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BAFF Suppresses IL-15 Expression in B Cells

Ning Ma,*‡,1 Chen Xing,*‡ He Xiao,*‡ Youdi He,‡ Gencheng Han,* Guojiang Chen,* Chunmei Hou,* Bernadette Marrero,§ Yujuan Wang,* Shengquan Zhang,‖ Beifen Shen,* Yan Li,* and Renxi Wang*

Clinical trials have shown that BAFF inhibitors do not reduce memory B cell levels but can reduce the number of mature B cells. It remains uncertain whether BAFF affects memory-maintaining cytokines such as IL-15. We found that BAFF suppressed IL-15 expression in B cells from lupus-like or experimental allergic encephalomyelitis mice. When BAFF was blocked with atacicept-IgG, IL-15 expression was upregulated in lupus-like or experimental allergic encephalomyelitis mice. Finally, we showed that BAFF suppressed IL-15 expression in transitional 2 B cells by reducing Foxo1 expression and inducing Foxo1 phosphorylation. This study suggests that BAFF suppresses IL-15 expression in autoimmune diseases, and this opens up the possible opportunity for the clinical application of BAFF- and IL-15–specific therapeutic agents. The Journal of Immunology, 2014, 192: 4192–4201.

Cell–activating factor is a crucial factor that regulates B cell maturation, survival, and function. An excess of BAFF has been shown to lead to the development of autoimmune disorders in animal models, and BAFF concentrations are known to be higher in patients with various autoimmune conditions compared with normal subjects (1). The pathogenic role of BAFF in systemic lupus erythematosus (SLE) was revealed in a study that showed BAFF-transgenic mice develop a lupus-like illness with the production of anti-DNA Abs and development of glomerulonephritis (2–4). Increased levels of BAFF reported in some studies correlate with disease activity (5). In multiple sclerosis (MS) patients, BAFF is expressed by astrocytes that are closely associated with BAFFR–expressing cells (6) within ectopic lymphoid follicles of the meninges (7). BAFF has been regarded as a new therapeutic target in many autoimmune diseases (8).

On March 9, 2011, the Food and Drug Administration approved belimumab, a fully human anti–BAFF mAb, as a new B cell–specific treatment for SLE. Belimumab selectively reduces the number of CD20+ naive B cells, activated B cells, and plasmablasts and results in a transient increase in the number of memory cells (9). Atacicept (TACI)-IgG shows similar clinical results to belimumab by binding a portion of the receptor TACI to block the effects of survival factors BAFF and a proliferating-inducing ligand (APRIL). It reduces levels of circulating mature B cells and plasma cells in the spleen and bone marrow as well as inhibiting T cell activation, but not memory B cells (10, 11). As long as the reactive memory is maintained, it is probably acceptable that in principle humoral immunological response can be restored from immunological memory (12).

IL-15 provided survival signals that maintain memory T and B cells in the absence of Ag (13–15). Therefore, we propose that BAFF suppresses memory B cells by downregulating IL-15 expression. In the current study, we found that BAFF suppressed IL-15 expression in B cells from lupus-like or experimental allergic encephalomyelitis (EAE) mice. When BAFF was blocked with TACI-IgG, IL-15 expression was upregulated. The study provides hints for the clinical application of BAFF- and IL-15–specific therapeutic agents.

Materials and Methods

Ethics committee approval

The blood samples were taken with the approval of the local ethical committee (Clinical Trial Center, Beijing Institute of Basic Medical Science), and all participants gave written informed consent to participate in the current study.

Care, use, and treatment of mice in this study were in strict agreement with international guidelines for the care and use of laboratory animals. This study was approved by Animal Ethics Committee of Beijing Institute of Basic Medical Sciences.

Peripheral blood from normal human subjects, MS patients, and SLE patients

Blood samples were obtained after the approval from the Beijing Institute of Basic Medical Sciences, consent from 15 normal human subjects and 11 SLE patients from Clinical Trial Center (Beijing 307 Hospital), and consent from 15 MS patients from Department of Neurology, Beijing Chaoyang Hospital.

Culture of human B cells

PBMCs were isolated from the heparin-treated whole blood by density gradient centrifugation, and CD19+ B cells were isolated using human CD19 MicroBeads (AutoMACS; Miltenyi Biotec). PBMC or CD19+ B cells were stimulated with 1 µg/ml LPS or 10 µg/ml goat F(ab’)2 anti-human IgM (Southern Biotechnology Associates, Birmingham, AL) to activate the cells in the presence of 50 ng/ml human rBAFF (PeproTech, Rocky Hill, NJ).
Mice

Nine-week-old C57BL/6, BALB/c mice, and (New Zealand Black/New Zealand White)F1 mice (Chinese Academy of Medical Sciences, Beijing, China) were bred in our animal facilities under specific pathogen-free conditions.

EAE induction

EAE induction was performed, as previously described (16). Briefly, C57BL/6 mice at 9 wk of age received a s.c. injection of 125 μg myelin oligodendrocyte glycoprotein (MOG)35–55 peptide (Mimotopes) emulsified 1:1 (v/v) in CFA containing 4 mg/ml Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI), to the base of the tail and both flanks. Pertussis toxin (300 ng pertussis toxin in PBS; List Biological) was injected i.p. at the time of induction, and a second dose was administered 3 d later. The same volume of CFA and pertussis toxin was injected into each mouse in the control group. Animals were weighed, monitored, and clinically assessed according to the following grading scale: 0 = no signs; 1 = distal tail weakness; 1.5 = tail weakness and some hind limb weakness; 2 = complete tail paralysis; 2.5 = complete tail paralysis and partial hind limb weakness; 3 = complete hind limb weakness; 3.5 = inability to right when placed on back or significant forelimb weakness; 4 = euthanized or spontaneous death (17, 18). Mice were euthanized if they lost 20% of their starting weight, displayed a clinical score of 3 for 72 h, or reached a clinical score of 3.5. Mice were examined for up to 21 d postimmunization. Number of mice per treatment group was 12.

Treatment of EAE or lupus-like mice with TACI-IgG and/or anti–IL-15 Ab

EAE and lupus-like mice were divided into the following six groups: 1, control mice; 2, untreated; 3, human IgG treated; 4, TACI-IgG treated; 5, TACI-IgG plus rabbit IgG treated; 6, TACI-IgG plus anti–IL-15. Twelve EAE mice per group were injected i.v. with 2 mg/kg TACI-IgG and/or 0.5 mg/kg anti-mouse IL-15 neutralizing Ab (R&D Systems) on days 4, 8, 12,
and 16 (one time per day) after EAE was induced with MOG33–55. Twelve (New Zealand Black/New Zealand White) F1 mice (lupus-like mice) per group were injected i.p. with 5 mg/kg TACI-IgG and/or 1 mg/kg rabbit anti-mouse IL-15 neutralizing Ab (R&D Systems) at 1, 2, 3, and 4 wk (twice per week) after the mice reached 6 mo of age.

**Isolation of memory B cells**

There is no marker for memory B cells in mice (12). Thus, we used a two-step procedure to isolate the memory B cells from six mice per group by memory B cell isolation kit (MACS Miltenyi Biotec; Order 130-095-838), according to the manufacturer’s protocol (Miltenyi Biotec). Briefly, we first depleted nonmemory B cells with nonmemory B cell biotin–Ab mixture and anti-biotin microbeads. Additional labeling was done with anti-IgG1 allophycocyanin and anti-IgG2ab allophycocyanin. The magnetically labeled cells were passed through a column placed in the magnetic field of a MACS separator. Secondly, anti-allophycocyanin microbeads were used for indirect magnetic labeling of memory B cells. Positive selection of memory B cells was sorted by the magnetic field of a MACS separator. Memory B cells were fluorescently stained with B220-VioBlue (130-094-287), CD38 PE (130-097-087), anti-IgG1 allophycocyanin, and anti-IgG2ab allophycocyanin, followed by FACS analysis. Flow cytometric analysis demonstrated >90% memory B cells (data not shown). Finally, we counted the absolute number of memory B cells per mouse.

**In vitro B cell cultures**

CD19+ B cells were isolated using mouse CD19 MicroBeads (AutoMACS; Miltenyi Biotec) and cultured in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 50 mM 2-ME. Cells were stimulated with 1 μg/ml LPS, 10 μg/ml goat F(ab)2 anti-mouse IgM (Southern Biotechnology Associates), MOG35–55 peptide (20 μg/ml precoated in 96-well plates), or dsDNA (20 μg/ml precoated in 96-well plates) in the presence of 50 ng/ml rBAFF (PeproTech).

**Cytometric analysis and intracellular cytokine staining**

All cell experiments were strictly prepared on ice, unless otherwise stated in other specific procedures. Cells (1 × 10^6 cells/sample) were washed with FACS staining buffer (PBS, 2% FBS or 1% BSA, 0.1% sodium azide). All samples were incubated with 2.4G2 anti-FcRs (BD Pharmingen), prior to

**FIGURE 2.** TACI-IgG treatment upregulated IL-15 expression in EAE mice. (A) TACI-IgG treatment upregulated memory B cell number in EAE mice. EAE were induced in female C57BL/6 mice at 9 wk of age by MOG35–55 peptide in CFA. Twelve EAE mice per group were injected i.v. with 2 mg/kg TACI-IgG and/or 0.5 mg/kg anti–IL-15 neutralizing Ab on days 4, 8, 12, and 16 (once per day) after EAE was induced. On day 21, mice were euthanized and the lymphocytes were collected from the spleen. Memory B cells were isolated from the spleen by a memory B cell isolation kit, according to the manufacturer’s protocol. The average total cell number in each spleen of the six mice per group is shown. The data represent at least three independent experiments (**p < 0.01, ***p < 0.001). (B) IL-15 levels were increased in the serum from TACI-IgG–treated EAE mice. The serum was collected from CFA-, IgG-, and TACI-IgG–treated mice (16 mice per group) on days 4–6 after therapy. IL-15 levels were determined by ELISA. The data represent at least four independent experiments (**p < 0.01). (C) IL-15 mRNA was increased in B cells of the LN and spleen from TACI-IgG–treated EAE mice. B cells were sorted from the LN and spleen in IgG- and TACI-IgG–treated EAE mice (16 mice per group) on days 4–6 after therapy and subjected to qPCR. The data represent at least six independent experiments (*p < 0.05, ***p < 0.001). (D–F) TACI-IgG treatment increased IL-15 expression in B cells. B cells were sorted by B220 beads, stained with anti-B220 and anti–IL-15, and then analyzed by FACS. The percentage of IL-15–expressing B cells, statistical analysis of the percentage of IL-15+ B cells, and the absolute number of IL-15+ B cells in each spleen are shown in (D), (E), and (F), respectively. The data represent at least four independent experiments (**p < 0.01).
incubation with other Abs diluted in FACS buffer supplemented with 2% anti-FcR Ab. For intracellular cytokine staining, 50 ng/ml PMA and 1 μg/ml ionomycin (all from Sigma-Aldrich) were added, and then 10 μg/ml brefeldin A and 2 μM monensin were added 3 h later. Another 3 h later, cells were collected and fixed for 20 min with 1 mL fixation buffer (Fix and Perm cell permeabilization kit; eBioscience). After washing, the fixed cells were stained. The samples were filtered immediately before analysis or cell sorting to remove remaining clumps. Dead cell exclusion/discrimination dye was used to eliminate dead cells from analysis and sorted. For nonfixed cells, we used DAPI, SYTOX Blue, Green, or Red; for fixed cells, we use LIVE/DEAD Fixable Blue, Green, or Red. Choice of dead cell exclusion dye depended on the color combination of fluorochromes within the sample. The following Abs were purchased from eBioscience: anti-mouse CD27, CD3, B220, CD19, CD93, CD21, CD23, and CD4 Abs. Anti-IL-15 Ab was purchased from R&D Systems. Data collection and analyses were performed on a FACSCalibur flow cytometer using CellQuest software.

Cytokine analysis by ELISA

The concentration of cytokines (IL-15, BAFF, and dsDNA) was measured by ELISA kits. Anti-mouse IL-15, BAFF, and dsDNA ELISA kits were purchased from R&D Systems. Briefly, diluted supernatants were added in triplicate to the plate for 1 h at 37°C. Then after washing, biotin rat anti-mouse IL-15, BAFF, and dsDNA (4 μg/ml) Abs were added to the plate and were incubated for another hour at 37°C. Thereafter, unbinding Abs were washed off, followed by addition of avidin-HRP (1/10000 diluted) (all Abs were obtained from eBioscience). Plates were incubated for 1 h at 37°C. Finally, the color was developed by incubation with o-phenylenediamine. The OD was read at 492 nm with an ELISA reader (Bio-Rad). Standard curves were established to quantitate the amounts of the respective cytokines.

Cell proliferation assays

Lymphocytes were collected from lymph nodes (LN) in different groups of EAE. The cells (5 × 10^5 cells/well) were stimulated for 72 h with 0, 1, 5, 15, and 30 μg/ml MOG35–55. Forty-eight hours later, the cultures were pulsed with ^3H-thymidine (0.5 μCi), and data were expressed as mean cpm ± SE of responses of five replicate cultures. The results were expressed as the stimulation index (cpm with Ag divided by cpm with medium alone). The data shown were from only one of the Ag concentrations; however, results were obtained with various concentrations of the Ags.

Quantitative PCR analysis

All RNA samples were DNA free. cDNA synthesis and quantitative PCR (qPCR) analyses were performed, as previously described (19). Each gene-specific primer pair used for qPCR analysis spanned at least an intron. Primers and probes used for qPCR were purchased from Applied Biosystems, and mRNA expression was normalized to the levels of β-actin gene.

B cell subpopulation sorting

For analysis of transitional B cells, multicolor flow cytometry (three, four, or five colors) was performed by gating on CD19^+CD9^3^ B cells that were either CD21^-/CD23^- (transitional 1 B [T1B] cells) or CD21^+CD23^- (transitional 2 B [T2B] cells). For analysis of mature B cells, multicolor flow cytometry (three, four, or five colors) was performed by gating on CD19^-CD9^3^- B cells that were either CD21^-/CD23^- (follicular B [FB] cells) or CD21^-CD23^- (marginal zone B [MZB] cells). All flow cytometry data were acquired with FACSCanTo, FACSCanToL, or FACSArray (BD Biosciences), gated on live lymphocyte-sized cells on the basis of forward and side scatter, and analyzed using FlowJo software (Tree Star, Ashland, OR). The following Abs were purchased from eBioscience: PerCP-conjugated anti-mouse CD19, FITC-conjugated anti-mouse CD93, PE-conjugated anti-mouse CD21, and allophycocyanin-conjugated anti-mouse CD23.

Reduced expression or overexpression of Foxo1

Foxo1-specific short hairpin RNA (shRNA) with GFP (Santa Cruz Biotechnology, Santa Cruz, CA) were transfected into CD19^- B cells. The B cells were stimulated for 72 h with 1 μg/ml LPS. The GFP^- Foxo1 was reduced and GFP^- T2B cells were sorted from cultured cells by flow cytometry. All RNA samples were DNA free. cDNA synthesis and qPCR analyses were done to detect Foxo1 and IL-15 expressions. Mouserecombinant Foxo1 construct pEGFP-N1/Foxo1 was generated by rPCR. Plasmid pEGFP-N1/Foxo1 was transduced into CD19^- B cells by Lipo- reactamine 2000 (Invitrogen, Carlsbad, CA). Cells were stimulated for 72 h with 1 μg/ml LPS and analyzed by FACS. Cells were also sorted by flow cytometry, cDNA synthesis and qPCR analyses were done to detect IL-15 expression.

IL-15 promoter reporting gene analysis

To clone series of the human IL-15 gene promoter and to construct six luciferase reporter gene vectors pGL3 containing IL-15 promoter regions, six DNA fragments of the 5'-flanking region of human IL-15 gene were isolated from genomic DNA of HaCaT cells by PCR with IL-15 promoter primer sets (Table I). Empty vector or pEGFP-N1/Foxo1 and luciferase reporter vector pGL3/LIL-15 promoter were cotransduced into 293T cells. Dual luciferase reporter gene expression was analyzed, and the results were shown as the ratio of firefly to Renilla luciferase activity.

Western blot analyses

CD19^- B cells were stimulated with 10 μg/ml goat F(ab')^2; anti-mouse IgM (Southern Biotechnology Associates) in the presence of 50 ng/ml human rBAFF (PeproTech). Cells were stimulated for 72 h. For Foxo1 phosphorylation analysis, CD19^- B cells were stimulated for 0, 30, and 60 min with 10 μg/ml goat F(ab')^2, anti-mouse IgM (Southern Biotechnology Associates) in the presence of 50 ng/ml human rBAFF (PeproTech). The whole-cell lysates were prepared. Blots were probed with anti-β-actin, Foxo1, and p-Foxo1 (Santa Cruz Biotechnology). Preimmune serum was used in parallel as controls and signals were detected with HRP-conjugated secondary F(ab')^2 (Zymed Laboratories, San Francisco, CA) using ECL system (Amersham, Arlington Heights, IL).

Statistics

Statistics were generated using t test in GraphPad Prism (version 5.0; GraphPad Software), and values are represented as mean ± SEM. Results were considered statistically significant at p < 0.05.
Results

TACI-IgG upregulated IL-15 in lupus-like and EAE mice

BAFF levels have been shown to be increased in many autoimmune diseases, such as SLE, MS, and their animal models (Supplemental Fig. 1). When BAFF was blocked with TACI-IgG, numbers of mature B cells (Supplemental Fig. 2A–C) and the titer of anti-dsDNA autoantibodies (Supplemental Fig. 2D) were decreased in lupus-like mice. Previous studies have shown that TACI-IgG does not reduce memory B cells (10, 11). However, there is no marker for memory B cells in mice (12). Thus, we used a two-step procedure to isolate the memory B cells from six mice per group with a memory B cell isolation kit. The absolute memory B cell number per mouse was counted, and the results suggested that TACI-IgG treatment significantly upregulated memory B cell number in lupus-like mice (Fig. 1A).

These results are in accordance with the clinical data suggesting that TACI-IgG does not reduce memory B cells (10, 11). However, there is no marker for memory B cells in mice (12). Thus, we used a two-step procedure to isolate the memory B cells from six mice per group with a memory B cell isolation kit. The absolute memory B cell number per mouse was counted, and the results suggested that TACI-IgG treatment significantly upregulated memory B cell number in lupus-like mice (Fig. 1A). These results are in accordance with the clinical data suggesting...
that the total number of circulating memory B cells is preserved (or sometimes even increased) in belimumab- or TACI-IgG–treated patients.

IL-15 is an important cytokine for the survival and maintenance of memory T and B cells (13–15). Thus, we propose that blocking BAFF with TACI-IgG upregulates memory B cells by inducing IL-15 in lupus-like mice. As expected, TACI-IgG treatment upregulated IL-15 expression in the serum (Fig. 1B), spleen, and LN (Fig. 1C) from lupus-like mice. Because BAFF, APRIL, and their receptors play important roles in the B cell arm of the immune system (20), we assessed whether TACI-IgG upregulated B cells to express IL-15 by qPCR and flow cytometry (FACS) analysis.

**FIGURE 6.** BAFF suppressed T2B cells to secrete IL-15. (A and B) BAFF suppressed IL-15 expression in T2B cells. Splenic B cells were sorted from CFA and EAE mice (6 mice per group) on day 21 after EAE induction and then stimulated for 72 h with precoated MOG35–55 with or without BAFF. The cells were stained with anti-CD19 or anti-CD93, anti-CD21, anti-CD23, and anti-IL-15 Abs. The percentage of IL-15–expressing T1B (CD19+CD93+CD212CD23+), T2B (CD19+CD93+CD21+CD23+), FB (CD19+CD93+CD21+CD23+), and MZB (CD19+CD93+CD21+CD23+) cells and statistical analysis for IL-15–expressing T2B cells are shown in (A) and (B), respectively. The data represent at least six independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001). (C) BAFF suppressed IL-15 mRNA expression in T2B cells. T1B, T2B, FB, and MZB were sorted (see Supplemental Fig. 4) from the medium and BAFF-treated B cells described as in (A) and subjected to qPCR. The data represent at least three independent experiments (****p < 0.0001). (D and E) BAFF induced B cell maturation. (D) Splenic B cells were sorted from CFA and EAE mice on day 21 after EAE induction and then stimulated for 72 h with precoated MOG35–55 with or without BAFF. Cells were stained with anti-CD19 or anti-CD93, anti-CD21, and anti-CD23; T1B (CD19+CD93+CD212CD23+), T2B (CD19+CD93+CD21+CD23+), FB (CD19+CD93+CD21+CD23+), and MZB (CD19+CD93+CD21+CD23+) cells (see Supplemental Fig. 4) were analyzed. The transforming ratio from T1B to T2B to FB cells was shown in (E). The data represent at least three independent experiments.
We showed that IL-15 expression was upregulated mainly in B cells from TACI-IgG–treated lupus-like mice (Fig. 1D–F).

To further prove the effect of TACI-IgG on IL-15 expression in B cells, TACI-IgG was also used to treat EAE mice, resulting in the reduction of B cell numbers (Supplemental Fig. 3A–C), clinical scores (Supplemental Fig. 3D), and autoantigen-induced immune response (Supplemental Fig. 3E). However, TACI-IgG treatment also upregulated both the memory B cell number (Fig. 2A) and IL-15 expression in the serum (Fig. 2B) of EAE mice. Further experiments showed that TACI-IgG induced B cells to upregulate the expression of IL-15 (Fig. 2C–F).

The evidence for the role of IL-15 in autoimmune condition is compelling. ELISA has demonstrated that IL-15 levels are increased in many autoimmune diseases, such as SLE, MS, and their animal models (Supplemental Fig. 1). qPCR and FACS analysis have further shown that IL-15 was upregulated mainly in the spleen in autoimmune diseases (Fig. 3). IL-15/IL-15R has been used as a therapeutic target in inflammatory autoimmune diseases (21). In addition, our results presented above suggest that TACI-IgG treatment upregulates memory B cells by inducing IL-15 in B cells. Thus, the combination of TACI-IgG and anti–IL-15 Ab was used to treat lupus-like and EAE mice. Once IL-15 was blocked with

![FIGURE 7. Foxo1 controlled IL-15 expression in T2B cells. (A) Foxo1 expression was positively associated with IL-15 level. T1B (CD19+CD93+CD21–CD23–), T2B (CD19+CD93+CD21+CD23–), FB (CD19+CD93–CD21+CD23+), and MZB (CD19+CD93–CD21–CD23–) cells were sorted by flow cytometry (see Supplemental Fig. 4) and subjected to qPCR. Foxo1 and IL-15 mRNA expressions were shown in the upper and lower panels, respectively. The data represent at least three independent experiments (**p < 0.0001). (B) IL-15 expression was reduced in Foxo1-specific shRNA-transfected T2B cells. CD19+ B cells were transfected with Foxo1-specific shRNA with GFP and then stimulated for 72 h with LPS. GFP+ (Foxo1 was reduced) and GFP– (Foxo1 was not reduced) T2B cells were sorted by flow cytometry and subjected to qPCR. Foxo1 and IL-15 mRNA expressions are shown in the upper and lower panels, respectively. The data represent at least four independent experiments (*p < 0.05). (C) Foxo1 overexpression induced IL-15 expression. CD19+ B cells were transduced with empty vector or pEGFP-N1/Foxo1 and then stimulated for 72 h with LPS. GFP+ B cells were analyzed and sorted by flow cytometry. Cells were gated on enhanced GFP with numbers in the quadrants indicating the percentage of IL-15–expressing B cells (upper panel), and the sorted cells were subjected to qPCR (lower panel). The data represent at least four independent experiments (**p < 0.0001). (D) Foxo1 directly regulated IL-15 promoter. Six DNA fragments (described in Materials and Methods) of the 5’-flanking region of human IL-15 gene were isolated from genomic DNA of HaCaT cells by PCR. Six luciferase reporter gene vectors pGL3 containing IL-15 promoter regions were constructed. Empty vector or pEGFP-N1/Foxo1 and luciferase reporter vector pGL3/IL-15 promoter were cotransduced into 293T cells. Dual luciferase reporter gene expression was analyzed, and the results were shown as the ratio of firefly to Renilla luciferase activity. The results for IL-15 F0 and IL-15 F3 are shown. The results of IL-15 F0 and F1 as well as IL-15 F2–F6 were similar. The data represent at least four independent experiments (**p < 0.0001).]

Table I. PCR primers’ sequences

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<tr>
<th>Common Downstream Primer</th>
<th>5’–CTCATTACTCGAGACCGCGTAA–3’</th>
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<tr>
<td>Primers’ Sequence</td>
<td>Production (bp)</td>
</tr>
<tr>
<td>F0</td>
<td>5’–ATGCAGTTATGGAGGGAGTGAG–3’</td>
</tr>
<tr>
<td>F1</td>
<td>5’–GATTCAGGCAGCAAGCAGAG–3’</td>
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<td>F2</td>
<td>5’–CTCCCGGATACGCGTGAAT–3’</td>
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<td>F5</td>
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</tr>
<tr>
<td>F6</td>
<td>5’–CTCAATATGATTACCAATGTTT–3’</td>
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F, front (upstream) primer.
neutralizing anti–IL-15 Ab, the memory B cell number was significantly reduced in TACI-IgG-treated lupus-like (Fig. 1A) and EAE (Fig. 2A) mice. This study provides hints for the clinical application of BAFF- and IL-15–specific therapeutic agents.

**BAFF suppressed IL-15 expression in B cells**

The above experiments demonstrated that IL-15 was upregulated mainly in the spleen but not in LN (Figs. 1C–F, 2C–F, 3). Thus, we focused on splenic B cells. To determine the effect of TACI ligand (BAFF or APRIL) on IL-15 expression in B cells, we used autoantigen dsDNA (precoated in 96-well plate) plus BAFF or APRIL to stimulate B cells from lupus-like mice. FACS assay showed that BAFF but not APRIL significantly suppressed IL-15 expression in dsDNA-stimulated B cells from lupus-like mice (Fig. 4A). Furthermore, qPCR assay also proved that BAFF but not APRIL significantly suppressed IL-15 mRNA expression in B cells (Fig. 4B).

To further prove the effect of BAFF on IL-15 expression in B cells, we used MOG35–55 (precoated in 96-well plates) plus BAFF to stimulate B cells from EAE mice. BAFF significantly suppressed IL-15 expression in MOG35–55-stimulated B cells from EAE mice (Fig. 4C, 4D). In comparison, BAFF also slightly suppressed IL-15 expression in B cells from wild-type (WT) mice (Fig. 4A, 4B). To further prove these results, we also used different conditions such as LPS or anti-IgM to stimulate B cells from WT mice. qPCR analysis showed that BAFF suppressed IL-15 expression in LPS- or anti-IgM–stimulated B cells from WT mice. Similarly, BAFF also suppressed IL-15 expression in anti-human IgM-stimulated B cells from healthy donors (Fig. 5C). These results suggest that BAFF suppresses IL-15 expression in B cells induced by different stimuli. Furthermore, BAFF suppresses the secretion of IL-15 by CD19+ B cells but not CD4+ T cells (Fig. 4C).

**BAFF suppressed T2B cells to secrete IL-15**

Next, we determined which population of splenic B cells was crucial in the BAFF suppression of IL-15. We used autoantigen MOG35–55 (precoated in 96-well plates) plus BAFF to stimulate B cells from control and EAE mice. Different populations of B cells were analyzed by FACS (Fig. 6A). The results showed that T2B cells expressed a high level of IL-15, whereas T1B cells, MZB cells, and FB cells expressed low levels of IL-15. Among these cell types, BAFF suppressed the expression of IL-15 from T2B cells (Fig. 6A, 6B). Furthermore, we sorted T1B, T2B, FB, and MZB cells from BAFF-treated B cells, as described in Fig. 6A, by FACS (Supplemental Fig. 4) and then used qPCR to detect IL-15 expression. The results suggest that IL-15 is expressed mainly in T2B cells but not in T1B, MZB, or FB cells (Fig. 6C). In addition, BAFF suppressed IL-15 expression in T2B cells (Fig. 6C). Meanwhile, we found that BAFF promoted the transformation of T1B cells into T2B cells and T2B cells into FB cells (Fig. 6D, 6E). These results suggest that BAFF suppresses T2B cells from secreting IL-15 and promotes the transformation of T2B cells into mature B cells.

**BAFF suppression of Foxo1 downregulates IL-15 expression in B cells**

Next, we examined which transcription factor is responsible for IL-15 expression in T2B cells. Foxo1 was mainly expressed in T2B cells but not in T1B, MZB, or FB cells (Fig. 7A, upper panel), which is in accordance with IL-15 expression (Fig. 7A, lower panel). We used Foxo1-specific shRNA to reduce Foxo1 expression in B cells (Fig. 7B, upper panel) and then sorted the T2B cells. qPCR analysis showed a lower IL-15 expression in Foxo1-reduced T2B cells than Foxo1-unreduced T2B cells (Fig. 7B, lower panel). When overexpressed in B cells, Foxo1 upregulated IL-15 mRNA (Fig. 7C, lower panel) and protein (Fig. 7C, upper panel) expression. These results suggest that Foxo1 regulates IL-15 expression in T2B cells. To prove that Foxo1 directly regulates IL-15 expression, we constructed six luciferase reporter gene vectors pGL3 containing IL-15 promoter regions. By dual luciferase reporter gene expression analysis, we found that Foxo1 bound the region...
from 1500 to 1800 bp upstream of the IL-15 promoter (Fig. 7D, Table I).

Subsequently, we detected whether BAFF suppressed Foxo1 expression. We used autoantigen dsDNA or MOG35–55 (precoated in 96-well plates) plus BAFF to stimulate B cells from lupus-like or EAE mice, respectively. BAFF significantly suppressed Foxo1 expression in dsDNA-stimulated B cells from lupus-like mice (Fig. 8A) and MOG35–55-stimulated B cells from EAE mice (Fig. 8B). To further prove the results, we used different conditions such as LPS or anti-IgM to stimulate B cells from WT mice. qPCR analysis showed that BAFF suppressed Foxo1 expression in LPS (Fig. 9A)- or anti-IgM (Fig. 9B)-stimulated B cells from WT mice. Similarly, BAFF also suppressed Foxo1 expression in anti-human IgM-stimulated B cells from healthy donors (Fig. 9C). These results suggest that BAFF suppresses Foxo1 expression induced by different stimuli. Further Western blot analysis also showed that BAFF suppressed Foxo1 expression in CD19+ B cells (Fig. 8C). Additionally, we found that BAFF induced Foxo1 phosphorylation (Fig. 8D), which suggests that BAFF could also reduce Foxo1 level in the nuclei by inducing Foxo1 phosphorylation. Furthermore, blocking BAFF with TACI-IgG induced Foxo1 expression in B cells from lupus-like (Fig. 8E) and EAE (Fig. 8F) mice. These results altogether suggest that BAFF suppresses IL-15 in B cells by downregulating Foxo1 expression.

Discussion

BAFF demonstrates sp. act. toward B cells and supports B cell proliferation, differentiation, and survival (22). BAFF transgenic mice harbor increased numbers of B220+ B cells and plasma cells in the spleen and LN; they also develop anti-dsDNA Abs, proteinuria, and glomerulonephritis consistent with a systemic lupus-like autoimmunity as they age (2–4). By contrast, BAFF knockout mice have a markedly reduced mature B cell population and decreased serum Ig levels (22, 23). This study demonstrates that BAFF is increased in autoimmune diseases, such as SLE, MS, and lupus-like and EAE animal models (Supplemental Fig. 1). A series of therapeutic agents that inhibit BAFF but not APRIL significantly suppress IL-15 expression in B cells main immune response (memory B cells) by regulating IL-15 expression in T2B cells and BAFF suppressed IL-15 expression in T2B cells (Fig. 5). These results suggest that BAFF suppresses late immune response (memory B cells) by regulating IL-15 expression when BAFF positively activates earlier stage B cells. The basic mechanism in the immune response is a self-limited process, and, based on this, the possible harmful effects are self-limited (30). CD4+ T cells can also mediate pathologic immune responses, such that Th1 cells self-limit their inflammatory activity by IFN-γ (31). When it expands B cell responses, BAFF limits the late response (memory B cells) by suppressing IL-15 expression in B cells.

TACI-IgG neutralizes both BAFF and APRIL. To determine the effect of BAFF and APRIL on IL-15 expression in B cells, we used autoantigen dsDNA (precoated in 96-well plates) plus BAFF or APRIL to stimulate B cells from lupus-like mice. The results suggest that BAFF but not APRIL significantly suppresses IL-15 expression in dsDNA-stimulated B cells from lupus-like mice (Fig. 4A). Moreover, we found that IL-15 was expressed mainly in T2B cells and BAFF suppressed IL-15 expression in these cells (Fig. 5). Thus, our data suggest that BAFF but not APRIL could suppress IL-15 expression in earlier B cells, such as T2B cells. This is in accordance with previous studies suggesting that the interaction of BAFF and BAFFR is essential for viability of B cells.

FIGURE 9. BAFF suppressed Foxo1 expression in B cells. Splenic CD19+ B cells were sorted using CD19 beads from naïve C57BL/6 mice and then stimulated for 48 h with LPS (A) or anti-IgM (B) with or without BAFF. (C) B cells from the peripheral blood cells of MS patients were sorted using CD19 beads and then stimulated for 48 h with anti-IgM with or without BAFF. (A–C) Cells were subjected to qPCR. The data represent at least four independent experiments (**p < 0.05, ***p < 0.01).
whereas APRIL-B cell maturation Ag and APRIL-TACI interactions are implicated more in later processes that culminate in Ab production (32–36).

A recent study has shown that Foxo3a expression does not change in B cell differentiation (37). Foxo1 can directly regulate the transcription of RAGs during B cell development (38). Our study showed that Foxo1 directly regulates the IL-15 gene transcription in T2B cells (Fig. 7). BAFF suppresses memory B cells by regulating the Foxo1–IL-15 signal pathway (Fig. 7) when it promoted B cell maturation (Fig. 6D, 6E). These results are consistent with a recent study showing that Foxo1 actively represses effectors or terminal differentiation processes to promote the memory CD8<sup>+</sup> T cell development (39).

In conclusion, BAFF suppressed IL-15 expression in T2B cells by downregulating the Foxo1 signaling pathway. Once BAFF was blocked with belimumab or TACI-IgG, Foxo1 and IL-15 levels increased, leading to an increase in the number of memory B cells in autoimmune diseases. As long as the immune memory B cells are maintained, humoral response can in principle be restored in autoimmune diseases. As long as the immune memory B cells (40), patients will become immunologically naive. As a result, it is necessary to use BAFF inhibitors in combination with anti–IL-15 Abs to treat autoimmune diseases. This provides hints for the clinical application of combination of BAFF and IL-15 inhibitor in human autoimmune diseases.

Disclosures

The authors have no financial conflicts of interest.

References


