optimal B-cell development.

Objective: We sought to elucidate the signaling pathways that are responsible for IL-10 production in B10 cells and *in vivo* mechanisms of how impaired B10 cell functions influence allergic and autoimmune responses.

Method: For *in vitro* assays, splenic CD1d^{hi}CD5⁺ B cells from BLNK-deficient (BLNK^{-/-}) mice were analyzed for intracellular signaling pathways and cytokine production.

Contact hypersensitivity (CHS) and experimental autoimmune encephalomyelitis were examined by using BLNK^{-/-} mice.

Results: Although the CD1d^{hi}CD5⁺ B-cell population was present in BLNK^{-/-} mice, IL-10 production was impaired both *in vitro* and *in vivo*. BLNK^{-/-} mice had exaggerated CHS and experimental autoimmune encephalomyelitis responses, which were normalized by adoptive transfer of splenic CD1d^{hi}CD5⁺ B cells from wild-type mice. In mice with CHS, BLNK^{-/-} mice exhibited decreased B-cell and regulatory T-cell percentages and increased CD8⁺ T-cell percentages in the skin and lymph nodes. *In vitro* BLNK was required for LPS-induced signal transducer and activator of transcription 3 phosphorylation in CD1d^{hi}CD5⁺ B cells. Finally, secreted IL-10 leads to autocrine expansion of IL-10-producing B cells.

Conclusion: BLNK serves as a critical signaling component for B10 cell function by mediating IL-10 production. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: B10 cell, IL-10, B-cell linker protein, signal transducer and activator of transcription 3

B cells play a central role in humoral immunity by producing antibodies while also exerting various functions essential for optimal immune responses, such as cytokine production and antigen presentation.¹ Through these functions, B cells generally enhance immune reactions, although studies have identified suppressive roles provided by “regulatory B cells.”²⁻⁶ Although there might be several regulatory B-cell subsets, recent studies have revealed that a specific CD1d^{hi}CD5⁺ B-cell population in the spleen potentially inhibits immune responses through the secretion of IL-10; these are termed B10 cells.^{3,4,7} B10 cells have been demonstrated to suppress murine allergic and autoimmune diseases, such as contact hypersensitivity (CHS),⁷ asthma,⁸ experimental autoimmune encephalomyelitis (EAE),⁹ lupus,¹⁰ and inflammatory bowel disease.¹¹

Regulatory activities of B10 cells are induced after cellular activation. B10 cells are currently defined as B cells that produce IL-10 after 5 hours of *in vitro* stimulation with LPS, phorbol 12-myristate 13-acetate (PMA), and ionomycin.³ Among these stimuli, LPS-induced Toll-like receptor (TLR) 4 signaling is considered critical.¹²⁻¹⁴ LPS stimulation initiates the production and secretion of IL-10, and restimulation through the B-cell antigen receptor (BCR), CD40, or both robustly enhances IL-10 production, suggesting that a 2-step process is required for optimal IL-10 production and B10 differentiation.¹²⁻¹⁴ Nonetheless, the precise signaling mechanisms responsible for the induction of B10 cells remain unknown.

These cellular signals are tightly mediated by arrays of cell-surface and intracellular molecules.¹⁵ The BCR signalosome is composed of B cell-specific/dominant molecules, including spleen tyrosine kinase (Syk), Bruton tyrosine kinase (Btk), and the B-cell linker protein (BLNK; also known as SLP65 and BASH).^{16,17} BLNK is a B cell-specific adaptor molecule that contains multiple protein-binding motifs. Activated Syk phosphorylates BLNK, allowing it to interact with Btk through its src homology 2 domain. Disturbed expression of B-cell signalosome molecules incurs severely altered development and function of B cells in mice and human subjects.¹⁸⁻²¹ Importantly, the loss of these molecules has been appreciated to affect other signaling pathways, including those downstream of LPS and CD40, suggesting diverse roles for this signalosome.^{16,17,19,21,22} BLNK-deficient (BLNK^{-/-}) B cells show diminished responses to LPS or anti-CD40 stimulation,^{16,17,19,21,22} suggesting the possibility that BLNK also serves as a critical signaling component in B10 cells.

In this study we sought to elucidate the signaling pathways responsible for IL-10 production in CD1d^{hi}CD5⁺ B10 cells.

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Abbreviations used

APC:	Allophycocyanin
BCR:	B-cell antigen receptor
BFA:	Brefeldin A
BLNK:	B-cell linker protein
Btk:	Bruton tyrosine kinase
CHS:	Contact hypersensitivity
DNFB:	2, 4-Dinitrofluorobenzene
EAE:	Experimental autoimmune encephalomyelitis
FITC:	Fluorescein isothiocyanate
H&E:	Hematoxylin and eosin
JAK:	Janus kinase
LN:	Lymph node
NF-κB:	Nuclear factor κB
PE:	R-phycoerythrin
PerCP:	Peridinin chlorophyll protein
PIB:	PMA, ionomycin, and BFA
PMA:	Phorbol 12-myristate 13-acetate
STAT:	Signal transducer and activator of transcription
Syk:	Spleen tyrosine kinase
TLR:	Toll-like receptor
Treg:	CD4 ⁺ CD25 ⁺ FoxP3 ⁺ regulatory T

BLNK was indispensable for LPS-induced IL-10 production through signal transducer and activator of transcription (STAT) 3 pathways in CD1d^{hi}CD5⁺ B cells. BLNK expression was also required for *in vivo* function of regulatory B cells because BLNK^{-/-} mice exhibited an augmented CHS response with expansion of IFN-γ-producing CD8⁺ T cells and decreased CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cell infiltration.

METHODS

The methods used in this study are described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS**The CD1d^{hi}CD5⁺ B cell subset is present in BLNK^{-/-} mice but is not capable of producing IL-10**

To assess whether BLNK expression influences the number, frequency, or both of regulatory B cells, we examined B-cell fractions in the spleens of BLNK^{-/-} mice. As reported previously,¹⁹ BLNK^{-/-} mice exhibited decreased percentages of mature B cells in the spleen compared with those seen in wild-type mice (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org), whereas ratios of transitional stage 1, transitional stage 2, and follicular B cells were normal (see [Fig E1, B](#)). By contrast, BLNK^{-/-} mice showed markedly increased percentages of CD1d^{hi}CD5⁺ B cells ([Fig 1, A](#)). Although total CD1d^{hi}CD5⁺ B-cell numbers were reduced in BLNK^{-/-} mice because of the decrease in total B-cell numbers, BLNK deficiency affected the development of CD1d^{hi}CD5⁺ B cells less than the development of follicular B cells. Additionally, the frequencies of marginal zone B cells and transitional stage 2 marginal zone precursor B cells, which have also been reported to contain IL-10-producing regulatory B cells, were also increased in BLNK^{-/-} mice (see [Fig E1, C and D](#)).

Next, we assessed whether BLNK expression influences the function of regulatory B cells. Splenic B cells from wild-type and BLNK^{-/-} mice were stimulated with LPS plus PMA, ionomycin,

and BFA (LPS plus PIB) to determine whether the absence of BLNK in B cells affects IL-10 production. The expression of the TLR4/MD2 complex was not different between wild-type and BLNK^{-/-} mice (see [Fig E1, E](#)). IL-10⁺ B cells comprised approximately 2% to 4% of total B cells in wild-type mice but were nearly absent in BLNK^{-/-} mice ([Fig 1, B](#)), indicating that BLNK is required for LPS+PIB-induced IL-10 production. T-cell subsets, including CD4⁺ T cells, CD8⁺ T cells, and Treg cells, were not altered in BLNK^{-/-} mice (see [Fig E1, F and G](#)).

Development of severe CHS and EAE in BLNK^{-/-} mice

Because IL-10 production induced by LPS plus PIB *in vitro* was abrogated in BLNK^{-/-} B cells, we investigated whether BLNK expression was important for the suppression of EAE and CHS. In the setting of CHS, BLNK^{-/-} mice displayed significantly enhanced swelling in challenged ears compared with that seen in wild-type mice ([Fig 2, A](#)). Histopathologic analysis also revealed that the degree of edema and cellular infiltration was markedly enhanced in BLNK^{-/-} mice compared with that seen in wild-type mice ([Fig 2, B](#)). Moreover, ear skin sections from BLNK^{-/-} mice exhibited an approximately 2-fold increase in CD3⁺ T-cell infiltration compared with that seen in wild-type mice ([Fig 2, C](#)).

In the setting of EAE, BLNK^{-/-} mice showed rapid disease progression and significantly higher disease scores than wild-type mice (see [Fig E2, A](#), in this article's Online Repository at www.jacionline.org). Inflammatory cell infiltration was also more extensive in the brains of BLNK^{-/-} mice (see [Fig E2, B](#)). On histopathologic analysis, inflammation and demyelination areas were more widespread in BLNK^{-/-} mice than in wild-type mice (see [Fig E2, C and D](#)). Thus BLNK deficiency results in more severe CHS and EAE responses.

BLNK deficiency affects CD8⁺ T-cell infiltration and cytokine production during the CHS response

Next, infiltrating cells and cytokine production were assessed in the inflammatory sites of CHS-induced mice. In the ear skin, the CD4⁺/CD8⁺ T-cell ratio and percentages of Treg cells were significantly decreased in BLNK^{-/-} mice relative to those seen in wild-type mice ([Fig 2, D and E](#)). CD19⁺ B-cell infiltration was substantially less in BLNK^{-/-} mice than in wild-type mice ([Fig 2, F](#)). In lymph nodes (LNs), the percentages of CD4⁺ T cells, CD8⁺ T cells, and Treg cells were comparable between wild-type and BLNK^{-/-} mice before elicitation ([Fig 2, G](#)). By contrast, similar to the skin, the percentage of CD8⁺ T cells was significantly increased in BLNK^{-/-} mice relative to that seen in wild-type mice in the draining LNs 48 hours after elicitation ([Fig 2, G](#)). Furthermore, BLNK^{-/-} mice also showed only a modest increase in Treg cell numbers in LNs compared with that seen in wild-type mice at 48 hours ([Fig 2, G](#)). To evaluate T_H1, T_H2, and T_H17 cytokine production in LNs, T cells were stimulated with PMA plus ionomycin for 5 hours *in vitro*, and intracellular staining of IFN-γ, IL-4, IL-10, and IL-17 was performed. The percentage of CD8⁺IFN-γ⁺ T cells was significantly increased in BLNK^{-/-} mice compared with wild-type mice ([Fig 2, H](#)). In contrast, IL-4, IL-10, and IL-17 were hardly increased in T cells from wild-type and BLNK^{-/-} mice (data not shown).

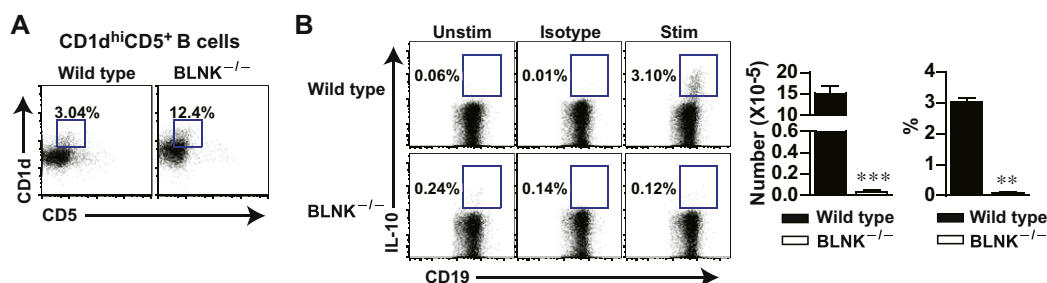


FIG 1. Loss of BLNK impairs IL-10 production in B cells. **A**, CD1d^{hi}CD5⁺CD19⁺ B-cell fractions in the spleen. **B**, Splenic B cells were treated with or without LPS plus PIB for 5 hours and then stained with anti-CD19 mAb. After permeabilization, the cells were stained with anti-IL-10 mAb or isotype control mAb. Percentages indicate IL-10⁺ B-cell proportions. Data were obtained from 3 or more mice in each group. ***P* < .01 and ****P* < .001.

Loss of BLNK attenuates IL-10 production in B cells *in vivo*

Next, we assessed mRNA expression of cytokines in splenic B cells from wild-type and BLNK^{-/-} mice. Overall, IL-10 was the only cytokine with significantly increased levels in B cells during the peak clinical phase, whereas IL-10 expression was significantly lower in B cells from BLNK^{-/-} mice than in those from wild-type mice (Fig 3 and see Fig E3 in this article's Online Repository at www.jacionline.org). IL-10 expression in draining LN B cells was also significantly lower in BLNK^{-/-} mice than in wild-type mice after CHS induction (see Fig E4 in this article's Online Repository at www.jacionline.org). Thus BLNK deficiency also represses IL-10 production in B cells *in vivo*.

BLNK expression in CD1d^{hi}CD5⁺ B cells is critical for the suppression of CHS and EAE

Adoptive transfer experiments were performed to directly confirm that exacerbated manifestations of CHS and EAE in BLNK^{-/-} mice arose from the absence of suppression provided by B10 cells. Splenic B cells from wild-type mice were sorted into CD1d^{hi}CD5⁺ and CD1d^{lo}CD5⁻ B cells (see Fig E4, A, in this article's Online Repository at www.jacionline.org). CHS response in BLNK^{-/-} mice was attenuated by adoptive transfer of CD1d^{hi}CD5⁺ B cells but not CD1d^{lo}CD5⁻ B cells (Fig 4, A). By contrast, when CD1d^{hi}CD5⁺ B cells from IL-10^{-/-} mice were transferred into BLNK^{-/-} mice, the severity and course of CHS were not altered (Fig 4, B). Although these results demonstrate that the exaggerated response in BLNK^{-/-} mice is due to the lack of CD1d^{hi}CD5⁺ B cells that secrete IL-10, whether this was a qualitative (insufficient IL-10 production by CD1d^{hi}CD5⁺ B cells) or quantitative (decreased numbers of CD1d^{hi}CD5⁺ B cells) defect in BLNK^{-/-} mice was not clear. Therefore additional B cells from BLNK^{-/-} mice were transferred into BLNK^{-/-} recipients to generate numbers of CD1d^{hi}CD5⁺ B cells equivalent to those found in wild-type mice, although this transfer did not alter the severity of CHS in BLNK^{-/-} mice (Fig 4, C). Therefore increased CHS severity in BLNK^{-/-} mice was due to impaired IL-10 production in B10 cells rather than merely a decreased number of B10 cells.

Likewise, the EAE reaction in BLNK^{-/-} mice was attenuated by adoptive transfer of wild-type CD1d^{hi}CD5⁺ B cells but not wild-type CD1d^{lo}CD5⁻ (see Fig E4, B), IL-10^{-/-}CD1d^{hi}CD5⁺ (see Fig E4, C), or BLNK^{-/-} (see Fig E4, D) B cells. Taken together, B10 cells are functionally impaired in BLNK^{-/-} mice.

Nuclear factor κ B signaling pathway is involved in the secretion of IL-10 in regulatory B cells

Because BLNK regulates multiple signaling molecules, we attempted to pinpoint the pathways responsible for IL-10 induction. Wild-type B cells were pretreated with various inhibitors and then stimulated with LPS plus PIB. IL-10 production was completely inhibited by the Btk inhibitor (-)-terreic acid and the nuclear factor κ B (NF- κ B) inhibitor Bay11-7082 (Fig 5, A and B). Additionally, the Src family kinase inhibitor PP2 and the Syk inhibitor Bay61-3606 significantly but not completely decreased the frequency of IL-10⁺ B cells. Furthermore, the p38 inhibitor SB202190 modestly reduced IL-10 production, whereas the c-Jun N-terminal kinase inhibitor did not. These results suggest that the B-cell signalosome and NF- κ B pathway are involved in LPS-induced IL-10 production in B cells.

Next, LPS-induced p38 and NF- κ B activation was examined. LPS stimulation did not induce p38 phosphorylation (data not shown) but did induce NF- κ B phosphorylation in B cells from wild-type and BLNK^{-/-} mice (Fig 5, C). Although NF- κ B was activated in total and CD1d^{hi}CD5⁺ B cells, NF- κ B phosphorylation was significantly lower in BLNK^{-/-} B cells than in wild-type counterparts (Fig 5, C and D). Nonetheless, the decrease in phospho-NF- κ B levels was less profound in CD1d^{hi}CD5⁺ B cells compared with total B cells. Thus, although BLNK can partially mediate LPS-induced NF- κ B activation, other pathways appear to be more critical for NF- κ B activation in B10 cells.

STAT3 phosphorylation is required for LPS-induced IL-10 production in B cells

Because the STAT pathway is closely associated with cytokine production, we hypothesized that IL-10 production in B10 cells was also related to BLNK-mediated STAT activation. To support this, preincubation with the STAT3 inhibitor S31-201 or STAT3 inhibitor VII completely abrogated IL-10 production in B cells stimulated with LPS or LPS plus PIB, whereas no decrease in IL-10 production was observed after treatment with either Janus kinase (JAK) inhibitor I or STAT5 inhibitor (Fig 6, A and B). Thus, STAT3 is required for LPS-induced IL-10 production, independently of JAK activation.

Next, we examined STAT3 phosphorylation in B-cell subsets. STAT3 was phosphorylated in CD1d^{hi}CD5⁺ but not in CD1d^{lo}CD5⁻ B cells from wild-type mice after treatment with LPS alone or LPS plus PMA (Fig 6, C). STAT3 phosphorylation was readily detectable in CD1d^{hi}CD5⁺ B cells after 30 minutes

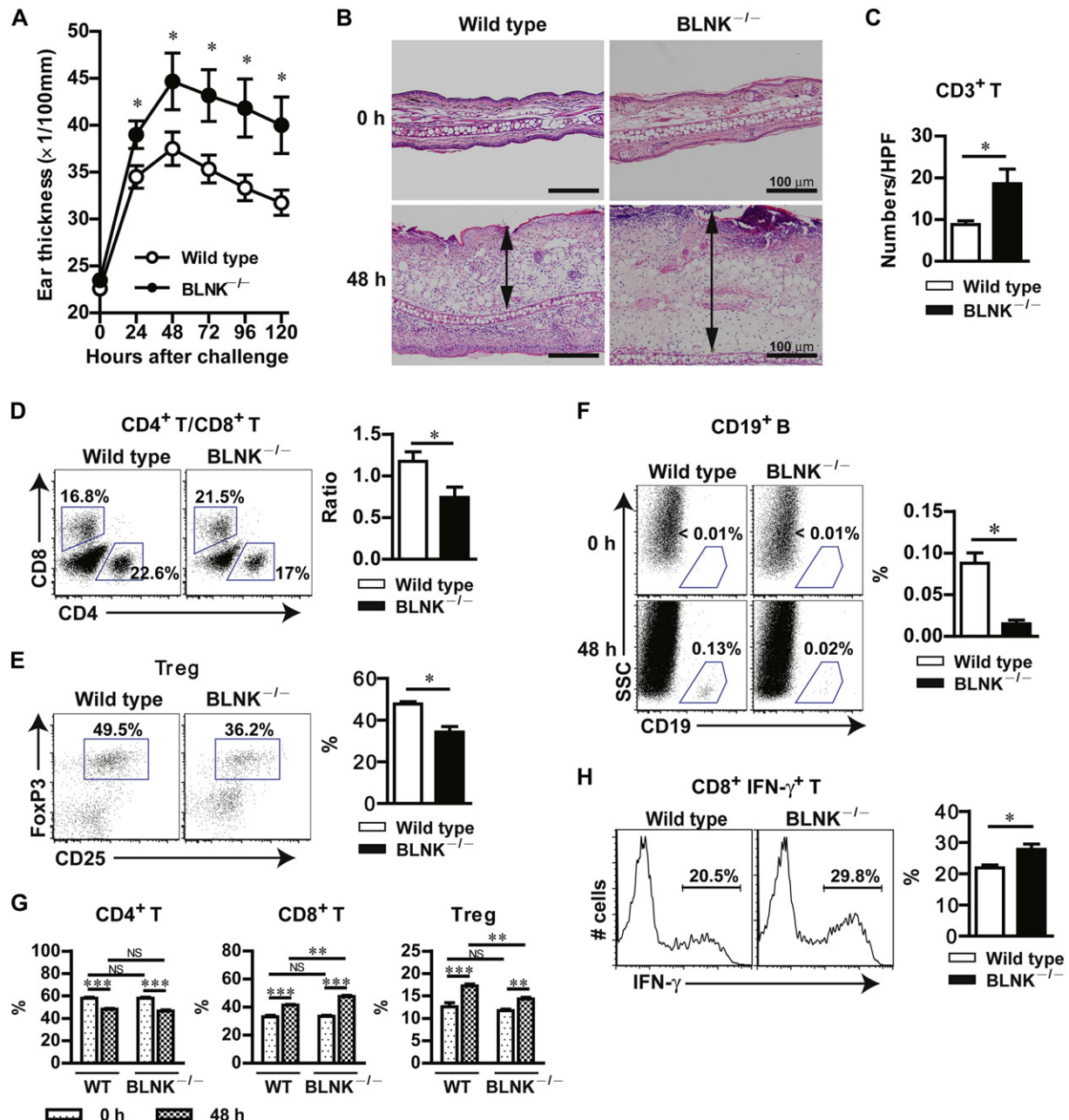


FIG 2. BLNK deficiency promotes CHS responses. **A**, Thickness of ear lobes. **B**, Representative histopathology. Sections were stained with hematoxylin and eosin. Original magnification $\times 100$. **C**, CD3⁺ T-cell infiltration in the ear section at 48 hours. **D–F**, Lymphocyte infiltration in the ear skin. SSC, Side scatter. **G** and **H**, CD4⁺ T cells, CD8⁺ T cells, and Treg cells (Fig 2, **G**) and CD8⁺IFN- γ ⁺ T cells (Fig 2, **H**) from LNs. Data were obtained from 5 mice in each group. * $P < .05$, ** $P < .01$, and *** $P < .001$. NS, Not significant.

and reached a maximum after 3 hours. CpG stimulation also induced STAT3 phosphorylation in CD1d^{hi}CD5⁺ B cells, whereas BCR stimulation or PMA treatment alone did not (data not shown). Therefore TLRs specifically induce STAT3 phosphorylation only in CD1d^{hi}CD5⁺ B cells.

STAT3 phosphorylation was also assessed in CD1d^{hi}CD5⁺ B cells from BLNK^{-/-} mice after LPS or LPS plus PMA stimulation. In contrast to wild-type CD1d^{hi}CD5⁺ B cells, no significant increase in STAT3 phosphorylation was detectable in BLNK^{-/-} CD1d^{hi}CD5⁺ B cells (Fig 6, **C** and **D**). Furthermore, STAT3

phosphorylation in wild-type CD1d^{hi}CD5⁺ B cells was diminished by (-)-terreic acid or BAY61-3606 but not by PP2 (Fig 6, **E** and **F**). Therefore, BLNK and Btk are critical for STAT3 activation in CD1d^{hi}CD5⁺ B cells.

BLNK is not involved in JAK-STAT signaling pathways engaged by cytokine stimulation

Because a previous study demonstrated that BLNK negatively regulates the JAK3-STAT5 signaling pathway,²³ dysregulated

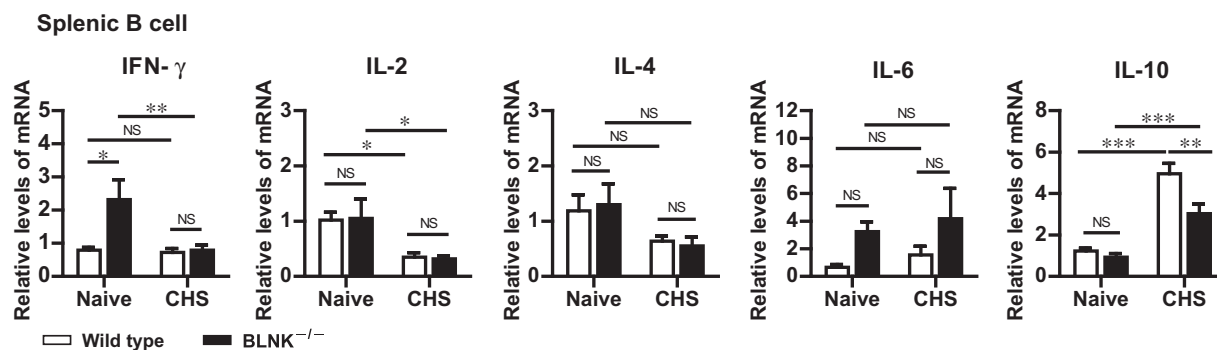


FIG 3. Cytokine expression in splenic B cells. mRNA levels of cytokines were analyzed by using RT-PCR. Data were obtained from 4 to 7 mice in each group. * $P < .05$, ** $P < .01$, and *** $P < .001$. NS, Not significant.

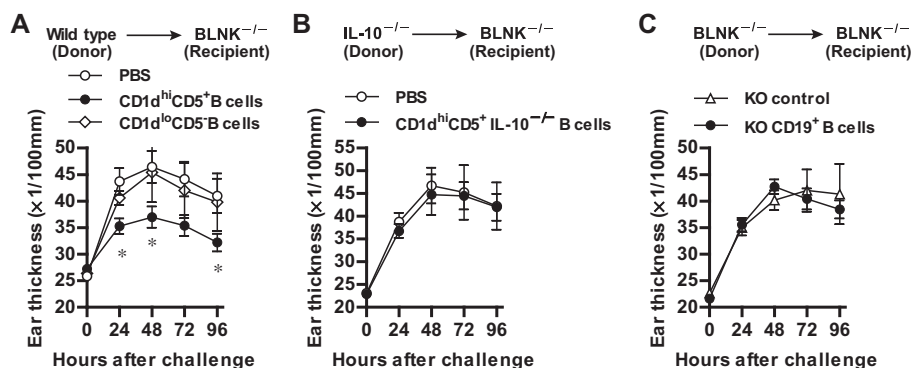


FIG 4. B10 cells attenuate CHS in an IL-10-dependent manner. **A** and **B**, B-cell subsets from wild-type ($n \geq 5$) or IL-10^{-/-} mice ($n \geq 5$) were transferred into BLNK^{-/-} mice before challenge. **C**, Splenic CD19⁺ B cells from naive BLNK^{-/-} mice ($n \geq 10$) were transferred into BLNK^{-/-} mice. KO, Knockout. Data were obtained from 5 mice in each group. * $P < .05$.

phosphorylation of STAT3 in BLNK^{-/-} B cells might be intrinsic, regardless of the stimulation type. To test this possibility, splenic B cells from wild-type and BLNK^{-/-} mice were treated with the known STAT3 activators IL-6, IL-10, or IL-21. STAT3 phosphorylation levels were equivalent between wild-type and BLNK^{-/-} B cells (see Fig E5 in this article's Online Repository at www.jacionline.org), indicating that BLNK is not required for JAK-mediated STAT3 phosphorylation. Thus, BLNK is specifically required for STAT3 phosphorylation induced by TLR stimulation in CD1d^{hi}CD5⁺ B cells.

IL-10-producing B-cell numbers are increased though an IL-10 autocrine loop

After LPS plus PMA stimulation, STAT3 phosphorylation increased to the maximum level compared with that seen with LPS stimulation alone. Increased STAT3 phosphorylation can be ascribed to further activation of B10 cells in response to IL-10 signaling. rIL-10 was added for 5 hours along with LPS plus PIB to explore this possibility. IL-10⁺ B-cell numbers increased to $6.3\% \pm 0.3\%$, which was up from $3.8\% \pm 0.3\%$ without exogenous IL-10 (Fig 7, A). This increase was equivalent to that seen after anti-CD40 stimulation for 48 hours when LPS plus PIB was added during the last 5 hours, a condition known to induce high frequencies of IL-10⁺ B cells (Fig 7, B).¹² rIL-10 stimulation in addition to this condition did not further increase the numbers/frequencies of IL-10⁺ B cells (see Fig E6, A, in this article's

Online Repository at www.jacionline.org). rIL-6 did not alter the frequency of IL-10⁺ B cells in either condition (Fig 7, A and B).

Percentages of IL-10⁺ B cells were not increased when B cells were pretreated with JAK inhibitor I (Fig 7, C), suggesting that this increase is dependent on JAK activation. In BLNK^{-/-} B cells, despite the addition of rIL-10 during LPS plus PIB stimulation, the frequency of IL-10⁺ B cells was not increased (see Fig E6, B). These results suggest that secreted IL-10 subsequently increased B10 cell numbers by activating the JAK-STAT3 pathway.

DISCUSSION

The results described herein demonstrate that LPS-induced IL-10 production in B10 cells requires STAT3 phosphorylation. LPS-induced STAT3 phosphorylation was restricted to CD1d^{hi}CD5⁺ B cells and dependent on BLNK expression. IL-10 secretion from CD1d^{hi}CD5⁺ B cells led to an autocrine increase in the percentage of IL-10-producing B cells. Thus this study elucidated key mechanisms underlying the induction of LPS-dependent IL-10 production specifically in B10 cells. BLNK expression was also required *in vivo* for regulatory B-cell suppression of CHS and EAE. In the setting of CHS, the profile of lymphocytes in the skin and LNs was significantly altered in BLNK^{-/-} mice; CD8⁺ T-cell percentages were significantly increased, whereas Treg cell percentages were significantly reduced. Therefore the study also demonstrated the mechanisms of how regulatory B cells contribute to the suppression of the CHS response.

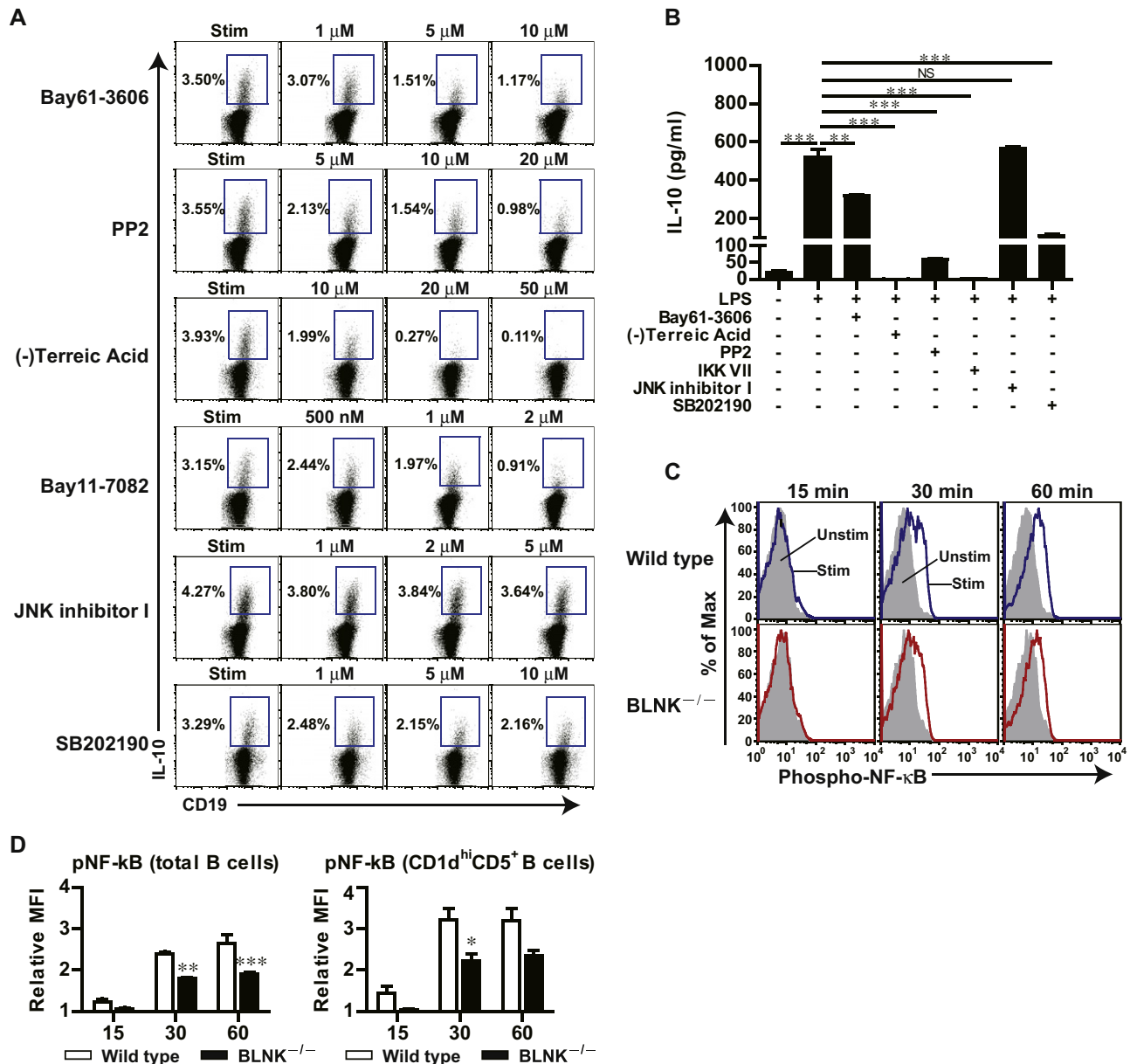


FIG 5. NF- κ B activation is required for IL-10 production. **A**, IL-10 production from a wild-type splenocyte was assessed as in Fig 1. **B**, IL-10 levels in supernatants were measured by means of ELISA. **C** and **D**, B cells were stimulated with LPS (10 μ g/mL). Fig 5, **C**, Representative histogram showing phospho-NF- κ B. Fig 5, **D**, Phospho-NF- κ B intensity was quantified as a ratio to the basal phosphorylation level. MFI, Mean fluorescence intensity. Data were obtained from 3 or more independent experiments. * P < .05, ** P < .01, and *** P < .001.

Several phenotypic subsets have been identified as regulatory B cells in mice.^{7,24-28} They share IL-10 secretion as a common mechanism of their potent inhibitory action. IL-10 production in regulatory B cells has been shown to require TLR stimulation.^{2,3,7,12,29} LPS stimulation, but not anti-IgM stimulation alone, induces an IL-10-producing B-cell population *in vitro*.^{7,12} MyD88-deficient and TLR2/4-deficient B cells cannot produce IL-10 *in vitro* and cannot suppress EAE *in vivo*.^{12,30} Nonetheless, activation through BCR and CD40 is also needed for the induction of IL-10-producing regulatory B cells to optimally suppress autoimmunity.^{2,13,14,31} Thus, a 2-step activation model has been proposed.^{13,14} In the first phase, TLR receptors

initiate IL-10 production by B cells. Subsequently, BCR or CD40 engagement amplifies the initial population of IL-10-producing B cells.^{14,32} We propose that the signaling mechanisms demonstrated here represent the first step in this process. The second step likely requires additional signaling molecules, such as STIM and TIM-1.^{13,33} BLNK can also be required for the second step because the B-cell signalosome is a critical mediator of calcium mobilization downstream of BCR ligation. Although *in vitro* stimulation with LPS might not completely mimic *in vivo* activation of regulatory B cells, BLNK deficiency also significantly reduced IL-10 transcription levels in splenic B cells *in vivo* compared with those seen in wild-type mice during

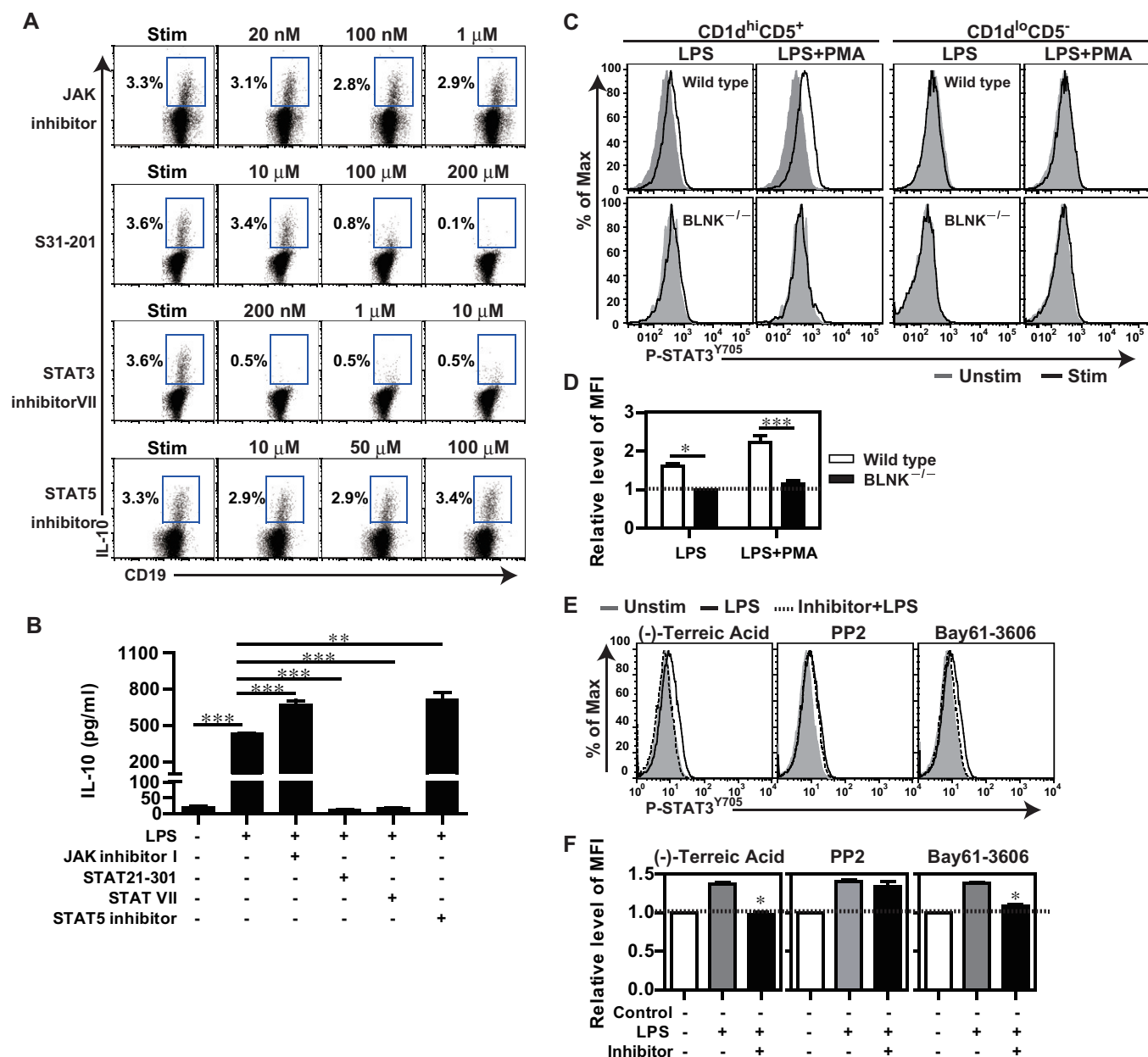


FIG 6. BLNK regulates STAT3 phosphorylation required for IL-10 production. **A** and **B**, IL-10 production (Fig 6, A) and IL-10 secretion (Fig 6, B) were processed, as in Fig 5, A and B. **C** and **D**, STAT3 phosphorylation (pSTAT3) after 3 hours of stimulation. Relative mean fluorescence intensity (MFI) levels of pSTAT3 in CD1d^{hi}CD5⁺ B cells are shown in Fig 6, D. Mean fluorescence intensity levels in unstimulated samples of respective groups were defined as 1.0. **E** and **F**, Representative histograms (Fig 6, E) and pSTAT3 levels (Fig 6, F) in wild-type CD1d^{hi}CD5⁺ B cells. Data were obtained from 3 or more independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$.

EAE and CHS responses. Nonetheless, IL-10 production by BLNK^{-/-} B cells was not completely abrogated in CHS-induced mice, suggesting that other stimuli that do not require BLNK expression can induce some IL-10 expression in B cells.

The initial phosphorylation of STAT3 induced by LPS was independent of JAK signaling but was dependent on BLNK and Btk. In addition to decreased B-cell responses to LPS in BLNK- and Btk-deficient/defective mice,^{21,34} studies have identified a critical role for Btk in B cells in TLR

signaling.³⁵⁻³⁸ Furthermore, Btk is necessary for LPS-induced IL-10 production in macrophages.³⁶ Tec family kinases have been shown to phosphorylate STAT3 and to associate with STAT3 through a Pleckstrin homology domain.^{39,40} Taken together, this study shows that the B-cell signalosome molecules Btk and BLNK mediate STAT3 phosphorylation in CD1d^{hi}CD5⁺ B cells and that this pathway appears to be essential for IL-10 production. Notably, impaired STAT3 phosphorylation has been reported in CD24^{hi}CD38^{hi} regulatory B cells from patients with lupus.⁴¹

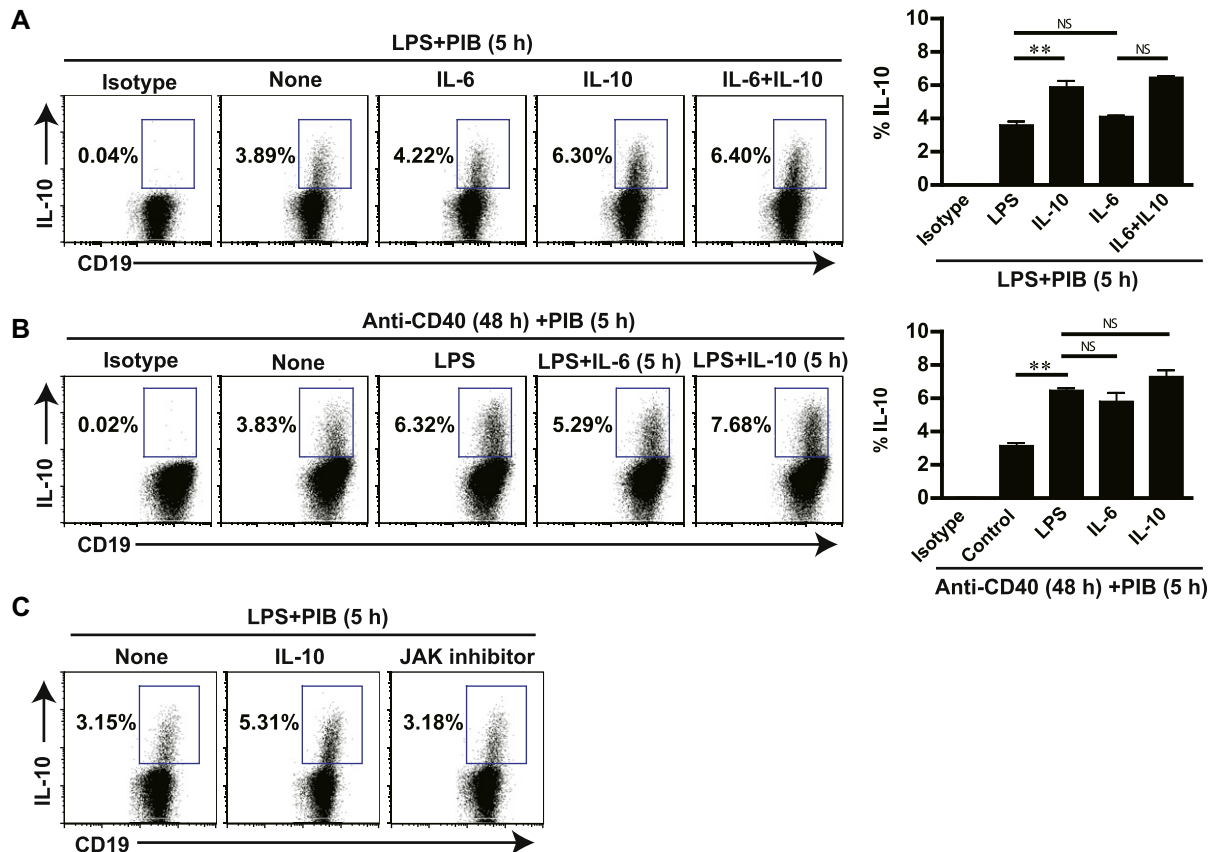


FIG 7. IL-10-producing B-cell numbers are increased by autocrine IL-10 and subsequent JAK-STAT3 pathway activation. **A-C**, Splenic B cells from wild-type mice were cultured in indicated conditions. IL-10 production was assessed as in Fig 1. Data were obtained from 3 independent experiments. ** $P < .01$, NS, Not significant.

IL-10 production by B cells is critical in immune functions, such as maintaining the balance between Treg cells and T_H1/T_H17 cells and suppressing antigen-presenting capacity in dendritic cells.⁵ Although we have reported that B10 cells are capable of suppressing CHS,⁷ the precise mechanism has remained unclear. Cutaneous B-cell infiltration was observed after elicitation, but BLNK^{-/-} mice showed decreased infiltration of B cells. In BLNK^{-/-} mice, despite of increased T-cell infiltration, the frequency of Treg cells was significantly decreased. IFN- γ -producing CD8⁺ T cells were significantly increased in LNs. Thus, regulatory B cells are likely to modulate T-cell function in the inflammatory sites and in LNs during the CHS response. In addition, the study suggests that IL-10 secretion from B cells also facilitates efficient expansion of IL-10-producing B cells. The addition of rIL-10 to B-cell cultures enhanced STAT3 phosphorylation at 3 hours, and after 5 hours, the frequency of IL-10-producing B cells was equivalent to that obtained by means of prolonged incubation with anti-CD40 mAb for 48 hours, followed by LPS plus PIB for 5 hours, by which IL-10⁺ B cells can be induced maximally *in vitro*. This might contribute to autocrine expansion of regulatory B-cell numbers *in vivo*. Nevertheless, because optimal B10 cell activation can require a multistep process, IL-10 stimulation alone might not be enough for initiating IL-10 production in B cells.

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Key messages

- Development of CHS and EAE was enhanced in BLNK^{-/-} mice because of defective IL-10 production in B cells.
- Impaired IL-10 production by B10 cells results in increased infiltration of CD8⁺ T cells and reduced infiltration of regulatory T cells in patients with CHS.
- BLNK is essential for LPS-induced IL-10 production by mediating STAT3 activation in B10 cells, and secreted IL-10 leads to autocrine expansion of B10 cells.

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METHODS

Mice

Wild-type C57BL/6 mice were obtained from Japan SLC. BLNK^{-/-} and IL-10^{-/-} mice were obtained from the Jackson Laboratory (Bar Harbor, Me). All mice were on the C57BL/6 background. For experiments, all mice used were 6 to 10 weeks of age and housed in a specific pathogen-free barrier facility. All studies and procedures were approved by the Animal Committees of Kanazawa University, Institute of Medical, Pharmaceutical and Health Sciences.

Flow cytometry, intracellular cytokine staining, and phosphorylation analysis

Cells were stained for multiparameter flow cytometric analysis on the FACSCanto II (BD Biosciences, San Jose, Calif). Fluorescein isothiocyanate (FITC)-, R-phycoerythrin (PE)-, peridinin chlorophyll protein (PerCP)-, PE-Cy5-, PE-Cy7-, allophycocyanin (APC)-, APC-Cy7-, or Pacific Blue-conjugated mAbs in this study include those to CD1d (1B1, Alexa Fluor 488, 1 ng/ μ L), CD3 (17A2, PerCP/Cy5.5, 0.8 ng/ μ L), CD4 (RM4-5, FITC, 1 ng/ μ L; APC/Cy7, 0.4 ng/ μ L), CD5 (53-7.3, PerCP/Cy5.5, 0.4 ng/ μ L), CD21/CD35 (7G6, FITC, 5 ng/ μ L), CD23 (B3B4, PE, 0.4 ng/ μ L), CD24 (M1/69, PE, 0.4 ng/ μ L), CD45R/B220 (RA3-6B2, PE or PE-Cy7, 0.4 ng/ μ L; PerCP, 1 ng/ μ L), IgM (R6-60.2, PerCP/Cy5.5, 0.4 ng/ μ L), Thy1.2 (53-2.1, PE, 0.4 ng/ μ L), and IL-4 (BVD4-1D11, PE, 2 ng/ μ L) from BD Pharmingen; phospho-NF- κ B (Ser536; 93-H1, 1:5 dilution) from Cell Signaling (Beverly, Mass); phospho-P38 (pT180/pY182; 36/p38, 1:5 dilution) and phospho-STAT3 (pY705; 4/P-STAT3, 1:5 dilution) from BD Biosciences; CD19 (1D3, PE/Cy5, 0.4 ng/ μ L), TLR4/MD2 (MTSS10, PE, 1 ng/ μ L), CD8 (53-6.7, Pacific Blue, 2 ng/ μ L), CD25 (PC61, PerCP/Cy5.5, 0.8 ng/ μ L), IgD (11-26, FITC or PE, 0.8 ng/ μ L), and forkhead box protein 3 (FoxP3; FJK-16s, PE, 2 ng/ μ L) from eBioscience (San Diego, Calif); and CD19 (6D5, PE/Cy7 or APC, 0.4 ng/ μ L), IFN- γ (XMG1.2, PE or FITC, 2 ng/ μ L), IL-10 (JES5-16E3, PE or APC, 2 ng/ μ L), and IL-17 (TC11-18H10.1, PE, 2 ng/ μ L) from BioLegend (San Diego, Calif). Background staining was assessed with a suitable isotype control mAb (2 ng/ μ L) from BioLegend, BD Pharmingen, or eBioscience. For cell-surface and intracellular cytokine staining, 2×10^6 cells of these reagents were used in a 100- μ L volume. Cells (0.5 – 2.0×10^6) were collected and assessed for levels of phosphorylation. Fc receptors were blocked with anti-mouse Fc receptor mAb (2.4G2; BD Pharmingen, 5 ng/ μ L).

For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences). For detection of phosphorylation, Lyse/Fix Buffer and Perm Buffer III in BD Phosflow were used. Dead cells were detected by using LIVE/DEAD Fixable Green or Aqua Dead Cell Kit (Invitrogen-Molecular Probes, Carlsbad, Calif) before cell-surface staining. Data were analyzed with FlowJo software (version 8.8.7; TreeStar, Ashland, Ore). All data were analyzed with FlowJo software (version 8.8.7).

B-cell stimulation

Splenic B cells were purified with anti-CD19 mAb-coated microbeads or the Pan B cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) or by means of cell sorting with the FACSARIA II (BD Bioscience) or JSAN (Bay Bioscience, Tokyo, Japan). B cells (2×10^6) were stimulated with LPS (10 μ g/mL; *Escherichia coli* serotype 0111: B4; Sigma-Aldrich, St Louis, Mo), PMA (50 ng/mL; Sigma-Aldrich), ionomycin (500 ng/mL; Sigma-Aldrich), and BFA (1 $\times$ solution; BioLegend) for 5 hours. In some experiments cells were incubated for 48 hours with anti-CD40 mAb (2 μ g/mL; HM40-3; BD Pharmingen) or plus recombinant IL-6 (rIL-6; 100 ng/mL; BD Pharmingen), rIL-10 (300 ng/mL, BD Pharmingen), or mouse rIL-21 (100 ng/mL, eBioscience). For inhibition experiments, cells were pretreated with the inhibitors Bay61-3606, PP2, (-)-terreic acid, Bay11-7082, IKK inhibitor VII, c-Jun N-terminal kinase inhibitor, SB202190, JAK inhibitor I, S31-201, STAT3 inhibitor VII, or STAT5 inhibitor (Calbiochem, San Diego, Calif) for 30 to 60 minutes.

CHS

Mice were sensitized with 0.5% 2, 4-dinitrofluorobenzene (DNFB; Sigma-Aldrich), in acetone and olive oil (4:1 vol/vol) on the shaved abdominal flank

skin for 2 consecutive days. Five days later, CHS was elicited by applying 0.25% DNFB on the right ear. As a control, the left ear was treated with acetone/olive oil alone. Ear thickness was measured in a blinded manner with a micrometer before and after the challenge every 24 hours for 120 hours. The degree of CHS was assessed as swelling of the hapten-challenged ear compared with that of the vehicle-treated ear and expressed in millimeters $\times 10^{-2}$ (mean $\pm$ SEM).

Skin single-cell suspension for flow cytometry

Mouse ears were minced and then digested in 7 mL of RPMI-10% FBS containing 2 mg/mL collagenase crude (Sigma-Aldrich), 1.5 mg/mL hyaluronidase (Sigma-Aldrich), and 0.03 mg/mL DNase I (Sigma-Aldrich) at 37°C for 90 minutes to prepare skin cell suspensions for flow cytometry. Digested cells were passed through a 70- μ m cell strainer (BD Biosciences) to generate single-cell suspensions. The cell suspension was centrifuged at 1500 rpm for 5 minutes. The pellet was resuspended in 70% Percoll solution (GE Healthcare, Fairfield, Conn) and then overlaid by 37% Percoll solution (GE Healthcare), followed by centrifugation at 500g for 20 minutes at room temperature. The cells were aspirated from the Percoll interface and passed through a 70- μ m cell strainer (BD Biosciences). Cells were harvested by means of centrifugation and washed with PBS plus 5% FBS.

EAE

EAE was induced by means of subcutaneous immunization with 100 μ g of MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK; NeoMPS) emulsified in complete Freund adjuvant containing 2 mg/mL heat-killed *Mycobacterium tuberculosis* H37RA (Sigma-Aldrich) on day 0. For secondary immunization, mice received 200 ng of pertussis toxin (Sigma-Aldrich) intraperitoneally in 0.5 mL of PBS on days 0 and 2. Clinical scores of EAE were assessed daily with a 0- to 6-point scoring system: 0, no signs; 1.0, flaccid tail; 2.0, impaired righting reflex and/or gait; 2.5, partial paralysis of hind limb; 3.0, full paralysis of 1 hind limb; 3.5, full paralysis of 1 hind limb with partial paralysis of second limb; 4.0, total hind-limb paralysis; 5.0, hind-limb paralysis with partial fore-limb paralysis; and 6.0, moribund station. Disease scores over the course of the 28-day experiments were totaled for each animal, and the mean for the experimental group was expressed as a cumulative EAE score.

Adoptive transfer of B cells

Purified splenic B cells (1×10^7) from BLNK^{-/-} mice were transferred into recipient BLNK^{-/-} mice by means of intravenous injection 1 day before CHS or EAE induction. Control mice were injected with PBS alone. CD1d^{hi}CD5⁺ or CD1d^{lo}CD5⁻ B cells were isolated from wild-type or IL-10^{-/-} splenocytes by using FACSARIA II or JSAN cell sorters with purities of approximately 95%. After isolation, CD1d^{hi}CD5⁺ or CD1d^{lo}CD5⁻ cells (1×10^6) were transferred intravenously into BLNK^{-/-} recipient mice 1 day before CHS or EAE induction. The control group (no transfer) received a PBS injection.

Histologic examination and immunohistochemical staining

Ear samples were taken before and 48 hours after DNFB challenge. Tissues were fixed in 4% formalin and then embedded in paraffin. Sections (6 μ m) were stained with hematoxylin and eosin (H&E) for assessing CHS-elicited ear pinnae and anti-CD3 antibody for the numbers of infiltrating T cells (Serotec, Oxford, United Kingdom). The stained cells were averaged in 5 high-power microscopic fields (0.07 mm²), and each section was examined independently by 2 investigators in a blinded fashion.

In the setting of EAE, mice were killed 18 days after MOG35–55 peptide treatment and perfused by means of intracardiac injection of 4% paraformaldehyde and 1% glutaraldehyde in PB buffer, processed, and blocked in paraffin wax. Transverse sections (6 μ m) of the spinal cord and brain were stained with H&E and Luxol Fast Blue. The number of inflammatory foci that contained at least 20 cells was counted in each H&E-stained section in a blinded fashion. When foci coalesced, estimates were made of the number of foci. Areas of demyelination were assessed for Luxol Fast Blue-stained sections.

RT-PCR and quantitative real-time PCR

Splenic B cells were harvested before and 48 hours after DNFB challenge or 18 days after MOG immunization. Isolated total RNA was reverse transcribed to cDNA by using a reverse transcription system with random hexamers (Promega, Madison, Wis). Quantitative real-time PCR was performed with the TaqMan system (Applied Biosystems) on an Mx3000P&Mx3005P Real-Time QPCR System (Applied Biosystems), according to the manufacturer's instructions. TaqMan probes and primers for IFN- γ , IL-2, IL-12, IL-4, IL-6, IL-10, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Applied Biosystems. Relative expression of real-time PCR products was determined by using the $\Delta\Delta$ cycle threshold technique.

ELISA

B cells were purified from splenocytes of wild-type mice. Purified B cells (2×10^6 cells/mL) were cultured with LPS (10 mg/mL, *Escherichia coli* serotype 0111: B4, Sigma) or LPS plus inhibitors for 48 hours in complete medium. IL-10 secretion in the culture supernatant fluid was determined by using an ELISA kit (Quantikine Mouse IL-10 Immunoassay; R&D Systems, Minneapolis, Minn).

Statistics

All data are shown as means $\pm$ SEMs. The significance of differences was determined by using the Student *t* test and 2-way ANOVA.

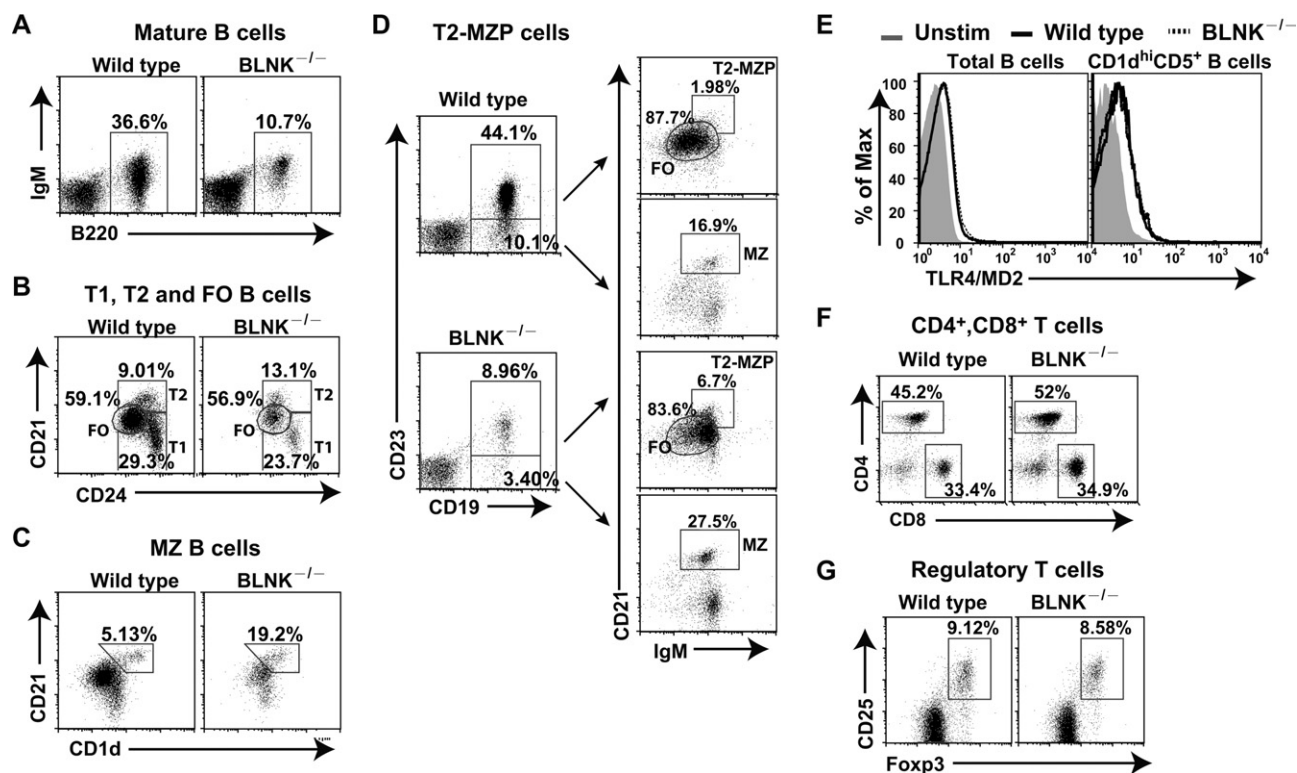


FIG E1. B-cell populations in BLNK^{-/-} mice. **A-D**, Splenocytes were stained as indicated in each panel and subjected to flow cytometric analysis. FO, Follicular; MZ, marginal zone; MZP, marginal zone precursor; T1, transitional stage 1; T2, transitional stage 2. Results were obtained from 3 or more mice in each group. **E**, TLR4/MD2 expression. Splenic B cells and CD1d^{hi}CD5⁺ B cells were stained with anti-TLR4/MD2 or isotype control mAb. **F** and **G**, T-cell subsets. Splenic T cells were stained as indicated. Data were obtained from 3 or more mice in each group.

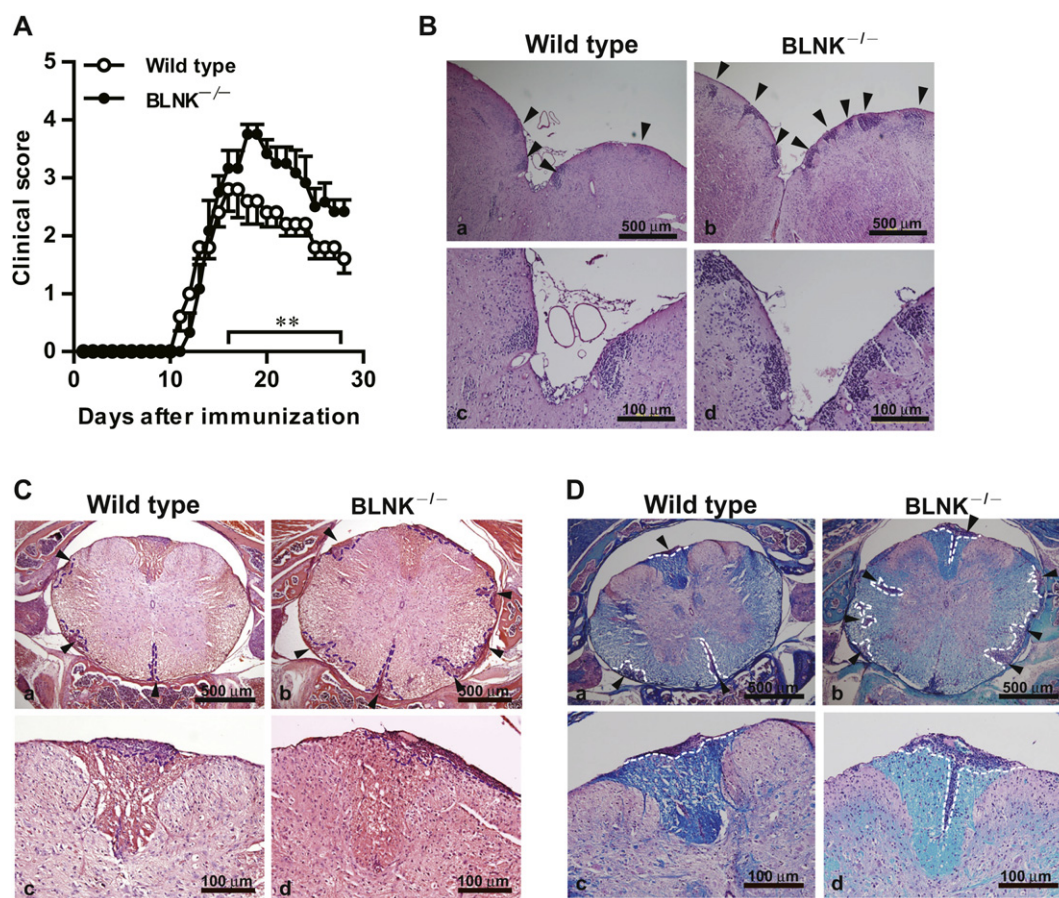


FIG E2. BLNK deficiency promotes EAE responses. **A**, Mean clinical score from 5 or more mice in each group. $**P < .01$. **B**, Inflammation reaction in brain sections was revealed by using H&E staining. **C** and **D**, Inflammation (H&E staining; Fig E2, **C**) and demyelination (Luxol Fast Blue staining; Fig E2, **D**) in spinal cord sections. Arrowheads indicate stained foci of inflammation and demyelination. Fig E2, **B-D**, Original magnification, upper panels, $\times 40$; lower panels, $\times 100$.

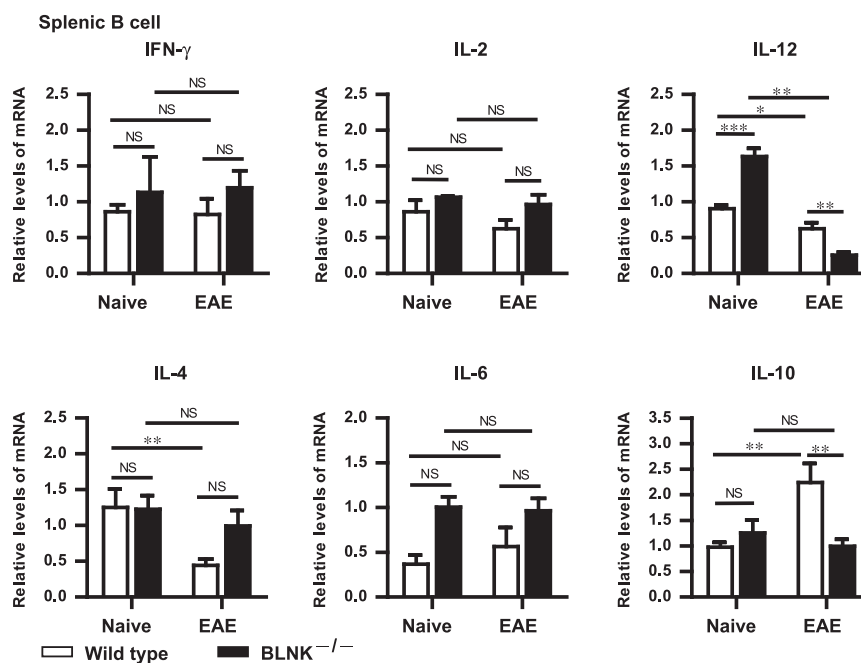


FIG E3. Cytokine production in splenic B cells during EAE responses. The mRNA levels of indicated cytokines were analyzed by using RT-PCR. Results were obtained from 4 to 6 mice. * $P < .05$, ** $P < .01$, and *** $P < .001$. NS, Not significant.

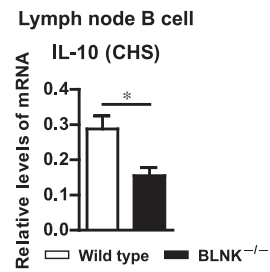


FIG E4. IL-10 production in B cells from draining LNs at 48 hours after CHS induction. mRNA levels of indicated cytokines were analyzed by using RT-PCR. Results were obtained from 5 to 6 mice. * $P < .05$.

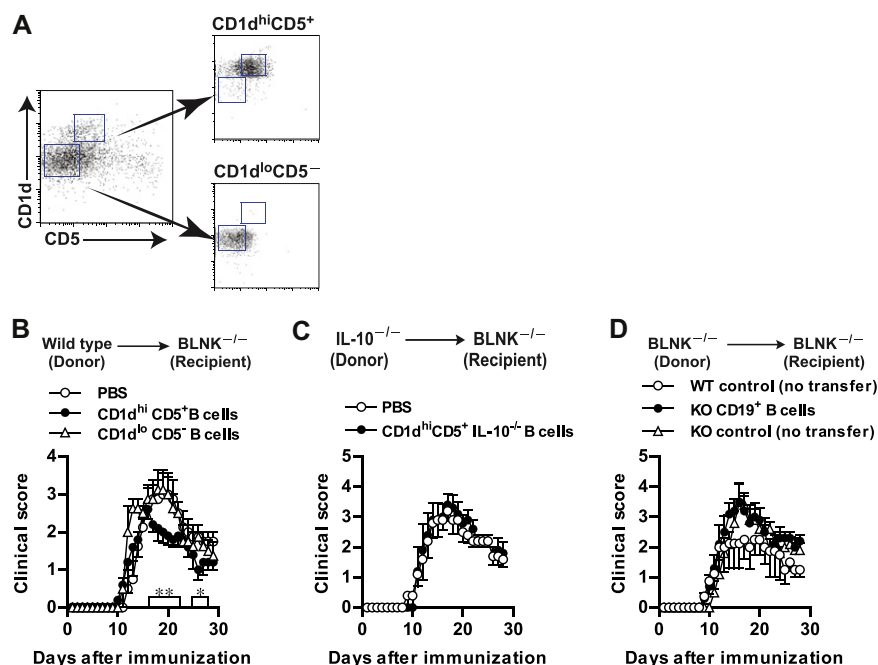


FIG E5. B10 cells attenuate EAE in an IL-10-dependent manner. **A**, Splenic CD19⁺ B cells were sorted into CD1d^{hi}CD5⁺ and CD1d^{lo}CD5⁻ B cells. **B** and **C**, Splenic CD1d^{hi}CD5⁺ or CD1d^{lo}CD5⁻ B cells from naive wild-type ($n \geq 5$) or IL-10^{-/-} ($n \geq 5$) mice were transferred into recipient BLNK^{-/-} mice before EAE induction. **D**, Splenic CD19⁺ B cells from naive BLNK^{-/-} mice ($n \geq 10$) were transferred into BLNK^{-/-} mice 1 day before immunization. KO, Knockout; WT, wild-type. Results were obtained from 4 to 6 mice. * $P < .05$ and ** $P < .01$.

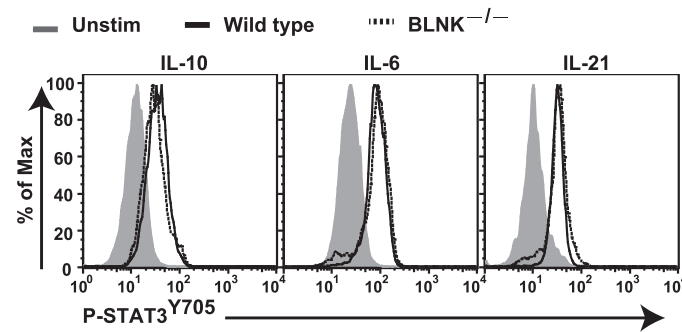


FIG E6. Cytokine-induced pSTAT3 in B cells. Splenocytes were treated with rIL-10 (300 ng/mL), rIL-6 (100 ng/mL), and rIL-21 (100 ng/mL) for 15 minutes. Data were obtained from 3 or more independent experiments.

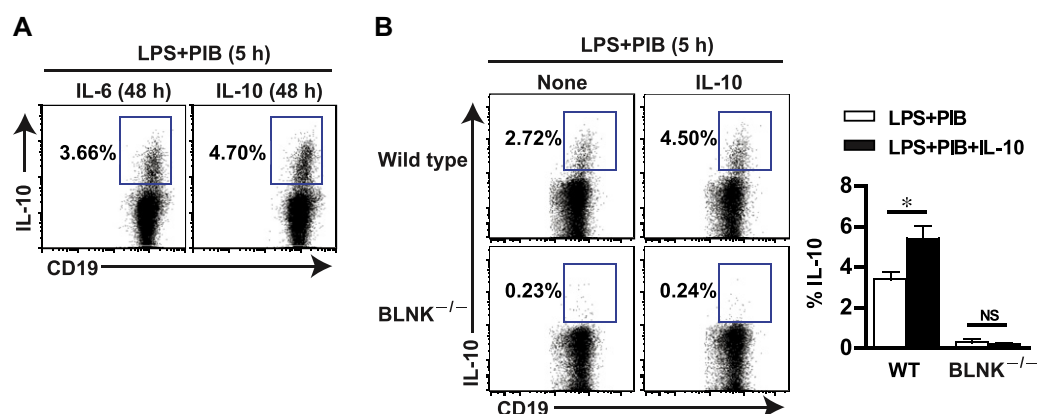


FIG E7. IL-10-producing B-cell numbers are increased by autocrine IL-10 and subsequent JAK-STAT3 pathway activation. **A**, Splenic B cells from wild-type mice were cultured in indicated conditions. **B**, Splenic B cells were stimulated by LPS plus PIB with or without rIL-10. Intracellular IL-10 production was assessed as in Fig 1. Data were obtained from 3 independent experiments. * $P < .05$. NS, Not significant.