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B cell activating factor (BAFF) induces the transcription of recombination-activating genes in transitional stage 1 B cells

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Abstract

A high level of receptor editing appeared to favor the loss of tolerance on an autoimmune background. However, the factors inducing imbalance of the receptor editing gene is still not clear. First, we used myelin oligodendrocyte glycoprotein (MOG) to induce experimental allergic encephalitis (EAE) in C57BL/6 mice. We found that recombination-activating genes (RAG) are expressed in periphery transitional stage 1 B (T1B) cells from EAE mice. In addition, RAG1 and RAG2 expression was up-regulated in T1B cells from the EAE model. At the same time, we also detected a level of the RAG gene in MS patients. The results were in accordance with those in the EAE mice model. Furthermore, we found that RAG1 and RAG2 increase was associated with BAFF. In vitro culture assay shows that BAFF up-regulated RAG1 and RAG2 mRNA expression in T1B cells from wild type mice. Block of BAFF with TACI-IgG reduced RAG1 and RAG2 expression in T1B cells from EAE mice. The study suggests that BAFF up-regulated the RAG expression to induce receptor editing in autoimmune diseases.

Key words: *BAFF, RAG, EAE.*

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Introduction

Multiple sclerosis (MS) is an inflammatory disease mediated mainly by T cells. Experimental allergic encephalomyelitis (EAE) is an animal model of brain inflammation. MOG-induced chronic EAE in C57BL/6 mice [1] is an animal model of brain inflammation like MS. Evidence from animal models also points to a role of B cells in addition to T cells in the development of the disease [2].

B cell activating factor (BAFF) is a member of the tumor necrosis factor ligand superfamily that demonstrates specific activity toward B cells, and supports B-cell proliferation, differentiation, maturation, survival and function [3]. An excess of BAFF leads to the development of autoimmune disorders in animal models, and high levels of BAFF have been detected in the serum of patients with various autoimmune conditions [4]. BAFF is expressed by astrocytes that are associated closely with BAFF-R-expressing cells [5] and within ectopic lymphoid follicles in the meninges [6] suggesting that BAFF plays also a potential pathogenic role in MS.

Autoreactive immature B cells were initially considered to be censored through either clonal deletion or anergy [7]. The investigators noticed that a small proportion of immature B cells harboring autoreactive immunoglobulin (Ig) transgenes did not succumb to clonal deletion but rather, through secondary V(D)J recombination events (refer to receptor editing) [8]. There are two mechanisms to explain the relationship of receptor editing and the generation of autoreactive B cells. When receptor editing leads to a productive rearrangement at the non-rearranged allele, the immature B cells will express two specificities: the original autoreactive specificity and a new potentially non-autoreactive specificity. It is conceivable that co-expression of non-autoreactive antigen receptors might promote

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the development of autoreactive B cells and prevent further censoring [9]. Other researchers propose an additional process whereby mature B cells can acquire a new autoreactivity by receptor editing (somatic mutation) [10].

Progress will be made in defining the molecular pathways that lead to the initiation or suppression of receptor editing. More attention is required in this area. Due to the important role of BAFF in autoimmune diseases, we propose that BAFF initiate RAG gene expression. Our data show that BAFF up-regulated recombination-activating genes (RAG) expressed in the periphery transitional stage 1 B (T1B) cells. Blockade of BAFF with TACI-IgG reduced RAG1 and RAG2 expression. The study suggests that BAFF up-regulated the RAG expression to induce receptor editing in autoimmune diseases.

Material and methods

Ethics Committee Approval

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

MS patients

Blood samples were obtained after the approval from the Beijing Institute of Basic Medical Sciences and consent from 15 untreated early relapsing-remitting MS patients from the Department of Neurology, Beijing Chaoyang Hospital and from 15 normal human subjects from the Beijing 307 Hospital.

EAE induction

At 9 weeks of age C57Bl/6 mice received a subcutaneous injection of 125 µg MOG35-55 peptide (Mimotopes, Australia) emulsified 1 : 1 (vol/vol) in Complete Freund's Adjuvant containing 4 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI), to both flanks and the base of the tail. Pertussis toxin (300 ng in PBS; List Biological, USA) was injected intraperitoneally at the time of induction and a second dose was administered three days later. Animals were weighed, monitored and clinically assessed according to the following grading scale: 0 = no symptoms; 1 = distal tail weakness; 1.5 = tail weakness and some hindlimb weakness; 2 = complete tail paralysis; 2.5 = complete tail paralysis and partial hindlimb weakness; 3 = complete hindlimb weakness; 3.5 = inability to right when placed on the back or significant forelimb weakness; 4 = = euthanize or spontaneous death [11, 12]. Mice were euthanized if they lost 20% of their starting weight, displayed a clinical score of 3 for 72 hours or reached a clinical score of 3.5. Mice were examined for up to 21 days post-immunization. Numbers of mice per treatment group: 12.

Treatment of EAE with TACI-IgG

Experimental allergic encephalomyelitis mice were divided into the following five groups: 1, none EAE (CFA) mice; 2, IgG-treated EAE mice; 3, TACI-IgG-treated EAE mice; twelve EAE mice per group were *i.v.* injected with 2 mg/kg TACI-IgG on day 4, 8, 12, 16 (one time per day) after EAE was induced with MOG33-55.

B-cell subpopulation sorting

For analysis of transitional B cells, multicolor flow cytometry (three, four, or five color) was performed by gating on $CD19^+CD93^+$B cells that were either $CD21^-/CD23^+$ (T1B cells) or $CD21^+CD23^+$ (T2B cells). For analysis of mature B cells, multicolor flow cytometry (three, four, or five color) was performed by gating on $CD19^+CD93^-$B cells that were either $CD21^+/CD23^+$ (Follicular B cells, FB cells) or $CD21^+CD23^-$ (Marginal zone B cells, MZB cells). All flow cytometry data were obtained using FACSCanto or FACSCantoII or FACSAria (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 antibodies were purchased from eBioscience: PerCP-conjugated anti-mouse CD19, FITC-conjugated anti-mouse CD93, PE-conjugated anti-mouse CD21, APC-conjugated anti-mouse CD23, PerCP-conjugated anti-human CD19, FITC-conjugated anti-human CD93, PE-conjugated anti-human CD21, APC-conjugated anti-human CD23.

In vitro T1B-cell cultures

T1B cells were isolated from wild type mice or healthy donors by flow cytometry and cultured in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), and 50 mM 2-mercapthoethanol. Cells were stimulated with 10 µg/ml Goat $F(ab')_2$ anti-mouse IgM or Goat $F(ab')_2$ anti-human IgM (SouthernBiotech, Birmingham, AL) in the presence of 50 ng/ml rBAFF (Peprotech, Rochy Hill, NJ).

Cytokine analysis by ELISA

The concentration of cytokines was measured by ELISA kits. Anti-mouse and human BAFF ELISA kits were purchased from R&D system. Briefly, diluted supernatants were added in triplicate to the plate for 1 h at 37°C. Then, after washing, biotin rat anti-mouse BAFF (4 µg/ml) Ab was added to the plate, and incubated for another hour at 37°C. Thereafter, unbinding antibody was washed away, followed by the addition of avidin-HRP (1/1000 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 amount of the respective cytokines.

qPCR analysis

All RNA samples were DNA free. cDNA synthesis and qPCR analyses were performed as described [13]. Each gene-specific primer pair used for qPCR analysis spans 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 and GAPDH genes.

Statistics

Statistics were generated using *t*-test in GraphPad Prism (version 5.0, GraphPad Software Inc., USA) and values are represented as mean ± standard error of the mean (SEM). Results were considered statistically significant at $p < 0.05$.

Results

High level of RAG gene in transitional stage 1 B (T1B) cells from EAE mice

MOG33-55 was used to induce the EAE model on the background of C57BL/6 mice. Lymphocytes were collected from the spleen of EAE mice on day 21 after EAE induction. $CD19^+CD93^+CD21^-CD23^+$ (T1B cells), $CD19^+CD93^+CD21^+CD23^+$ (T2B cells), $CD19^+CD93^-CD21^+CD23^+$ (Follicular B cells, FB cells), or $CD19^+CD93^-CD21^+CD23^-$ (Marginal zone B cells, MZB cells) were sorted by flow cytometry. These sorted cells were subject to qPCR. In T2B cells, FB cells and MZ B cells, RAG1 and RAG2 expression is very low so that they cannot almost be significantly shown in Fig. 1. As compared to T2B cells, FB cells, MZ B cells and total B cells, T1B cells expressed a high level of RAG1 and RAG2 (Fig. 1). These results suggest that RAG1 and RAG2 are mainly expressed in T1B cells.

We sorted T1B cells from the spleen of control (only injection of CFA) and EAE mice on day 21 after EAE induction. T1B cells were subject to qPCR. The results demonstrate that as compared with control mice, EAE mice induced a high level of RAG1 and RAG2 mRNA (Fig. 2A). At the same time, we detected a level of RAG1 and RAG2 mRNA in T1B cells from healthy donors and MS patients. We found that MS patients expressed higher levels of RAG1 and RAG2 mRNA than healthy donors (Fig. 2B). These results suggest a higher level of RAG1 and RAG2 mRNA in autoimmune diseases.

BAFF up-regulated RAG gene expression in T1B cells

Various autoimmune conditions caused high levels of BAFF which has been regarded as a new therapeutic target in autoimmune diseases [14]. Our data demonstrate that BAFF is increased in EAE mice (Fig. 3A) and MS patients (Fig. 3B).

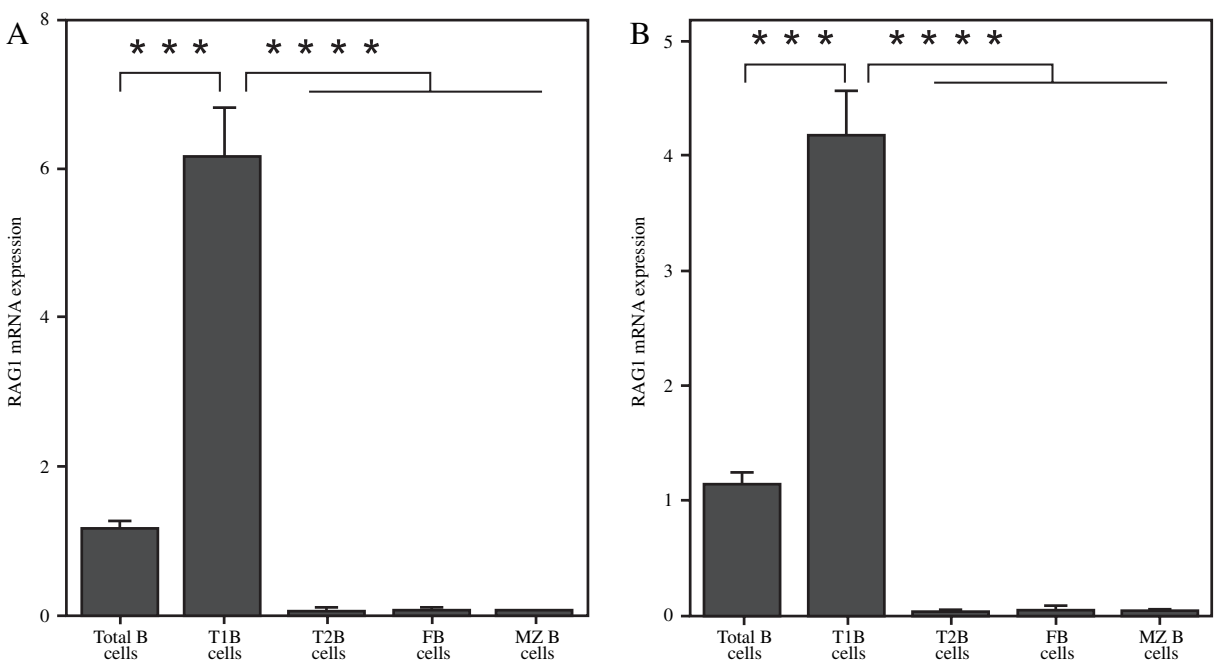

Fig. 1. Transitional stage 1 B (T1B) cells expressed a high level of RAG gene. An EAE model on the background of C57BL/6 mice was induced by injecting MOG33-55 in CFA. Lymphocytes were collected from the spleen of EAE mice on day 21 after EAE induction. $CD19^+CD93^+CD21^-CD23^+$ (T1B cells), $CD19^+CD93^+CD21^+CD23^+$ (T2B cells), $CD19^+CD93^-CD21^+CD23^+$ (Follicular B cells, FB cells), or $CD19^+CD93^-CD21^+CD23^-$ (Marginal zone B cells, MZB cells) were sorted by flow cytometry. These sorted cells were subject to qPCR. The data represent at least four independent experiments (***$P < 0.001$, ****$P < 0.0001$).

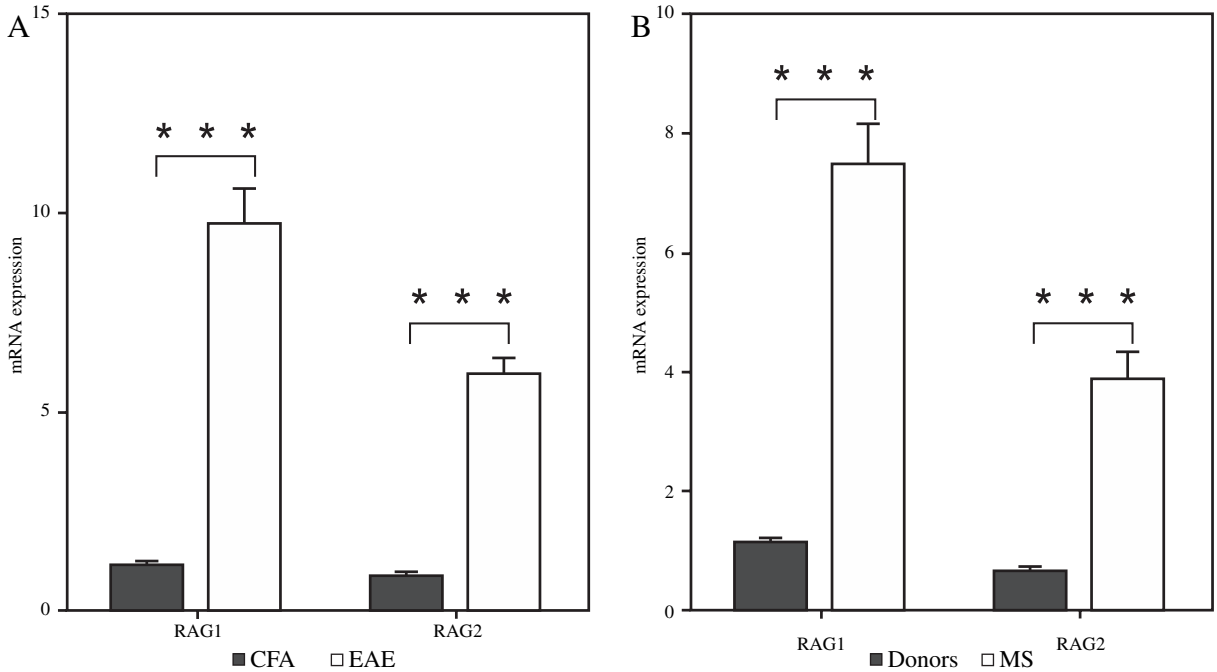

Fig. 2. High level of RAG gene in MS and EAE. (A) EAE up-regulated RAG gene expression. T1B cells were sorted from the spleen of control (only injection of CFA) and EAE mice on day 21 after EAE induction. T1B cells were subject to qPCR. (B) MS up-regulated RAG gene expression. T1B cells were sorted from periphery blood cells in healthy donors and MS patients. T1B cells were subject to qPCR. The data represent at least five independent experiments (***$P < 0.001$)

Next, we examined whether BAFF induced RAG gene expression in T1B cells from mice. First, we sorted T1B cells from wild type mice. T1B cells were cultured with anti-mouse IgM and BAFF for 48 hours and then subject to qPCR. The results suggest that BAFF induced RAG gene expression (Fig. 3C). At the same time, we detected whether BAFF induced RAG gene expression in T1B cells from healthy donors. T1B cells were sorted, cultured with anti-human IgM and BAFF for 48 hours and then subject to qPCR. The results suggest that BAFF induced RAG gene expression in human T1B cells (Fig. 3D).

Blockade of BAFF with TACI-IgG reduced RAG1 and RAG2 expression

BAFF inhibitor atacicept (TACI-IgG) shows similar clinical results as belimumab (anti-BAFF antibody) by the binding portion of a receptor TACI to block the effect of BAFF and APRIL. It reduces circulating mature B-cell and plasma cell levels in the spleen and bone marrow, inhibits T-cell activation [15]. The EAE model on the background of C57BL/6 mice was induced with MOG33-55 in CFA and then treated with TACI-IgG described in Materials and methods. T1B cells were sorted from the CFA alone injection group, IgG or TACI-IgG-treated EAE and subject to qPCR. Compared with the CFA control group, IgG-treated EAE mice induced the high level of RAG1 and RAG2 mRNA expression. Compared with IgG-treated, TACI-IgG-treated EAE mice reduced RAG1 and RAG2 mRNA expression (Fig. 4). The results suggest that blockade of BAFF with TACI-IgG reduced RAG1 and RAG2 expression.

The above results altogether suggest that BAFF up-regulated RAG gene expression to induce receptor editing in autoimmune diseases.

Discussion

Receptor editing appears to be an important normal mechanism to purge the B-cell repertoire of autoreactivity. If a developing B cell expresses a heavy chain/light chain combination that recognized an autoantigen with sufficient affinity, it can be signaled to continue to express the Ig gene recombination machinery, including the RAG1 and RAG2 genes. It thereby undergoes further gene rearrangements that replace either the light chain or the heavy chain variable regions, so that a new B cell receptor that is not autoreactive is produced. This process can also lead to the development of mature B cells co-expressing autoreactive and non-autoreactive specificities and, thus, is a potential source of autoantibodies.

In the setting of autoimmune disease, particularly lupus, the characterization of Ig gene usage by an autoantibody producing B cells has shown increased receptor editing [16, 17]. Although several laboratories have demonstrated the

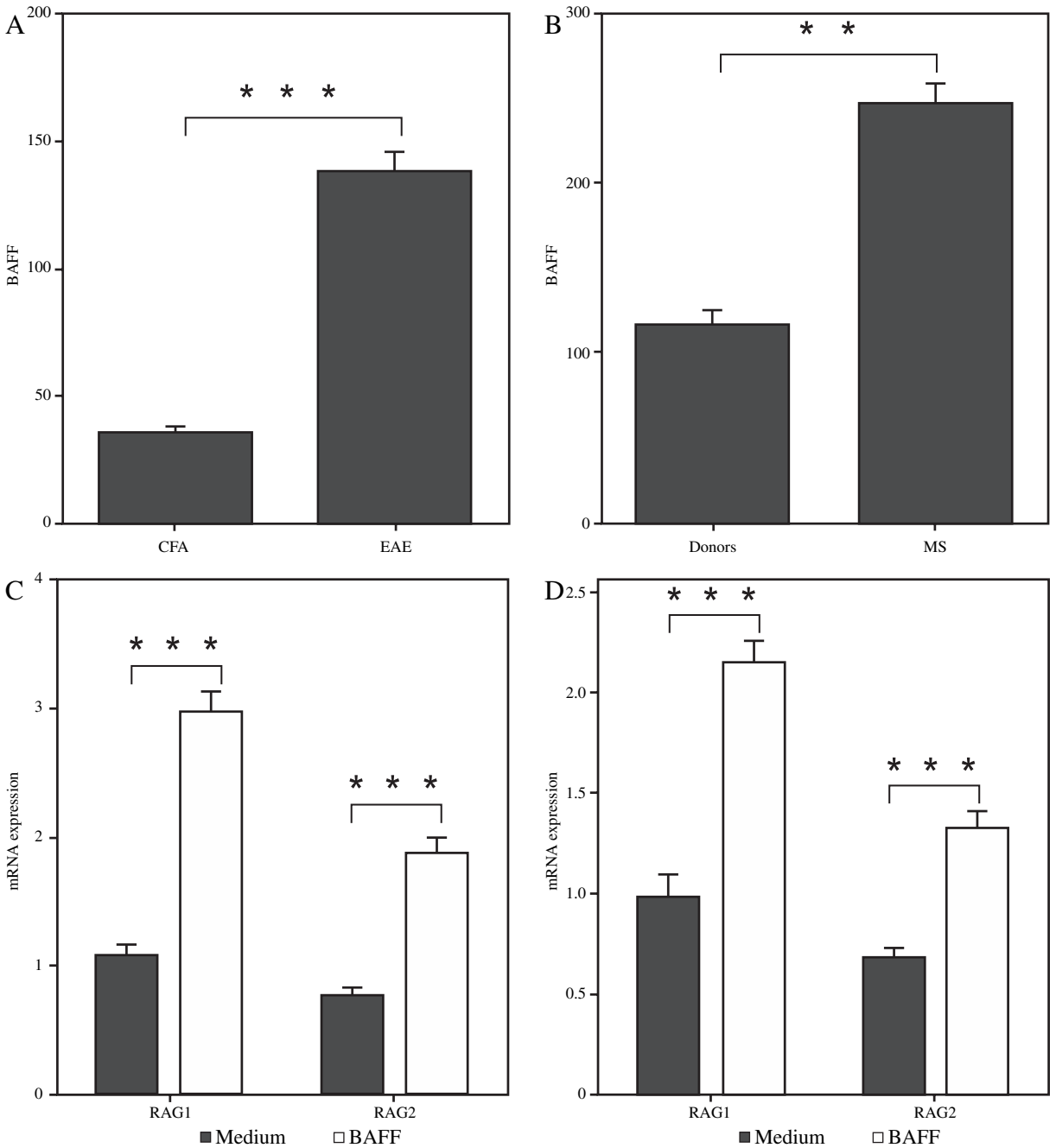

Fig. 3. BAFF up-regulated RAG gene expression in T1B cells. (A) BAFF increased in EAE mice. The BAFF level in the serum from CFA and EAE mice was determined by ELISA. (B) BAFF increased in MS patients. The BAFF level in the serum from healthy donors and MS patients was determined by ELISA. (C) BAFF up-regulated RAG gene expression in murine T1B cells. T1B cells were sorted from wild type mice. T1B cells were cultured with anti-mouse IgM and BAFF for 48 hours and then subject to qPCR. (D) BAFF up-regulated RAG gene expression in human T1B cells. T1B cells were sorted from healthy donors. T1B cells were cultured with anti-human IgM and BAFF for 48 hours and then subject to qPCR. The data represent at least four independent experiments (**$P < 0.01$, ***$P < 0.001$)

expression of the RAG genes in the spleen and lymph nodes, particularly after an antigenic challenge, much of this phenomenon has been explained by the peripheralization of immature B cells [18]. We sorted transitional stage 1 (T1B) cells ($CD19^+CD93^+CD21^-CD23^+$), transitional stage 2 (T2B) cells ($CD19^+CD93^+CD21^+CD23^+$), Follicular B (FB) cells ($CD19^+CD93^-CD21^+CD23^+$), and Marginal zone (MZ) B cells ($CD19^+CD93^-CD21^+CD23^-$). By analyzing

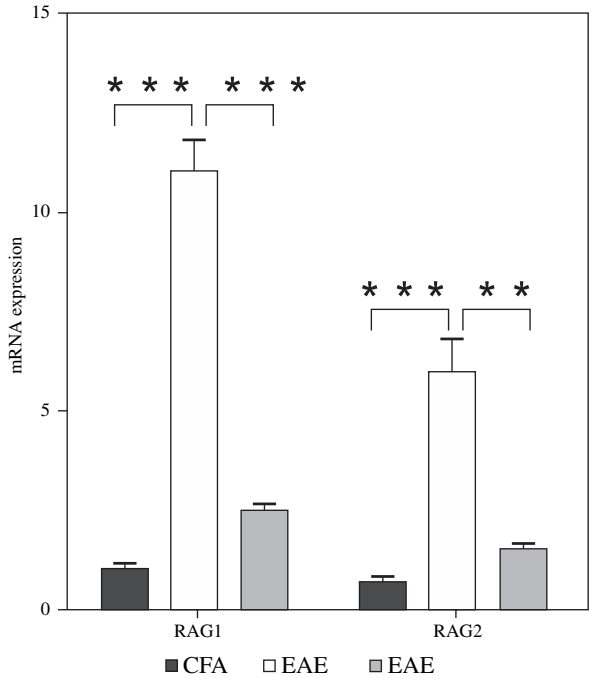

Fig. 4. Blockade of BAFF with TACI-IgG reversed RAG gene expression in EAE mice. EAE was induced in 9-week-old C57BL/6 mice by MOG35-55 peptide in CFA. Twelve EAE mice per group were i.v. injected with 2 mg/kg TACI-IgG (BAFF inhibitor) or IgG (control for TACI-IgG) on day 4, 8, 12, 16 (one time per day) after EAE was induced. Mice were examined for up to 21 days post-immunization. TACI-IgG-treatment significantly reduced EAE development, compared with IgG-treated group (data not shown, $p < 0.05$). On day 21, mice were killed. T1B cells were sorted from spleen and subject to qPCR. The data represent at least four independent experiments (**$P < 0.01$, ***$P < 0.001$)

the transcription of RAG1 and RAG2, we found that RAG1 and RAG2 were mainly expressed in T1B cells (Fig. 1). Furthermore, compared with control mice, EAE mice induced a high level of RAG1 and RAG2 mRNA (Fig. 2A) in T1B cells. In addition, MS patients also expressed higher levels of RAG1 and RAG2 mRNA in T1B cells than healthy donors (Fig. 2B). Thus, our data proved that rag gene is expressed in immature B cells. This is in accord with previous studies suggesting that dysfunctional editing or receptor revision has a role in the context of autoantibody disease and lymphocyte neoplasia raised by the apparent examples of revision and re-editing reported in systemic lupus erythematosus [19], rheumatoid arthritis [20] and thyroiditis [21].

Progress has also been made in defining the molecular pathways that lead to the initiation or suppression of receptor editing. However, it is clear that more attention is required in this area. Specifically, we need to identify how the effectors influence receptor editing for example, the transcription level of the rag. Various autoimmune conditions caused high levels of BAFF which has been regarded as a new therapeutic target in autoimmune diseases [14]. Our data demonstrate that BAFF *in vitro* induced RAG gene expression in T1B cells from mice (Fig. 3). Blockade of BAFF with TACI-IgG reduced the RAG1 and RAG2 expression in the EAE model. These results altogether suggest that BAFF up-regulated RAG gene expression to induce receptor editing in autoimmune diseases. The signaling cascades must ultimately regulate the level and activity of transcription factors that, in turn, modulate rag expression. Nuclear factor κB (NF-κB) transcription factors also appear to have a role in regulating receptor editing. Inhibition of NF-κB activity in immature B cells prevents RAG transcription [22]. BAFF activates a canonical NF-κB signaling pathway leading to the nuclear translocation of p50, c-Rel and p65 [23]. Thus, BAFF may induce RAG gene expression by activating NF-κB signaling pathway.

A high level of receptor editing appeared to favor the loss of tolerance on an autoimmune background. RAG1 and RAG2 expression was up-regulated in T1B cells from the EAE model or MS patients. Further we demonstrate that RAG1 and RAG2 increase is associated with BAFF. Blockade of BAFF with TACI-IgG reduced RAG1 and RAG2 expression. The study suggests that BAFF up-regulated RAG expression to induce receptor editing in autoimmune diseases.

The authors declare no conflict of interests.

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