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Regulation of B cell activating factor (BAFF) receptor expression by NF-κB signaling in rheumatoid arthritis B cells

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Abbreviations: APRIL, a proliferation-inducing ligand; BAFF, B cell activation factor; BAFF-R, BAFF receptor; BCMA, B cell maturation antigen; BCR, B cell receptor; MMP, matrix metalloproteinase; OA, osteoarthritis; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis; TACI, transmembrane activator and calcium modulator and cyclophilin ligand interactor

Abstract

B cells play an important role in the pathogenesis of rheumatoid arthritis (RA). High levels of B cell activating factor (BAFF) are detected in autoimmune diseases. BAFF and BAFF receptor (BAFF-R) are expressed in B and T cells of RA synovium. The study was undertaken to identify the NF-κB signal pathway involved in the induction of BAFF-R in human B cells. Immunohistochemical staining of NF-κB p65, NF-κB p50, BAFF, and BAFF-R was performed on sections of synovium from severe and mild RA and osteoarthritis (OA) patients. Peripheral blood mononuclear cells (PBMCs) were isolated from control and RA patients and B cells were isolated from controls. BAFF-R was analyzed by flow cytometry, realtime PCR and confocal staining after treatment with NF-κB inhibitors. NF-κB p65, NF-κB p50, BAFF, and BAFF-R were highly expressed in severe RA synovium relative to mild RA synovium or OA synovium. BAFF-R expression was reduced by NF-κB inhibitors in PBMCs and B cells from normal controls. We also showed reduction in expression of BAFF-R via inhibition of the NF-κB pathway in PBMCs of RA patients. BAFF/BAFF-R signaling is an important mechanism of pathogenesis in RA and that BAFF-R reduction by NF-κB blocking therapy is another choice for controlling B cells in autoimmune diseases such as RA.

Keywords: B-cell activation factor receptor; B-cell activating factor; B-lymphocytes; NF-kappa B; rheumatoid arthritis

Introduction

B cell activating factor (BAFF), a member of the tumor necrosis factor family and also known as BLys, TALL-1, zTNF4, or THANK, is a critical cytokine in peripheral B cell survival during primary development and homeostasis (Rahman *et al.*, 2003; Matsushita *et al.*, 2007). BAFF is mainly secreted by monocytes, dendritic cells, and T cells. (Rahman *et al.*, 2003; Rahman and Manser, 2004; Zhang *et al.*, 2005; Matsushita *et al.*, 2007). BAFF stimulation also results in prolonged survival of mature resting B cells (Do *et al.*, 2000; Gross *et al.*, 2001). The addition of BAFF to cultures of B cells augments class switching of those cells from IgM to IgG (Litinskiy *et al.*, 2002).

In humans, high levels of BAFF are detectable in the blood of a percentage of patients with autoimmune diseases, particularly systemic lupus erythematosus and Sjögren's syndrome (Nakajima *et al.*, 2007; Groom *et al.*, 2002) Controlling BAFF may be a potential treatment in B cell-related autoimmune diseases.

BAFF binds to three receptors, BAFF-R, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and B cell maturation Ag (BCMA) (Rahman *et al.*, 2003; Rahman and Manser, 2004; Zhang *et al.*, 2005; Matsushita *et al.*, 2007; Kim *et al.*, 2009). TACI and BCMA also bind a closely related cytokine, a proliferation-inducing ligand (APRIL). Although all three receptors bind to BAFF, BAFF-R appears to be the most important receptor for mature B cell survival

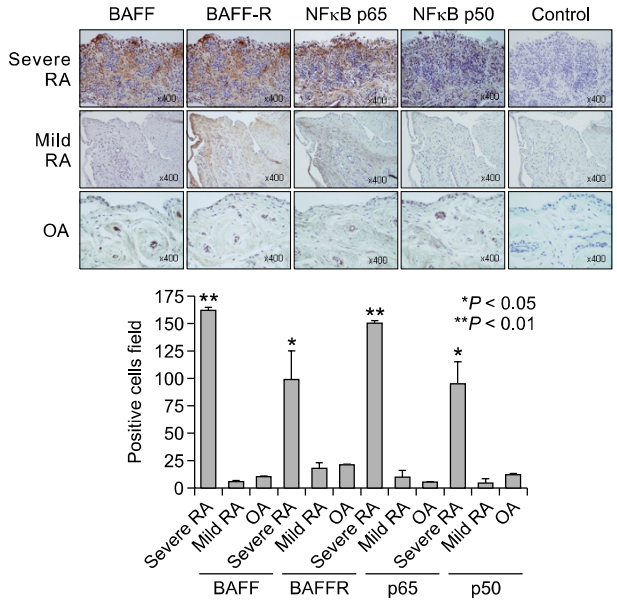

Figure 1. Expression of BAFF, BAFF-R, NF-κB p65, and NF-κB p50 are significantly increased in RA synovium. Synovial tissue sections were analyzed using immunohistochemistry and monoclonal antibodies to BAFF, BAFF-R, NF-κB p65 and NF-κB p50.

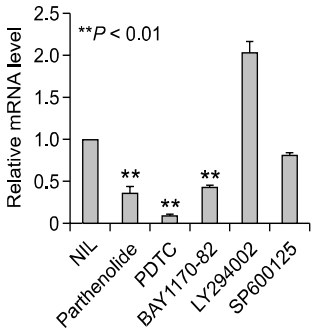

Figure 2. Effects of signal pathway inhibitors on BAFF-R expression in B cells isolated from healthy volunteers. PBMCs were cultured for 24 h with parthenolide (10 μM), PDTC (0.5 μM), BAY1170-82 (10 μM), LY294002 (1 μM), or SP900125 (10 μM). Analysis of BAFF-R expression was performed using real time PCR. The results were confirmed by four independent experiments.

and homeostasis in peripheral B cells. BAFF-R is the only known BAFF receptor detectable on most mature B cells. Furthermore, anti-BAFF-R monoclonal antibodies completely inhibited BAFF-mediated costimulation of B cells (Ng *et al.*, 2004). A/WySnJ mice, containing a mutation in BAFF-R, exhibit a phenotype that is similar to BAFF-deficient mice (Mayne *et al.*, 2008).

Nuclear factor (NF)-κB controls the expression of cytokines IL-1β and TNFα, the essential mediators of inflammation in rheumatoid arthritis (RA). Many conventional anti-inflammatory and antirheumatic drugs, including glucocorticoids, aspirin, sodium salicylate and sulfasalazine, are inhibitors, albeit not very potent ones, of NF-κB activation. Some studies have shown that more specific inhibitors of NF-κB reduced the severity of arthritis and inhibited the production of IL-1β and TNFα in several arthritis animal models (Miagkov *et al.*, 1998; Tomita *et al.*, 1999; Makarov, 2001; Jung *et al.*, 2010).

Following B lymphocyte depletion with the anti-CD20 antibody rituximab in patients with RA, a positive clinical response occurred that correlated with a significant drop in the levels of C-reactive protein and autoantibodies (Cambridge *et al.*, 2003). In addition, cells of the synovial sublining expressed BAFF. BAFF and BAFF-R were expressed in B and T cells extracted from RA synovium (Nakajima *et al.*, 2007). These results showed a role for B lymphocytes in the pathogenesis of RA. Inhibition of NF-κB reduced production of inflammatory cytokines IL-1 and TNFα in the RA model. NF-κB might also control B cell function via BAFF and BAFF-R. This result would suggest that not only T helper cells but also B cells are connected by NF-κB pathways in RA.

Excessive BAFF signaling through BAFF-R results in prolonged B cell survival and costimulates B and T cells. Instead of blocking BAFF-R or decreasing BAFF, reduction of BAFF-R numbers would also, theoretically, reduce the effects of BAFF-BAFF-R signaling in inflammatory autoimmune diseases.

We redefined the expression of BAFF-R in RA synovium and investigated the relation of BAFF-R and NF-κB. We show the possibility of controlling the amounts of BAFF-R and reducing the effects of BAFF-R signaling through NF-κB inhibition.

Results

Increased expression of BAFF-R in the RA synovium

To examine BAFF and BAFF-R expression in synovium, samples of severe RA, mild RA and OA synovium were immunostained with specific antibodies for these proteins. BAFF and BAFF-R were expressed more strongly in synovial tissue sections derived from patients with severe RA than in sections from patients with mild RA or OA. To determine NF-κB p65 and NF-κB p50 expression, tissues were immunostained with specific antibodies for these proteins. The immunostaining patterns of NF-κB p65 and NF-κB p50 were similar to those for BAFF-R (Figure 1).

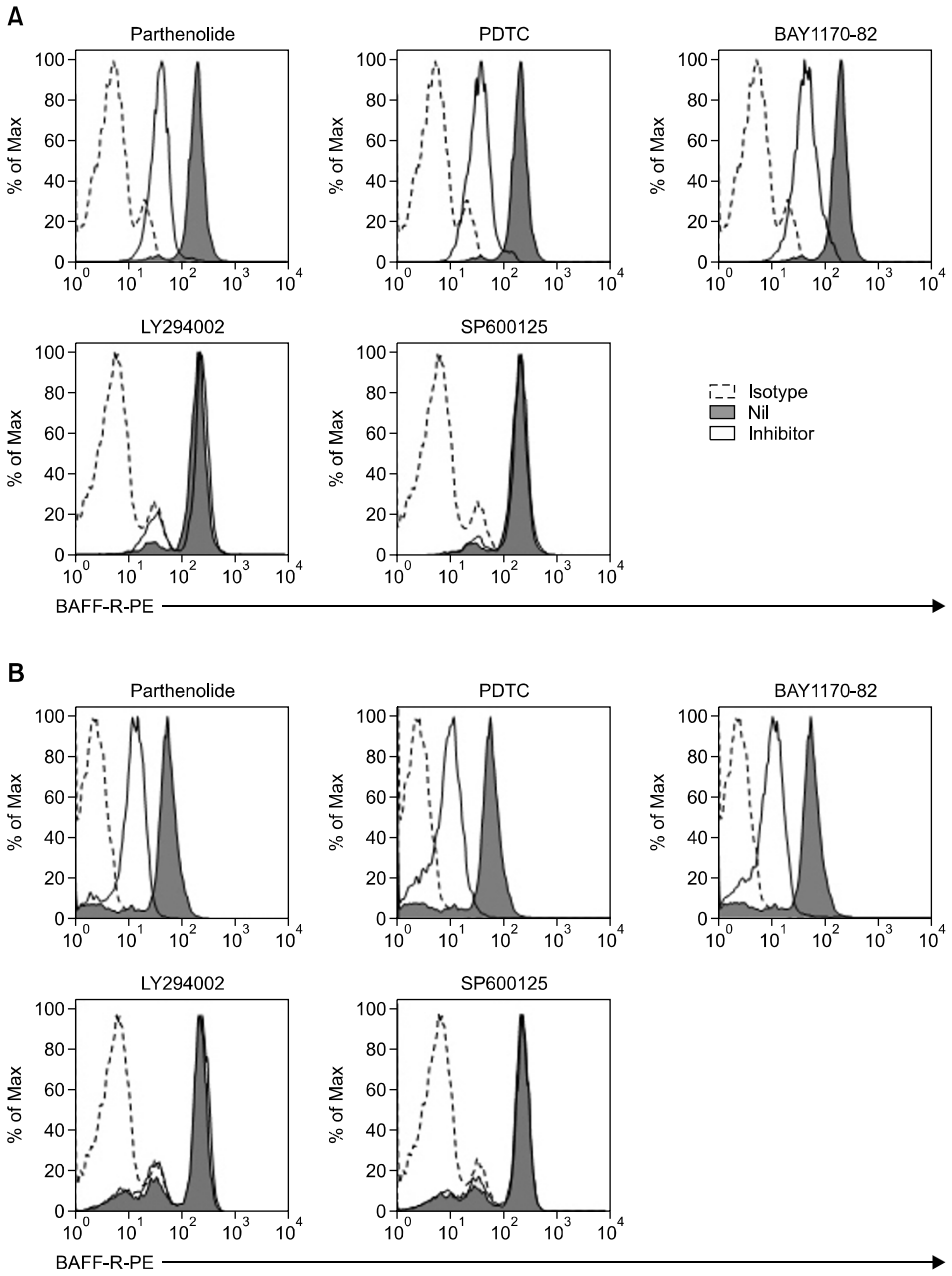

Figure 3. Effects of signal pathway inhibitors on BAFF-R expression on PBMC (A) and B cells (B) isolated from healthy controls. Cells were cultured with parthenolide (10 μM), PDTC (0.5 μM), BAY1170-82 (10 μM), LY294002 (1 μM), or SP900125 (10 μM) for 24 h. Analysis of BAFF-R expression was performed using FACS. The results were confirmed by four independent experiments.

Effect of inhibition of signaling pathways on expression of BAFF-R in healthy PBMCs

To determine the effect on BAFF-R expression of inhibition of several signal pathways, PBMCs isolated from healthy volunteers were cultured for 24 h with parthenolide 10 μM, PDTC 0.5 μM, BAY1170-82 10 μM, LY294002 1 μM, or SP900125 10 μM, and the level of BAFF-R mRNA expression was measured by real time PCR. BAFF-R was reduced by NF-κB inhibitors parthenolide, PDTC, and BAY1170-82 (Figure 2). And the above conditions, cells were stained with PE-conjugated anti-human BAFF-R. We did not stain for B cells because BAFF-R is expressed mainly on B cells. The expression of BAFF-R was reduced by the NF-κB inhibitors. In contrast, LY294002, a phosphatidylinositol-3 kinase inhibitor, and SP900125, a

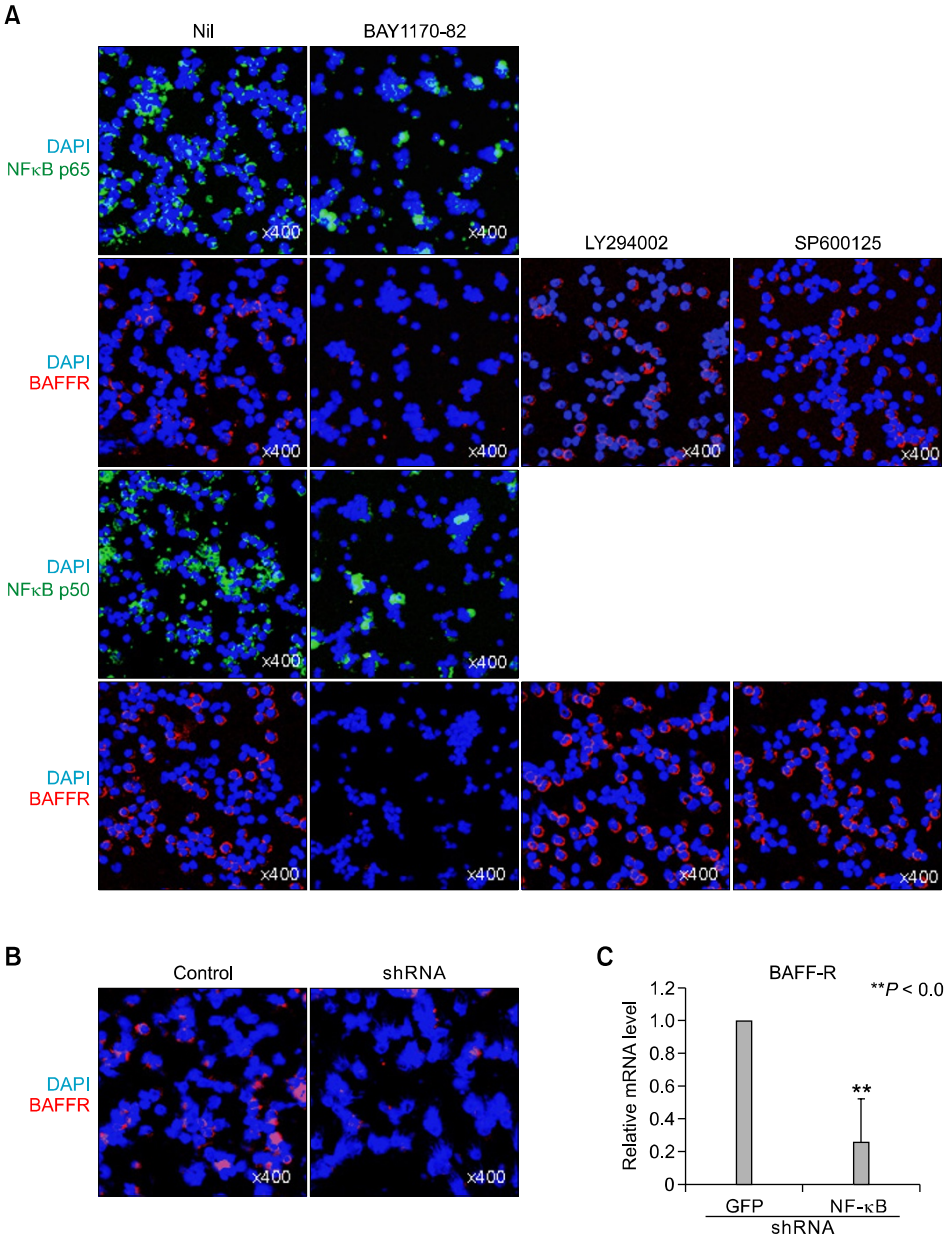

Figure 4. (A) B cells cultured with BAY1170-82 (10 LY294002 (1 or SP900125 (10 for 24h were examined bv immunofluorescence staining of BAFF-R, NF-p65 and NF-p50. (B and C) B cells treated with shRNAs (2 g/ml) to NF-κB or GFP (negative control) were analyzed by confocal microscopy (B) and real-time PCR (C). $^{**}P < 0.01$.

JNK inhibitor, had no effect on BAFF-R expression (Figure 3A). Next, we isolated peripheral B cells using B cell isolation kit II and cultured them with parthenolide 10 μM, PDTC 0.5 μM, BAY1170-82 10 μM, LY294002 1 μM, or SP900125 10 μM. After 24 h, cells were stained with PE-conjugated anti-human BAFF-R. There was also a tendency towards a reduced expression of BAFF-R on B cells stimulated with NF-κB inhibitors (Figure 3B). The results were confirmd by four independent experiments. Next, we were checked the level of NF-κB of B cells after treatement of NF-κB inhibitors by confocal microscopy. Expression of both NF-κB p65 and p50 was reduced in the BAY1170-82 treated B cells compared with that non treated B cells (Figure 4A). In the same conditions, expression of BAFF-R was reduced but LY294002 and SP600125 were not reduced the expression of BAFF-R. These results demonstrated that the reduction of BAFF-R expression was derived from

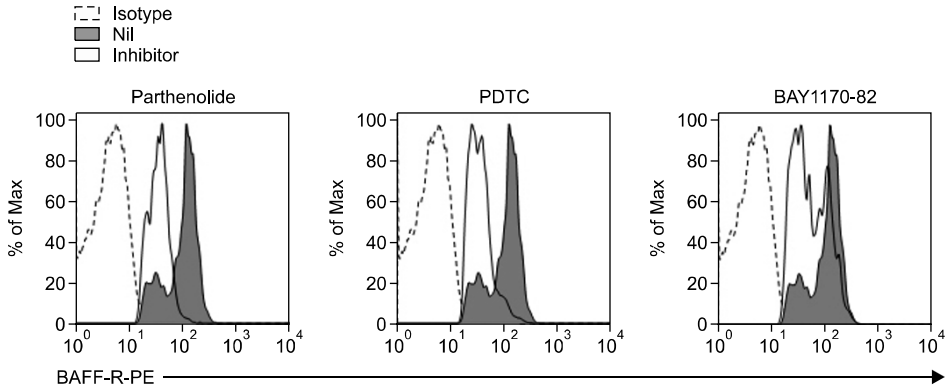

Figure 5. Effects of NF-κB pathway inhibitors on BAFF-R expression in PBMCs isolated from RA patients. PBMCs were cultured with parthenolide (10 μM), PDTC (0.5 μM), or BAY1170-82 (10 μM) for 24 h. Analysis of BAFF-R expression was performed using FACS. The results were confirmed by four independent experiments.

specific inhibition of NF-κB signaling pathway.

And then we focused on the NF-κB shRNA for specific inhibition of NF-κB pathway. The confocal result showed that NF-κB ShRNA treated B cells were reduced BAFF-R compared with control ShRNA treated B cells (Figure 4B). And real time PCR result showed reduction of BAFF-R on NF-κB shRNA treated B cells (Figure 4C).

Effect of NF-κB inhibition on expression of BAFF-R in cells from RA patient

We assessed the response of BAFF-R expression in RA PBMCs to NF-κB signal inhibitors. Isolated PBMCs from RA patients were cultured for 24 h with parthenolide 10 μM, PDTC 0.5 μM, or BAY1170-82 10 μM, then cells were stained with PE anti-human BAFF-R and analyzed. BAFF-R expression was also significantly decreased compared with control in cells cultured with NF-κB signal inhibitors (Figure 5). The results were confirmed by four independent experiments.

Discussion

We demonstrated that inhibition of NF-κB promotes decreased expression of BAFF-R in human PBMC and peripheral B cells. We also showed that inhibition of NF-κB leads to decreased expression of BAFF-R in PBMCs from RA patients.

NF-κB plays a pivotal role in inflammation by virtue of its ability to induce transcription of an array of proinflammatory genes (Baeuerle and Henkel, 1994). In RA, NF-κB is activated within the synovial tissue and contributes to cytokine production, adhesion molecule expression, and matrix metalloproteinase (MMP) activation (Aupperle *et al.*, 2001).

BAFF is involved in the ectopic GC reaction in the RA synovium, and might contribute to the pathogenesis of RA, and BAFF and BAFF-R are widely expressed in the RA synovium (Nakajima *et al.*, 2007). The primary role of BAFF in inflamed tissues is to support local B cell and T cell survival and activation. Blocking BAFF severely inhibits inflammation in an in vivo mouse model of RA (Wang *et al.*, 2001).

We confirmed the higher expression of NF-κB (p65 and p50), BAFF, and BAFF-R in severe RA synovium compared with mild RA and OA synovium. These data suggest that NF-κB-targeted therapeutics might be effective in inflammatory diseases such as RA.

Because NF-κB controls the expression of Th1 cytokines IL-2, IFNγ, and IL-12, activation of NF-κB should facilitate Th1 subset development. However, NF-κB is also important in B cells via B cell receptor (BCR) and BAFF-R signaling. In B cells, both BCR and BAFF-R promote B cell survival through NF-κB activation (Patke *et al.*, 2004). BCR signaling induces NF-κB activation by the canonical pathway. T1 B cells are the most sensitive and have been shown to die in response to BCR cross-linking: this negative selection weeds out self-reactive B cells. T2 B cells gain a second advantage for survival by responding to BAFF. T2 B cells express higher levels of BAFF-R and display enhanced responsiveness to BAFF relative to T1 B cells (Castro *et al.*, 2009).

In the canonical NF-κB pathway of BCR signaling, the IKK protein complex is critical for regulating NF-κB pathway activation. The IKK complex includes three important subunits: the catalytic subunits IKKα and IKKβ (also known as IKK1 and IKK2) and the regulatory subunit IKKγ (also known as NEMO). Degradation of IκB kinase proteins allows translocation of active c-Rel dimers to the

nucleus (Patke *et al.*, 2004).

However, downstream mediators of BAFF-R activation include both the canonical (classic, NF-κB1) and alternative (noncanonical, NF-κB2) NF-κB pathways (Fu *et al.*, 2009). In the alternative pathway, activation of the IKK complex induces processing of precursor p100 into p52, resulting in NF-κB subunit dimeric partners that migrate from the cytoplasm into the nucleus. In the nucleus, BAFF-R associates with IKKγ and promotes histone H3 phosphorylation. BAFF-R, IKKβ, and NF-κB/c-Rel form a nuclear complex binding to NF-κB target gene promoters and regulating transcription of these genes (Patke *et al.*, 2004).

A shared outcome of both BCR and BAFF-R signaling is NF-κB activation. Although signaling through either the BCR or BAFF-R is individually capable of promoting B cell survival, the simultaneous stimulation of both pathways has a synergistic effect on proliferation and maintenance (Patke *et al.*, 2004; Castro *et al.*, 2009). One mechanism could be the sequential induction of one pathway by the other in a positive feedback loop. This appears to be at least partially the case, because BAFF-R expression is upregulated in response to IgM stimulation in T2 and mature B cells, but not in T1 cells (Mackay and Browning, 2002).

Our results suggest that NF-κB blocking therapy could control B cells via the reduction of BAFF-R expression.

By what mechanism does NF-κB control BAFF-R expression? Castro *et al.* demonstrated that BCR signaling induced more long-term c-Rel activation in T2 and mature B cells than in T1 B cells, leading to increased expression of antiapoptotic genes as well as prosurvival BAFF-R and its downstream substrate p100 (NF-κB2) (Castro *et al.*, 2009). BCR signaling induces activation of nuclear c-Rel in T2 and follicular B cells. Activated nuclear c-Rel then regulates BCR-inducible expression of BAFF-R and substrate p100 of the noncanonical NF-κB pathway, thereby providing additional antiapoptotic functions in T2 B cells (Castro *et al.*, 2009).

Fu *et al.* found that nuclear BAFF-R associates with the NF-κB component c-Rel and binds to the NF-κB binding site in the promoters of NF-κB target genes such as BAFF, CD154, Bcl-xL, IL-8, and Bfl-1/A1, regulating the transcription of these genes (Fu *et al.*, 2009). This finding indicates that BAFF-R can promote B cell survival and proliferation by directly functioning as a transcription cofactor with other NF-κB transcription factors and possibly regulating transcription. Thus, NF-κB blocking therapy might reduce BAFF secretion.

In short, the anti-inflammatory mechanisms of NF-κB blocking therapy are complex and omnidirectional. The mechanisms relate to T cells, proinflammatory cytokines, autoantibody production, and B cell survival through BAFF and BAFF-R signaling. NF-κB blocking therapy is a promising method for solving the complex pathogenesis of RA.

Methods

Immunohistochemistry of synovial tissues

Immunohistochemical staining of NF-κB p65, NF-κB p50, BAFF-R, and BAFF was performed on sections of synovium. The synovium samples were obtained from RA and osteoarthritis (OA) patients, fixed with 4% paraformaldehyde solution overnight at 4°C, dehydrated with alcohol, washed, embedded in paraffin, and sectioned into 7 μm slices. The sections were incubated overnight at 4°C with primary anti-NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-NF-κB p50 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-BAFF-R (R&D Systems, Minneapolis, MN), anti-BAFF (Abcam, Cambridge, MA) antibodies, or irrelevant primary isotype-specific antibodies as a negative control. Slides were incubated with biotinylated anti-mouse IgG as secondary antibody for 20 min and then incubated with streptavidin-peroxidase complex (Vector Laboratories, Peterborough, UK) for 1 h followed by incubation with 3,3-diaminobenzidine (Dako, Glostrup, Denmark). The sections were counterstained with hematoxylin. Slides were mounted in permanent mounting media (Dako, Glostrup, Denmark). Samples were photographed with an Olympus photomicroscope (Tokyo, Japan) (Richardson *et al.*, 2008).

Cells and reagents

Peripheral blood was obtained with a heparin-treated syringe. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden). B cell isolation kit II was used as recommended by the manufacturer (Miltenyi Biotec, Auburn, CA). CD19+ B cells were greater than 95%. The purified cells were washed and suspended in RPMI 1640 medium (GibcoBRL, Carlsbad, CA) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (GibcoBRL) that had been inactivated by heating to 56°C for 1 h. The cell suspension was dispensed into 96-well plates (Nunc, Roskilde, Denmark) and incubated at 37°C in 5% CO_2. Pyrrolidine dithiocarbamate (PDTC; 0.5 μM) (Zhou *et al.*, 2008) was purchased from Sigma-Aldrich (St. Louis, MO). Parthenolide (10 μM) (Sheehan *et al.*, 2002), LY294002 (1 μM) (Ikezoe *et al.*, 2007), and BAY 11-7082 (10 μM) (O'Sullivan and Thomas, 2002) were purchased from Calbiochem (Schwalbach, Germany). SP600125 (10 μM) (Xie *et al.*, 2004) was purchased from A.G. Scientific (San Diego, CA).

Real-time polymerase chain reaction with SYBR Green

mRNA was extracted using RNAzol B (BioTex Labs, San Antonio, TX) according to the manufacturer's instructions. Reverse transcription of 2 μg of total mRNA was conducted at 42°C using the Superscript Reverse Transcription system (Takara, Shiga, Japan). Polymerase chain reaction (PCR) amplification of cDNA aliquots was performed by adding 2.5 mM dNTPs and 2.5 U Taq DNA polymerase (Takara), and human BAFF-R was amplified using the sense primer 5'- AGA CAA GGA CGC CCC AGA GCC C-3' and the anti-sense primer 5'- GTG GGG TGG TTC CTG GGT CTT C -3' in a Light Cycler TM (Roche Diagnostics Mannheim, Germany). The relative expression levels were calculated by normalizing the BAFF-R levels to the endogenously expressed housekeeping gene (β-actin). Melting curve analysis was performed immediately after the amplification protocol under the following conditions: 0 s (hold time) at 95°C, 15s at 65°C, and 0 s (hold time) at 95°C. The temperature change rate was 20°C/s except in the final step, when it was 0.1°C/s. The crossing point (Cp) was defined as the maximum of the second derivative from the fluorescence curve.

NF-κB shRNA

Human NF-κB shRNA was designed by Sigma-Aldrich. The sequence is CCGGCGAATGA CAGAGGCGTGTAT ACTCGAGTATACACGCCTCTGTCATTCGTTTTT. Human CD19+ B cells were plated in 24-well plates were transfected with 1 μg or 2 μg of shRNA using polyMAG and Magneto FACTOR Plate of Chemicell (Berlin,Germany) according the manufacturer's protocol.

Flow cytometry

PBMCs were incubated with human IgG to block the Fc receptor and then incubated with phycoerythrin (PE)-conjugated anti-human BAFF-R (eBioscience, San Diego, CA) for 30 min at 4°C. After washes, the cells were resuspended in phosphate-buffered saline and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Diego, CA).

Confocal microscopy

Cytospins were made on the poly lysine-coated (Sigma-Aldrich) slides. Cells were fixed with acetone and blocked with 10% goat serum for 30 min at room temperature and stained with primary and secondary antibodies for overnight incubation at 4°C. Primary antibodies anti-Human CD268 (BAFF Receptor) PE (eBioscience) and NF-κB p65, p50 and the secondary antibody goat anti-rabbit IgG -FITC (Santa cruz) were used at 1:100 and 1:100 dilutions, respectively. Confocal images were acquired using a LSM 510 confocal microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

The results are expressed as the mean ± SEM. Statistical analysis was performed using Student's *t* test and Wilcoxon signed-rank test. *P* values < 0.05 were regarded as significant.

Acknowledgements

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