The Unknown Aspect of BAFF: Inducing IL-35 Production by a CD5<sup>+</sup>CD1d<sup>hi</sup>FcγRIIb<sup>hi</sup> Regulatory B-Cell Subset in Lupus

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IL-35 is a critical immunosuppressive cytokine that plays an important role in various autoimmune diseases. The purpose of this study was to determine whether BAFF, a key pathogenic factor in systemic lupus erythematosus, also a dichotomous regulator for B-cell immune responses, has an effect on IL-35-producing regulatory B cells and their underlying mechanisms in lupus. We found that exogenous BAFF could induce IL-35 production by splenic B cells from MRL-Faslpr/lpr mice. BAFF-induced IL-35-producing regulatory B cells were mainly from the marginal zone B-cell subset and exhibited a CD5<sup>+</sup>CD1d<sup>hi</sup>FcγRIIb<sup>hi</sup> phenotype. These IL-35-producing regulatory B-cell subsets exhibited regulatory effects on both CD4<sup>+</sup>CD25<sup>-</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. We further identified that BAFF-TACI interaction could induce the production of IL-35 through the classical NF-κB pathway. In vivo study also showed that BAFF could facilitate IL-35 secretion in marginal zone B cells, whereas anti-BAFF treatment could decrease the frequency of IL-35-producing regulatory B cells in MRL-Faslpr/lpr mice. We showed that BAFF could induce IL-35 production by a unique CD5<sup>+</sup>CD1d<sup>hi</sup>FcγRIIb<sup>hi</sup> regulatory B-cell subset mainly through TACI activation in lupus, providing an advanced understanding of the regulatory effect of BAFF in autoimmune diseases.


INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease with substantial morbidity and increasing mortality rate (Sabahi and Anolik, 2006). The critical role for B cells in the pathogenesis of SLE has long been investigated (Kaul et al., 2016; Lisnevskai et al., 2014). Meanwhile, BAFF, a crucial factor for B-cell maturation and survival, has been reported to increase significantly in SLE patients and promote B-cell activation and proliferation (Vincent et al., 2013). The discovery of belimumab, a human anti-BAFF monoclonal antibody approved by the US Food and Drug Administration for the treatment of SLE, marks a major advance in the management of this complex disease. Despite the success of belimumab in the phase III trials, more than 40% of belimumab-treated patients in these clinical trials failed to complete an effective clinical response (Furie et al., 2011; Navarre et al., 2011; Stohl, 2013). Increasing evidence showed that BAFF is not only a proinflammatory and prosurvival cytokine, it also exhibits unexpected immune regulatory functions. For example, Walters et al. (2009) found that BAFF could promote the expansion of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs), which further suppressed T-cell responses in BAFF-transgenic mice (Walters et al., 2009). Yang et al. (2010) also showed that BAFF could induce the generation of IL-10–producing B cells, which possess regulatory functions in a model of collagen-induced arthritis (Yang et al., 2010). Thus, given limited anti-BAFF therapeutic efficacy, an advance in our understanding of the regulatory role of BAFF in the induction of immunosuppressive cytokines and regulatory immune cells in autoimmune diseases, especially in SLE, is of great necessity.

IL-35, a heterodimeric IL-12 family member composed of p35 (IL-12p35) and Ebi3, is a newly identified critical immunosuppressive cytokine that exerts crucial regulatory effects on autoimmune and infectious diseases (Devergne et al., 1997, 2017; Shen et al., 2014; Wang et al., 2014). Besides Tregs (Collison et al., 2007, 2010), recent studies showed that some B-cell subsets could also produce IL-35. For example, CD138<sup>+</sup> plasma cells were the main source of B-cell–derived IL-35 in response to infection with Salmonella species (Shen et al., 2014). CD1d<sup>+</sup>CD5<sup>+</sup> B cells were also reported to produce IL-35 and contribute to the

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Abbreviations: Breg, regulatory B cell; FO, follicular; IHC, immunohistochemistry; MZ, marginal zone; PBS, phosphate buffered saline; SLE, systemic lupus erythematosus; Treg, regulatory T cell; WT, wild type.

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pathogenesis of pancreatic cancer (Plyayeva-Gupta et al., 2016). Conversely, IL-35 could further convert B lymphocytes to IL-35—producing B regulatory cells (Bregs), orchestrating a positive feedback loop (Egwuagu et al., 2015). Several proof-of-principle studies have identified that rIL-35 and autologous IL-35—producing Breg therapies are significantly effective in a variety of autoimmune disease models, including SLE (Cai et al., 2015; Li et al., 2016; Niedbala et al., 2007; Wang et al., 2014). Given the capability of BAFF in the induction of IL-10—producing B cells in certain autoimmune diseases, whether BAFF could also induce IL-35—producing regulatory B-cell subset(s) in lupus remains to be elucidated.

Here, we showed that BAFF could induce IL-35 production in a specific B-cell subset that displays a CD5⁺CD1dhiFcγRIIbhi phenotype in MRL-FasIpr/lpr mice. Moreover, these BAFF-induced CD5⁺CD1dhiFcγRIIbhi B cells were primarily from a marginal zone (MZ) B-cell subset and exhibited regulatory effects on both CD4⁺CD25⁺ T cells and CD4⁺CD25⁺ Treg cells. Mechanistically, BAFF-TACI (calcium modulator and cyclophilin ligand interactor) interaction promoted the production of IL-35 through the classical NF-κB1 pathway. Anti-BAFF treatment resulted in a dramatic decrease of IL-35—producing B cells in MRL-FasIpr/lpr mice in vivo (summarized in Supplementary Figure S1 online). Collectively, our study identified that BAFF is an important physiological factor in the induction of IL-35 production by a previously unreported regulatory B-cell subset in lupus, providing an advanced understanding of the unknown regulatory effect of BAFF in autoimmune diseases.

RESULTS
Exogenous BAFF induces IL-35 production by splenic B cells derived from MRL-FasIpr/lpr mice in culture
To investigate the effect of BAFF on B-cell IL-35 production in lupus, splenic MZ and follicular (FO) B cells from MRL-FasIpr/lpr mice, which particularly express high levels of BAFF receptors BAFF-R and TACI (Vincent et al., 2013), were incubated with varying concentrations of recombinant BAFF from 5 to 100 ng/ml for 60 hours. We showed that BAFF treatment significantly increased the expression of p35 and EB13, which achieved the maximum secretion level at 20 ng/ml. Although when further increasing the concentration p35 and EB13 expression gradually decreased, they remained higher than in the untreated group (Figure 1a and b). Western blotting result and flow cytometric analysis also showed higher p35 and EB13 production in splenic MZ/FO B cells in MRL-FasIpr/lpr mice compared with the wild-type WT group (see Supplementary Figure S2a and b online).

Our study further showed that the percentage and count of p35⁺EB13⁺ B cells were substantially increased from 1.76 ± 0.14 to 6.33 ± 0.62 and from 0.27 ± 0.04 (×10⁴) to 1.09 ± 0.15 (×10⁵), respectively, when treated with BAFF 20 ng/ml, respectively (Figure 1c), which was further confirmed by immunofluorescence (Figure 1f). In line with these findings, higher levels of p35 and EB13 mRNA were detected in the BAFF-stimulated group (Figure 1d). Finally, we observed elevated levels of IL-35 in culture supernatant with BAFF 20 ng/ml stimulation (Figure 1e). Collectively, these data indicated that BAFF could promote IL-35 production in splenic MZ/FO B cells derived from MRL-FasIpr/lpr mice.

BAFF-induced IL-35—producing B cells exhibit a CD5⁺CD1dhiFcγRIIbhi phenotype and are mainly from the MZ B-cell subset
We next assessed the membrane molecule expressions of BAFF-induced IL-35—producing B cells. After stimulation with BAFF 20 ng/ml, p35⁺EB13⁺ B cells exhibited higher fluorescence intensity of CD5, CD1d, TACI, IgM, and FcγRIIb, whereas CD138 remained unchanged compared with p35⁺EB13⁻ B cells (Figure 2a). We observed that FcγRIIb was overexpressed on 92.6% of p35⁺EB13⁺ B cells, whereas the percentages of CD5⁺CD1dhi, CD5⁺FcγRIIbhi, and CD1d⁻FcγRIIbhi were 74.8%, 70%, and 65%, respectively (Figure 2b). Furthermore, 96% of p35⁺EB13⁺ B cells were FcγRIIbhi within the CD5⁺CD1dhi population, and approximately 79.7% were CD1dhi within the CD5⁺FcγRIIbhi population (Figure 2c), indicating that BAFF-induced IL-35—producing B cells exhibit a unique CD5⁺CD1dhiFcγRIIbhi phenotype. Conversely, different B-cell subsets were gated and assessed for the frequency of p35⁺EB13⁺ B cells, which also showed that CD5⁺CD1dhiFcγRIIbhi B cells secreted the overwhelming majority of IL-35 with stimulation of BAFF (Figure 2d). These data showed that BAFF-induced IL-35—producing B-cell subsets with a CD5⁺CD1dhiFcγRIIbhi phenotype are completely different from IgM⁺CD138⁺TACICXCR4⁺CD1d⁻Tim1⁺ plasma cells, which produce IL-10 and IL-35 during Salmonella infection (Shen et al., 2014). Furthermore, we found that the frequency of CD5⁺CD1dhiFcγRIIbhi B cells also increased when stimulated with BAFF (Figure 2e). Together, these data suggested that CD5⁺CD1dhiFcγRIIbhi B cells were the major BAFF-induced IL-35—producing B-cell subset.

To identify the B-cell subsets from which BAFF-induced IL-35—producing B cells were derived, sorting-purified splenic MZ or FO B cells from MRL-FasIpr/lpr mice were incubated with BAFF 20 ng/ml. Flow cytometry analysis showed that the percentages of p35⁺EB13⁺ B cells were much higher in MZ B cells compared with FO B cells, whether treated with BAFF or not (Figure 2f). Consistent with this observation, the frequency of CD5⁺CD1dhiFcγRIIbhi B cells significantly increased in MZ B cells upon BAFF stimulation (P < 0.05), whereas FO B cells did not exhibit similar differences (Figure 2g). Meanwhile, a significant increase of IL-35 production was detected in the culture supernatant of BAFF-treated MZ B cells (Figure 2h). Conversely, p35⁺EB13⁺ B cells were gated and assessed for CD21 or CD23 expression. We observed that the p35⁺EB13⁺ B cells exhibited high expression of CD21 but not CD23, a phenotype similar to MZ B cells (Figure 2i). Collectively, these data indicated that the MZ B-cell subset may be the main source of BAFF-induced IL-35—producing CD5⁺CD1dhiFcγRIIbhi B cells.

IL-35 contributes to the regulatory functions of BAFF-induced CD5⁺CD1dhiFcγRIIbhi B cells in vitro
We then evaluated whether BAFF-induced CD5⁺CD1dhiFcγRIIbhi B cells possess regulatory functions. Our results showed that purified CD5⁺CD1dhiFcγRIIbhi B cells remarkably suppressed the secretion of IFN-γ by CD4⁺CD25⁻ T cells, which can be weakened partly by neutralizing IL-35
Figure 1. BAFF promotes the production of IL-35 in B cells derived from MRL-Fas<sup>lp/lpr</sup> mice in vitro. (a, b) Splenic MZ/FO B cells from MRL-Fas<sup>lp/lpr</sup> mice were sorted and cultured with BAFF for 60 hours, and the expressions of p35 and EBI3 were detected by Western blots. (c) Flow cytometric analysis of Y Zhang et al. BAFF Induces IL-35-Producing Bregs in Lupus. Journal of Investigative Dermatology (2017), Volume 137 2534.
The secretion of tumor necrosis factor-α also showed a similar trend, even though it had no statistical significance when neutralizing IL-35 in culture (Figure 3b). Meanwhile, exogenous IL-35 showed significant inhibitory effects on IFN-γ and tumor necrosis factor-α production in CD4+CD25+ T cells (Figure 3a and b), indicating that IL-35 contributed to the suppressive function of CD5+CD1dhiFcγRIIbhi B cells on CD4+CD25+ T cells.

Moreover, CD5+CD1dhiFcγRIIbhi B cells could increase the percentage of CD4+CD25+ Tregs and promote IL-35 secretion in CD4+ T cells. Accordingly, IL-35 blockade significantly reversed the regulatory functions of CD5+CD1dhiFcγRIIbhi B cells on Tregs (Figure 3c and d). Taken together, our data showed that BAFF-induced CD5+CD1dhiFcγRIIbhi B cells have regulatory functions on both CD4+CD25+ T cells and CD4+CD25+ Tregs, partly through the secretion of IL-35. We observed that BAFF could induce splenic MZ/FO B cells to produce IL-10 (see Supplementary Figure S2d), suggesting that IL-35 and IL-10 may contribute to the regulatory functions of CD5+CD1dhiFcγRIIbhi B cells together.

**BAFF-TACI interaction induces the production of IL-35 through classical NF-kB1 pathway**

The signaling of the BAFF system in SLE pathogenesis has been elaborated previously (Vincent et al., 2014). To ascertain which receptor mainly contributes to this effect, splenic MZ B cells were pretreated with anti-BAFFR or anti-TACI antibodies before BAFF stimulation. Blocking TACI resulted in significant reduction of p35 and EBI3 expression, although their expressions were only slightly decreased when BAFFR blockage was used (Figure 4a and b). Similar results were obtained by flow cytometric analysis (Figure 4c). The secretion level of IL-35 was reduced significantly in the culture supernatant of both anti-BAFFR and anti-TACI antibody–treated groups (Figure 4d). It is well characterized that BAFFR is essential for the survival and proliferation of MZ B cells (Mackay and Schneider, 2009), supporting a speculation that the reduced IL-35 level in the BAFFR-blocking group may be linked with impaired B-cell viability rather than a direct effect on IL-35 production.

We next investigated whether the NF-kB signal pathway is involved in the process of BAFF-induced IL-35 production. MZ B cells were pretreated with BMS 345541, which selectively inhibited the total NF-kB pathway, and the intracellular protein level of p35 and EBI3 declined significantly (Figure 4e), suggesting that the NF-kB pathway participated in BAFF-induced IL-35 production. Furthermore, when we pretreated MZ B cells with different concentrations of MLN120B, which selectively inhibit the NF-kB1 pathway, the expression of p35 and EBI3 was significantly reduced (Figures 5e). Consistently, the frequency of p35+EBI3+ B cells and the extracellular level of IL-35 were obviously reduced when both total NF-kB and classical NF-kB1 signaling pathways were blocked (Figure 4f and g). Collectively, these results indicated that BAFF-TACI interaction induces MZ B cells to produce IL-35 mainly through the classical NF-kB1 pathway.

**BAFF induces IL-35 production by splenic B cells in vivo**

We next investigated whether BAFF enables B cells to produce IL-35 in lupus in vivo. We observed that the serum BAFF and IL-35 levels in MRL-Fas+/−/− mice were 361.5 ± 38.16 pg/ml and 95.43 ± 12.48 pg/ml, respectively. Although their levels in MRL/MpJ mice used as WT controls were 70.02 ± 20.26 pg/ml and 8.73 ± 4.49 pg/ml. Compared with WT mice, MRL-Fas+/−/− mice had distinctly elevated serum BAFF and IL-35 levels (Figure 5a). The serum IL-35 level correlated positively with serum BAFF level (Figure 5b).

Ultimately, we also observed that the proportion of CD4+CD25+Foxp3+ Tregs and CD4+CD35+EBI3+ T cells was significantly reduced frequencies of Tregs and CD4+CD35+EBI3+ T cells compared with PBS-injected MRL-Fas+/−/− mice (see Supplementary Figure S5 online). The expression levels of BAFF, IL-35, and IL-35+CD1dhiFcγRIIbhi B cells increased in the cutaneous lesions of both lupus-prone mice and SLE patients.

![Image](https://example.com/image.png)

**Figure 3a.** The secretion of tumor necrosis factor-α also showed a similar trend, even though it had no statistical significance when neutralizing IL-35 in culture (Figure 3b). Meanwhile, exogenous IL-35 showed significant inhibitory effects on IFN-γ and tumor necrosis factor-α production in CD4+CD25+ T cells (Figure 3a and b), indicating that IL-35 contributed to the suppressive function of CD5+CD1dhiFcγRIIbhi B cells on CD4+CD25+ T cells.

**Figure 3b.** Real-time PCR analysis of p35 and EBI3 mRNA in splenic MZ/FO B cells. (e) ELISA measurement of IL-35 secretion in culture supernatants. (f) Immunofluorescence staining of p35, EBI3, and DAPI in MZ/FO B cells. Original magnification ×400, scale bar = 20 μm. Data are representative of at least triple independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. h, hour; FO, follicular; MZ, marginal zone.
Figure 2. BAFF-induced IL-35-producing B cells express high levels of CD5, CD1d, and FcγRIIb and are mainly from the MZ B-cell subset. (a) p35+EBI3+ and p35+EBI3+B cells were gated and assessed for the discrepant expression of cell surface marks. The red line indicates p35+EBI3+B cells, and the black line indicates p35+EBI3+B cells. (b) The percentage of FcγRIIbhi, CD5+CD1dhi, CD5+FcγRIIbhi, and CD1dhiFcγRIIbhi B-cell subsets in p35+EBI3+ and p35+EBI3+B cells. (c) Within the p35+EBI3+B-cell gate, CD5+CD1dhi B cells were assessed for FcγRIIb expression, and CD5+FcγRIIbhi B cells were assessed for CD1d expression. (d) Various B-cell subsets were gated and assessed for the expression of p35 and EBI3. (e) Flow cytometric analysis of the percentage of CD5+CD1dhiFcγRIIbhi B cells treated with or without BAFF 20 ng/ml. (f) The frequencies of splenic p35+EBI3+B cells detected by flow cytometry analysis. (g) Flow cytometric analysis of the percentage of CD5+CD1dhiFcγRIIbhi B cells in MZ or FO B cells. (h) The secretion level of IL-35 in the culture supernatants by ELISA. (i) p35+EBI3+ and p35+EBI3+B cells from BAFF-induced MZ/FO B cells were gated, and the expression of CD21 and CD23 was assessed. Data are representative of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. FO, follicular; MZ, marginal zone; ns, not significant.
correlation analysis) (Figure 5e). Meanwhile, BAFF and p35 (Figure S6 online), which is consistent with previous observation in spleen (see Supplementary Figure S7 online). The frequencies of IFN-γ– and tumor necrosis factor-α–expressing CD4+ T cells were detected by flow cytometry. (c, d) Flow cytometric analysis of the frequencies of CD4+CD25+ and CD4+ p35+EBI3+ T cells. Data are representative of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.

We next evaluated whether anti-BAFF treatment could affect the expression levels of BAFF, IL-35 and IL-35-producing CD5+CD1dhiFcγRIIhbhi B cells in the cutaneous lesions of MRL-Fas1pr/pr mice. We finally compared the expression levels of IL-35 and IL-35-producing CD5+CD1dhiFcγRIIhbhi B cells in the cutaneous lesions of MRL-Fas1pr/pr mice resulted in significantly reduced expression of BAFF, EBI3, p35, CD19, CD5, CD1d, and FcγRIIb compared with those in PBS control (Figure 5e). However, intraleisional anti-BAFF treatment did not exhibit significant effects on the clinical appearance of skin lesions (data not shown), possibly because of a relatively short period of therapy. The results of both systemic and intraleisional anti-BAFF treatment suggested that a concentration-dependent effect of anti-BAFF treatment on the expression levels of IL-35 and IL-35-producing CD5+CD1dhiFcγRIIhbhi B cells in the cutaneous lesions might exist. We finally compared the expression levels of BAFF, IL-35, and IL-35–producing CD5+CD1dhiFcγRIIhbhi B cells in the normal skin of human healthy control subjects and cutaneous lesions of SLE patients. Overall, 25 specimens of cutaneous lesions from SLE patients and seven normal skin specimens from healthy control subjects were collected. We found that the expressions of BAFF, EBI3, and p35 were significantly increased in the cutaneous lesions of SLE patients, whereas they were rarely detected in the normal skin of healthy control subjects (Figure 6a). Prominent B-cell infiltrates were also detected in the upper dermis proximal to the cutaneous lesions of SLE patients (Figure 6a). Furthermore, the cutaneous lesions of SLE patients were characterized by higher levels of CD5, CD1d, and FcγRIIb than those of healthy control subjects (Figure 6a). There are also strong correlations between EBI3 and p35 (r = 0.772, P < 0.0001, Pearson
correlation analysis) (Figure 6b), BAFF and p35 ($r = 0.883, P < 0.0001$), and BAFF and EBI3 ($r = 0.673, P = 0.0002$) (Figure 6c and d), which further strengthened the evidence that BAFF is positively related to IL-35 in the supernatants. (e) The expression level of p35 and EBI3 protein in MZ B cells treated with different NF-κB pathway inhibitors. (f) The frequency of p35 $^+$EBI3 $^+$ B cells in MZ B cells treated with different NF-κB pathway inhibitors. (g) ELISA measurement of IL-35 secretion in culture supernatants. Data are representative of at least three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. M, mol/L; MZ, marginal zone; ns, not significant.

**DISCUSSION**

In this study, we showed that BAFF could induce IL-35 production in a specific B-cell subset primarily from MZ B cells. As a potent B-cell activator, excessive BAFF signals promote the survival of self-reactive B cells and induce the differentiation of MZ B cells into plasma cells in SLE (Mackay and Schneider, 2008, 2009). Although belimumab depletes B cells through BAFF neutralization, only modest efficacy in some SLE patients has been achieved in randomized clinical trials (Furie et al., 2011; Manzi et al., 2012; Navarra et al., 2011; Stohl, 2013). Recent studies have shown that BAFF could induce the production of IL-10 both in normal regulatory B cells and chronic lymphocytic leukemia B cells. Saulep-Easton et al., 2016; Yang et al., 2010 suggested that possible immune regulatory functions of BAFF may exist on B cells. Nevertheless, there are no reports and literature of the relevant studies on the association between IL-35, the recently discovered immunosuppressive cytokine, and BAFF, the critical pathogenic factor in SLE. Thus, our study showed the unknown immune regulatory effect of BAFF via inducing IL-35 production in SLE, which could partly explain the unsatisfactory clinical efficacy of BAFF-targeted therapy. Whether BAFF enables the induction of IL-35—producing
Figure 5. BAFF promotes the production of IL-35 in B cells in vivo. (a) Serum levels of BAFF and IL-35 in wild-type control (n = 5) and MRL-Fas\textsuperscript{+/-} mice (n = 10) by ELISA. (b) Correlation between serum BAFF and IL-35 in MRL-Fas\textsuperscript{+/-} mice (Pearson correlation analysis). (c) Splenic CD19\textsuperscript{+} B cells were gated and assessed for the expression of p35 and EBI3. (d) Within the CD19\textsuperscript{+} B-cell gate, the percentage of CD5\textsuperscript{+}CD1d\textsuperscript{hi}FcyRIIb\textsuperscript{hi} B cells was assessed. (e) The effect of intralesional injection of anti-BAFF mAb on the expression of BAFF, EBI3, p35, CD19, CD5, CD1d, and FcyRIIb in the cutaneous lesions of MRL-Fas\textsuperscript{+/-} mice. Representative images of IHC staining of BAFF, EBI3, p35, CD19, CD5, CD1d, and FcyRIIb in the cutaneous lesions (ear and back) of MRL-Fas\textsuperscript{+/-} mice 3 days after intralesional injection of anti-BAFF mAb or PBS (n = 5 in each group) and IHC result correlation analysis (Pearson correlation analysis) of EBI3 and p35, BAFF and p35, and BAFF and EBI3 in the cutaneous lesions of MRL-Fas\textsuperscript{+/-} mice. Scale bars = 20 μm. *P < 0.05, ***P < 0.001. HE, hematoxylin and eosin; IHC, immunohistochemistry; IOD, integral optical density; PBS, phosphate buffered saline; WT, wild type.
Figure 6. The expression levels of BAFF, IL-35, and IL-35-producing CD5^+CD1d^hiFcγRIIb^hi B cells in the cutaneous lesions of human SLE patients and healthy control subjects. (a) Representative images of IHC staining of BAFF, EBI3, p35, CD19, CD5, CD1d, and FcγRIIb in the cutaneous lesions of SLE patients (n = 25) and the normal skin of healthy control subjects (n = 7). Figure panel pairs represent images taken with different zooming options. Scale bars = 20 um. (b–d) IHC result correlation analysis (Pearson correlation analysis) of (b) EBI3 and p35, (c) BAFF and p35, and (d) BAFF and EBI3. *P < 0.05. HE, hematoxylin and eosin; IHC, immunohistochemistry; IOD, integral optical density; SLE, systemic lupus erythematosus.
B cells and IL-35 production in other types of autoimmune diseases, inflammatory diseases, and cancers still needs further investigation. Additionally, given that BAFF induces IL-35 production under only certain concentration ranges, an advanced understanding of the relationship between IL-35 production and serum BAFF levels in different individual lupus animal models and patients also needs to be elucidated.

We next identified that this BAFF-induced IL-35—producing B-cell subset displayed a unique CD5^+CD1d^hiFcγRIIb^hi phenotype and exhibited regulatory effects on both CD4^+CD25^+ T cells and CD4^+CD25^+ Treg cells. However, whether these IL-35—producing Bregs could promote the differentiation and proliferation of Tregs in SLE patients still needs to be elucidated because a recent publication suggested that the level of IL-35 expression in SLE patients is not sufficient to induce the production Tregs because of low expression of the IL-35 receptor (gp130) on CD4^+ T helper cells (Cai et al., 2015). It is generally accepted that Breg cells are not lineage specific but might be differentiated from various B-cell subsets in response to suitable environmental stimuli at different B-cell development stages (Rosser and Mauri, 2015). CD138^+ plasma cells were also the main source of B-cell—derived IL-35 and IL-10 during experimental autoimmune encephalomyelitis (Shen et al., 2014). In addition, lipopolysaccharide and/or rili-35 treatment could induce IL-35—producing Breg cells with a phenotype of CD5^+CD19^+B220^lo in C57BL/6 mice (Wang et al., 2014). The identified CD5^+CD1d^hiFcγRIIb^hi IL-35—producing Breg cell subset is obviously distinct from those previously reported IL-35—producing Bregs. More than 90% of these Breg cell populations highly express FcγRIIb on their surfaces. FcγRIIb has long been known to be the only inhibitory Fc receptor expressed on B cells (Smith and Clatworthy, 2010), and soluble FcγRIIb treatment has been shown to be effective in SLE mouse models (Wewitzke et al., 2008). Given the characteristic high expression pattern of FcγRIIb on these cell subsets, whether its overexpression contributes to IL-35 production remains to be addressed. Furthermore, because these CD5^+CD1d^hiFcγRIIb^hi Breg cell subsets share some of the surface markers (CD5 and CD1d) with IL-10—producing Breg cells previously described (Yanaba et al., 2008; Yoshizaki et al., 2012), whether the CD5^+CD1d^hiFcγRIIb^hi B cells and IL-10—producing Bregs belong to the same subset of Breg cells or distinct Breg subsets needs to be further investigated.

We next tried to explore the possible mechanism of BAFF-induced IL-35 production in these Breg cell subsets. Our study showed that BAFF—TACI interaction could induce the production of IL-35 through the classical NF-κB1 pathway. As we know, TACI, one of the three receptors (BAFFR, TACI, and BCMA) for BAFF, has both positive and negative roles in the regulation of B-cell development (Mackay and Schneider, 2008; Zhang et al., 2015). Despite extensive studies showing that BAFF signaling through TACI rather than BAFF-R may contribute to SLE development (Figgett et al., 2015; Jacobs et al., 2013, 2015, 2016), in vitro studies have shown that activation of TACI decreases the numbers of B cells, and TACI^−/− mice have dramatically increased numbers of B cells and finally develop lupus-like disease, indicating an inhibitory effect of TACI on B cells in vivo (Seshasayee et al., 2003; Yan et al., 2001). Nevertheless, how TACI negatively regulates B cells remains elusive. Our findings that IL-35 production was mainly through TACI activation may provide a possible explanation for the underlying mechanism of TACI-mediated negative regulatory effect. Although previous research suggested the possible regulation of NF-κB on EB13 or p35 transcription in other immune cells (e.g., dendritic cells) (Kollet and Petro, 2006; Wirtz et al., 2005), whether a similar transcriptional regulation mode exists in these IL-35—producing Breg cells remains to be elucidated. Moreover, whether IL-35 production induced by TACI activation could further affect the expression level of TACI on these Bregs, that is, the possible existence of a feedback loop between TACI signaling and IL-35 production, still needs further exploration.

Finally, we showed that the expression of BAFF and IL-35 were significantly increased in the cutaneous lesions of both human and mouse lupus and that BAFF is positively related to IL-35—producing CD5^+CD1d^hiFcγRIIb^hi B cells. Blocking BAFF within cutaneous lesions resulted in significantly reduced IL-35—producing CD5^+CD1d^hiFcγRIIb^hi B cells. Even though the effectiveness of B-cell depletion therapy in the cutaneous lesions of SLE is still a matter of debate (Chan et al., 1999; Hofmann et al., 2013) and needs to be further evaluated, our study showed an alternative visual for understanding and interpreting the aspect of BAFF in cutaneous lesions of SLE.

In conclusion, we showed that BAFF could induce IL-35 production by a unique CD5^+CD1d^hiFcγRIIb^hi Breg cell subset mainly through TACI activation in lupus, providing an advanced understanding of the unknown regulatory effect of BAFF in autoimmune diseases.

**MATERIALS AND METHODS**

**Human sample collection, mice, and reagents**

A total of 25 cutaneous lesions of SLE and seven normal skin samples of healthy control subjects were collected from patients who were treated as outpatients of the Dermatology Department, Affiliated Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Retrospective clinicopathological data of these patients were obtained simultaneously. SLE was defined by satisfying at least 4 of 11 American College of Rheumatology revised criteria (Hochberg, 1997). The use of these specimens was approved by the Ethics Committee of Huazhong University of Science and Technology. Specifically, written informed patient consent is not needed because the archival material obtained from these patients is for diagnostic purposes, and we use it only for retrospective and observational study. This study complied with the Declaration of Helsinki. Mature MRL/MpJ and MRL/MpJ-Fas^−/−/Fas^−/− mice were kindly provided by Department of Dermatology, Second Xiangya Hospital, Central South University and Hunan Key Laboratory of Medical Epigenomics, Changsha, Hunan, China. All animal studies were approved by the Committee on Animal Handling of Huazhong University of Science and Technology. Anti-BAFF mAb (Sandy-2, AdipoGen, San Diego, CA) was intravenously injected at dose of 200 μg per mouse. For the purposes of analyzing cutaneous lesions in MRL-Fas^−/− and MRL-Fas^−/−/Fas^−/− mice, each animal received 10 μg of anti-BAFF mAb or PBS through intradermal injection in skin lesions of the ear and back.
symmetrically; 3 days after injection, the cutaneous lesions were obtained for IHC analysis.

Cell extract and culture
Splenic B cells were all purified by magnetic isolation with Marginal Zone and Follicular B Cell Isolation Kit (130-100-366, Miltenyi Biotec, Germany), and the purity was greater than 95%. RPMI medium 1640, supplemented with 15% fetal calf serum, L-glutamine and penicillin/streptomycin were used for all cell cultures. The following reagents were used: recombinant mouse BAFF (Q9WU72, R&D Systems, Minneapolis, MN), varied from 5 to 100 ng/ml; lipopolysaccharide, 1 µg/ml; anti-CD40, 5 µg/ml; anti-BAFF-R mAb (9B9, AdipoGen), 2 µg/ml and anti-TACI mAb (Q9ET35, R&D systems), 5 µg/ml; and BMS 345541(ab144822, Abcam, Cambridge, UK) and MLN120B (HY-15473, MedChem Express, Monmouth Junction, NJ).

Antibodies and flow cytometric analysis
Purified B cells were cultured for 72 hours. Phorbol 12-myristate 13-acetate (50 ng/ml), ionomin (500 ng/ml), and GolgiStop were added to the culture for the last 5 hours. The following antibodies were used: anti-mouse EBI3 (IS502022) and p35 (27537) (both from R&D Systems); anti-mouse CD32b (AT130-2), CD23 (B3B4), BAFFR (eBio7H22-E16), TACI (eBio8F10-3), and Foxp3 (FJK-16s) (all from BD Biosciences, San Diego, CA); anti-mouse CD1d (1B1) and CD138 (53-7.3), CD21/CD35 (eBio8D9), and CD4 (GK1.5) (all from BD Biosciences, San Jose, CA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

T-cell activation assay
Purified MZ B cells from MRL-Faslpr/lpr mice were stimulated with recombinant mouse BAFF 20 ng/ml, antigen-presenting cells (APCs), and GolgiStop were added to the culture for the last 5 hours. The following reagents were used: recombinant mouse BAFF (Q9WU72, R&D Systems, Minneapolis, MN), varied from 5 to 100 ng/ml; lipopolysaccharide, 1 µg/ml; anti-CD40, 5 µg/ml; anti-BAFF-R mAb (9B9, AdipoGen), 2 µg/ml and anti-TACI mAb (Q9ET35, R&D systems), 5 µg/ml; and BMS 345541(ab144822, Abcam, Cambridge, UK) and MLN120B (HY-15473, MedChem Express, Monmouth Junction, NJ).

Western blotting
Cultured B cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml), ionomin (500 ng/ml), and GolgiStop for the last 5 hours before extracting cell lysates. The following primary antibodies were used: rabbit anti-EBI3 (ab124694, Abcam), rabbit anti-IL12A (ab131039, Abcam), β-actin (C-4, Santa Cruz), and β-tubulin (D-10, Santa Cruz Biotechnology, Dallas, TX).

Statistical analysis
Data are shown as mean ± standard error of the mean. Statistical significance was performed by unpaired Student’s t tests. Values of P less than 0.05 were considered significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.07.843.


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