

Expression and function of TNF family member B cell-activating factor in the development of autoimmune arthritis

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Keywords: apoptosis, autoimmunity, B lymphocytes, cytokine, dendritic cells

Abstract

B cell-activating factor (BAFF), a member of tumor necrosis factor family cytokines, has been shown to enhance the maturation and survival of peripheral B cells. While BAFF is implicated in regulating B cell function and autoimmunity, its role in the development of autoimmune arthritis has not been fully clarified. Using a collagen-induced arthritis (CIA) mouse model, we detected dysregulated expression of BAFF and its receptors in the peripheral lymphoid organs during arthritis induction. Elevated serum levels of BAFF were closely correlated with increased levels of anti-collagen antibodies during the CIA progression. Moreover, dendritic cells (DCs) and macrophages were found to express high amount of BAFF proteins at the acute and chronic stages of CIA, respectively. In cultures, recombinant BAFF suppressed apoptosis of splenic B cells from arthritic mice, and DC-induced B cell proliferation was specifically blocked by soluble decoy receptor B cell maturation antigen–Fc. These findings suggest that overproduction of BAFF by DCs and macrophages may play a crucial role in the pathogenesis of experimental arthritis.

Introduction

The tumor necrosis factor (TNF) cytokine superfamily plays a central role in immune regulation by modulating lymphocyte proliferation and apoptosis, which are important factors in maintaining immune homeostasis and self-tolerance (1). B cell-activating factor (BAFF; also known as B lymphocyte stimulator, THANK, TALL-1 and zTNF4) is a recently discovered TNF family cytokine that is essential for the development of mature B cell populations (2). A type II membrane protein, BAFF binds to three receptors, named BCMA (B cell maturation antigen), TACI (transmembrane activator and CAML interactor) and BAFF-R (BAFF receptor), on mature B cells in the peripheral immune system and promotes the activation and survival of B cells, both *in vitro* and *in vivo* (3, 4). Recent studies show that BAFF induces proliferation of anti-IgM-stimulated peripheral B lymphocytes in culture, while *in vivo* administration of recombinant BAFF protein to normal mice leads to increased levels of B cell production and a polyclonal hypergammaglobulinemia (5). APRIL (a proliferation-inducing ligand), another TNF family cytokine that shares high sequence homology with BAFF and binds to

receptors, TACI and BCMA, has also been shown to enhance the proliferation of peripheral B and T cells in cultures (6, 7).

Recent gene-targeting studies have suggested a critical role for BAFF in autoimmune disease. BAFF over-expression in transgenic mice results in increased numbers of mature B cells, enlarged lymphoid organs and hypergammaglobulinemia associated with autoimmune-like manifestations resembling systemic lupus erythematosus (SLE) (4, 8). In human SLE patients, BAFF serum levels are elevated, correlating with high titers of anti-dsDNA antibody (9). Similarly, serum levels of BAFF are higher in patients with rheumatoid arthritis (RA) than in healthy individuals (10). A common autoimmune disease, RA is characterized by chronic inflammation of multiple joints and destruction of cartilage and bone (11). Salient features of the disease include the presence of circulating auto-antibody, dysregulated lymphocyte activation and linkage to MHC class II (11). In recent years, animal models of human RA have provided insights into the pathogenesis of the disease. Of these models, the collagen-induced arthritis (CIA) mouse has been the best characterized and most widely accepted (12).

Involving both B and T cell autoimmune responses, CIA resembles human RA in both cellular and humoral dysfunctions (13). Most reports have centered on the role of T cell-mediated, antigen-specific responses and cytokine production, but the finding that B cell-deficient mice do not develop CIA demonstrates that B cells also play a pivotal role in the development of this autoimmune arthritis (14). Neutralization of BAFF by administering soluble receptor TACI-Fc to CIA mice inhibits both the production of collagen-specific antibodies and the progression of arthritis (15). These findings, suggesting that B cell dysregulation in CIA may be BAFF mediated, raise questions concerning the level and source of BAFF production and its possible functions both in the initiation of experimental autoimmune arthritis and during the evolution of the disease.

In the present study, using the CIA mouse model, we have examined the role of BAFF in the induction of autoimmune arthritis. Our findings of increased BAFF expression in dendritic cells (DCs) and macrophages suggest the possible cellular sources of BAFF overproduction during disease progression. Increased BAFF production by DCs occurs at the early stage of arthritis development whereas macrophage-derived BAFF contributes most significantly to the later phase of disease progression. Furthermore, co-culture studies of B cells and DCs have revealed a potent role for DC-secreted BAFF in enhancing peripheral B cell proliferation and survival. These results provide new insights into the function of BAFF in the development of autoimmune arthritis, suggesting possible therapeutic strategies to arrest or slow down disease progression.

Methods

Mice

DBA/1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a pathogen-free animal facility at the University of Hong Kong. All experimental procedures were approved by the University Committee on the Use of Live Animals in Teaching and Research.

CIA induction

Male DBA/1 mice at 10 weeks of age were immunized intradermally at the base of the tail with 200 µg of bovine collagen type II (CII; Sigma, MO, USA) dissolved in 100 µl of 0.05 M acetic acid and mixed with an equal volume of CFA (Difco, MI, USA). Three weeks later, the animals were re-immunized with 200 µg of CII emulsified in incomplete Freund's adjuvant (IFA) (Difco). Following the same protocol, adjuvant-treated littermates given either PBS or chicken egg ovalbumin in place of CII served as controls. Mice were observed three times per week for signs of joint inflammation and scored for clinical signs as follows: 0, normal with no erythema and swelling; 1, mild swelling confined to the ankle joint; 2, mild swelling extending from the ankle to metatarsal or metacarpal joints; 3, moderate swelling extending from the ankle to the metatarsophalangeal or metacarpophalangeal joints, and 4, severe swelling extending from the ankle to the digits and resulting in ankylosis and loss of joint movement. Thus, maximum arthritis score per paw was 4, and the maximal disease score per mouse was 16.

After sacrifice, joint tissues were harvested, fixed in 10% formaldehyde/PBS for 72 h and decalcified using EDTA for 3 weeks. Tissue samples were sectioned at 4-µm thickness and stained with hematoxylin/eosin for histological assessment. Some joints were homogenized for RNA preparation.

Evaluation of serum BAFF and anti-CII antibody levels

ELISA kits for measurement of the soluble form of BAFF were obtained from Alexis Co. (Lausen, Switzerland) and assays were performed according to the manufacturer's instructions. Briefly, anti-mouse mAb to BAFF (5A8) was used as capture antibody (5 µg ml⁻¹) to coat a 96-well ELISA microplate (Nunc Maxi-Sorp Plate, Roskilde, Denmark) overnight at 4°C. After blocking, protein A-agarose pre-absorbed serum samples were added to the ELISA plate with serial dilutions. Recombinant mouse BAFF was used as standard. After washing, the plate was further incubated with biotin-conjugated detection antibody to BAFF (1C9) for 1 h at 37°C. After additional washing, the plate was incubated with HRP-conjugated streptavidin. The reaction was finally developed by tetramethylbenzidine and read in a Vmax microplate reader. A standard curve using serial dilutions of recombinant BAFF (10, 5, 2.5, 1.35, 0.625, 0.3125 and 0 ng ml⁻¹) was incorporated into each assay. Serum anti-CII antibody levels were measured by a standard ELISA. Briefly, a 96-well Maxi-Sorp Plate (Nunc) was coated with purified CII peptide (5 µg ml⁻¹; Sigma) in 100 µl coating buffer (0.05 M carbonate, pH 9.6; Merck, Whitehouse Station, NJ, USA) overnight at 4°C. Plates were washed with PBS/0.05% Tween 20 (Sigma) and blocked with blocking buffer (0.5% gelatin/0.5% BSA/0.05% Tween 20 in PBS) for 1 h at room temperature. Subsequently, plates were washed and incubated with sample sera in serial dilutions at 37°C for 2 h. After washing, plates were incubated with HRP-conjugated anti-mouse IgG (1 : 1000; Zymed, South San Francisco, CA, USA) for 1 h and then developed with freshly prepared *p*-nitrophenyl phosphate (Sigma) in substrate buffer (0.1 M diethanolamine buffer, pH 10.3) for 30 min at room temperature. The absorbance was read at 405 nm using a Vmax microplate reader.

Preparation of cell suspensions

Cell suspensions from the spleen (SP) and bone marrow (BM) of adult mice were prepared by mechanical disruption of the tissue through a 70-µm-pore size cell strainer. Red blood cells were lysed by incubating cells in 1 ml of ACK buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA) for 3 min on ice. After the lysis of erythrocytes, the number of nucleated cells was determined using a hemacytometer. Aliquots of cell samples were either assayed immediately or incubated at 37°C in a humidified atmosphere with 5% CO₂, as described (16).

Immunostaining

For multiple-color flow cytometric analysis, cell samples were stained for phenotypic markers: B220 (RA3-6B2), IgM (R6-60.2), IgD (AMS 9.1), HSA (M1/69), CD4 (GK1.5), CD8 (53-6.7), Gr-1 (RB6-8C5), CD11c (HL3) and CD11b (M1/70). Cells were incubated with combinations of labeled antibodies in PBS containing 2% FCS. All antibodies, directly conjugated

with FITC, PE and Cy-Chrome™, were obtained from PharMingen (San Diego, CA, USA).

Flow analysis and cell sorting

Immunostained cells were analyzed with an EPICS-Altra flow cytometer (Beckman Coulter, Fullerton, CA, USA). A minimum of 10 000 events per sample were collected from various phenotypically defined cell sub-populations. Cell debris and clumps were excluded by setting a gate on forward scatter versus side scatter, as described (16). Splenic IgM⁺ B cells, CD11c⁺ DCs and CD11c⁻ CD11b⁺ macrophages and BM IgM⁺ B cells from acute CIA mice and adjuvant-treated controls were purified by cell sorting. When re-analyzed after sorting, sorted cell fractions were routinely >96% pure.

Cell cultures

Purified splenic IgM⁺ B cells were cultured for 5 days in complete RPMI-1640 medium supplemented with 2% heat-inactivated FCS unless otherwise specified. Recombinant BAFF and BCMA-Fc (Alexis Co.) were added to cultures at 200 ng ml⁻¹. Cell viability was determined daily by trypan blue staining using a hemacytometer. In separate experiments, splenic IgM⁺ B cells and CD11c⁺ DCs purified from acute CIA mice and adjuvant-treated controls were cultured in separate compartments of Transwells (Costar, Corning, NY, USA). DCs (1 × 10⁵) in a volume of 100 µl in the upper chamber were cultured with B cells (2 × 10⁵) in 600 µl in the lower chamber in the presence or absence of BCMA-Fc (200 ng ml⁻¹) for 72 h. Cultures were performed in triplicate, and results are presented as the mean ± SD of each triplicate.

Generation of DCs

Immature and mature DCs were generated from BM cell suspensions of normal DBA/1 mice. Briefly, BM cells, after depletion of erythrocytes, were seeded at 2 × 10⁶ per 100-mm dish in RPMI-1640 complete media with recombinant murine granulocyte macrophage colony-stimulating factor (20 ng ml⁻¹). On day 3 of culture, floating cells were gently removed and fresh media added. On day 6, non-adherent and loosely adherent cells were collected as immature DCs. Flow cytometric analysis showed that these cell samples comprised >90% CD11c⁺ cells. To prepare mature DCs, immature DCs were further incubated in the presence of LPS (1 µg ml⁻¹) for 24 h either with or without CII (50 µg ml⁻¹). Overnight incubation of immature DCs with LPS, either with or without CII, produced mature DCs characterized by increased surface expression of class II MHC, CD40 and CD80.

Apoptotic assay

Freshly prepared splenic cell suspensions were cultured in serum-free RPMI-1640 medium either with or without recombinant BAFF (200 ng ml⁻¹). After 10 h of culture, cell samples were phenotypically labeled and re-suspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂). Two microliters of FITC-Annexin V (PharMingen) was added and incubated for 15 min in the dark at room temperature before flow cytometric analysis, as described (16). In separate experiments, splenic cell suspensions from

acute CIA mice were cultured in 2% FCS/RPMI-1640 media with either BAFF or BAFF plus BCMA-Fc for various time intervals. Then, the numbers of viable cells recovered from each well of triplicate cultures were enumerated, followed by immunophenotypic staining and flow cytometric analysis.

Proliferation assay

A total of 2.5 × 10⁵ splenic B cells purified from CIA and control mice were added to each well of a 96-well plate with the following treatments: medium only (RPMI-1640 + 2% FCS), BAFF (200 ng ml⁻¹), anti-IgM (2.5 µg ml⁻¹), BAFF (200 ng ml⁻¹) plus anti-IgM (2.5 µg ml⁻¹) and BAFF (200 ng ml⁻¹) with BCMA-Fc (200 ng ml⁻¹). In separate experiments, splenic CD11c⁺ DCs purified by cell sorting were co-cultured with B cells in the absence or presence of BCMA-Fc (200 ng ml⁻¹). After incubation for 72 h at 37°C, cell samples were pulsed with [³H]thymidine ([³H]TdR) (25 µCi per well) for the final 16 h of culture and harvested using a Packard harvester. [³H]TdR incorporation was measured using a liquid scintillation counter (Beckman LS5801).

Fluorescence microscopy

Freshly prepared splenic cell suspensions from arthritic mice at an acute stage and adjuvant-treated controls were immunostained with PE-CD11c. DCs were purified by cell sorting and then fixed before cytospin preparation. Slides were stained with FITC-anti-mouse BAFF mAb (Alexis Co.) and isotype control antibody, respectively. After extensive washing, slides were examined and photographed using a Leica DMR microscope.

Semi-quantitative reverse transcription-PCR analysis

Total RNA was isolated from BM, SP, PBMC as well as cell sorting-purified cell fractions (B cells, DCs and macrophages) of CIA mice and adjuvant-treated controls using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Joint tissues were homogenized with a polytron homogenizer on ice for subsequent RNA extraction. Samples were processed according to the manufacturer's protocol. RNA was precipitated by adding isopropanol. RNA was pelleted at 12 000 × g for 10 min at 4°C, and washed with 70% ethanol. After drying in the vacuum concentrator (5301; Eppendorf, Hamburg, Germany) at 30°C, the pellet was re-suspended in diethylpyrocarbonate-treated water, and the quality of RNA isolation was confirmed by 1.5% agarose gel in Tris acetate EDTA (TAE) buffer. cDNA was synthesized with random primers and MML V-RT as described by the manufacturer (Invitrogen). PCRs were performed semi-quantitatively by varying the number of amplification cycles and performing dilutional analysis so that there was a linear relationship between the amount of cDNA used and the intensity of the PCR product. BAFF, APRIL, BCMA, BAFF-3, TACI and β-actin sequences were amplified by PCR using the following gene-specific primers (Invitrogen): BAFF (347 bp), 5'-TGTTGTCCAGCAGTTTCAC-3' and 5'-CTG CAG ACA GTC TTG AAT GA-3'; APRIL (700 bp), 5'-AGC CTC ATC TCC AGG CCA GAT-3' and 5'-AAA CCC CAG GAA TGT TCC ATG CG-3'; TACI (263 bp), 5'-GCG CAC CTG TAG AGA CTT C-3' and 5'-GCC TCA ATC CTG GAC CAT G-3'; BAFF-R (605 bp), 5'-GCC CAG ACT CGG AAC TGT CCC A-3' and

5'-GCC CAG TAG AGA TCC CTG GGT TCC-3'; BCMA (304 bp), 5'-GGC GCA ACA GTG TTT CCA CA-3' and 5'-CTC GGT GTC GGC CTT GTC CA-3'; β -actin (166 bp), 5'-GCA TTG CTG ACA GGA TGC-3' and 5'-CCT GCT TGC TGA TCC ACA TC-3'. PCR reactions were performed using 0.5 mM of each deoxynucleoside triphosphate and 0.5 μ M sense and anti-sense primers with 1 U *Taq* polymerase (Promega) under the following conditions: BAFF, 55°C annealing temperature, 35 cycles; APRIL, 57°C annealing temperature, 38 cycles; TACI, 50°C annealing temperature, 35 cycles; BAFF-R, 55°C annealing temperature, 35 cycles; BCMA, 55°C annealing temperature, 35 cycles, and β -actin, 50°C annealing temperature, 25 cycles. All reactions were performed using a thermal cycler for PCR (RoboCycler Gradient 96; Stratagene). The reverse transcription (RT)-PCR products were separated on 1.5% agarose gel in 1 \times TAE buffer, and visualized by 1 μ M ethidium bromide. The gels were then photographed. The authenticity of the RT-PCR products was verified by sequencing. All experiments were repeated at least three times. Controls were performed by applying the whole PCR master mix without cDNA. Base pair length was determined by using A DNA marker (Invitrogen).

Statistical analysis

Data are presented as the mean \pm SD where appropriate. The Mann-Whitney two-sample rank test was used to compare clinical scores and Student's *t*-test was used for comparing BAFF and anti-CII antibody levels in mouse sera. The one-way analysis of variance was performed to determine the correlation between the levels of BAFF and anti-CII antibody during the progression of CIA pathogenesis. For each test, *P* values < 0.05 were considered statistically significant.

Results

Dysregulated expression of BAFF and its receptors during arthritis development

To study the potential role of BAFF in the pathogenesis of autoimmune arthritis, we produced the experimental model of CIA in DBA/1 mice by immunizing the mice with bovine CII in CFA and challenging them with CII in IFA 3 weeks later. Acute arthritic symptoms usually developed 2 weeks after the second immunization and progressed rapidly to reach a mean clinical score of \sim 14. Histological examination of joint tissue in CIA mice at an acute stage (2–8 weeks after the second immunization) revealed structural features of severe arthritis including pronounced synovial hyperplasia, ankylosis and cartilage erosion. Intense inflammation, osteolysis and joint destruction were observed at a chronic stage, 12–16 weeks after the second immunization. However, adjuvant-treated control mice did not show any clinical symptoms of arthritis.

In examining BAFF gene expression, we first analyzed lymphoid organs of normal mice by semi-quantitative RT-PCR. BAFF transcripts were readily detected in the BM and SP. In CIA mice, BAFF mRNA expression was markedly increased in the SP but showed no alteration in BM (Fig. 1A). Consistent with previous findings, BAFF mRNA was not detectable in sorting-purified IgM⁺ splenic B cells (17). PCR products for

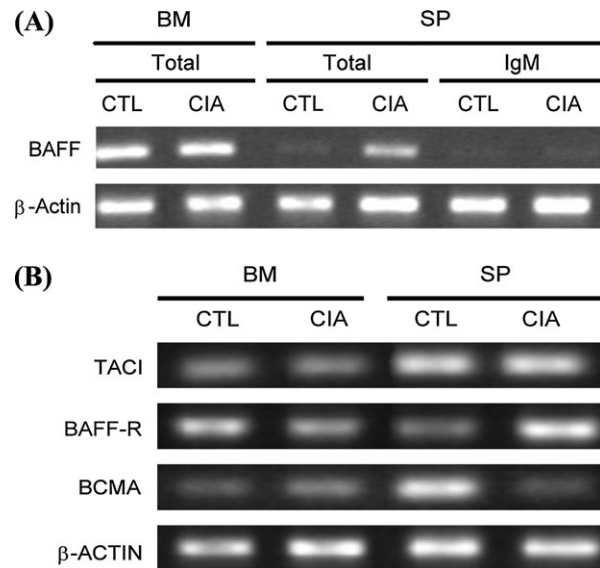


Fig. 1. Expression of BAFF and its receptors during arthritis development by semi-quantitative RT-PCR analysis. (A) RNA was isolated from total BM, SP and sorting-purified splenic IgM⁺ B cells (purity >97%) of adjuvant-treated controls (CTL) and CIA mice (CIA). Expression of BAFF did not show any change in the BM whereas levels of BAFF mRNA were markedly up-regulated in the SP of CIA mice. Moreover, BAFF transcripts were not detectable in splenic B cells. (B) Expression of BAFF-Rs was examined among IgM⁺ B cells purified from the BM and SP of adjuvant-treated controls (CTL) and CIA mice (CIA). All three receptors did not show any significant changes among BM B cells. However, levels of BAFF-R mRNA were highly up-regulated while BCMA expression was down-regulated in SP B cells from CIA mice.

BAFF were excised and their identity was confirmed by sequencing analysis (data not shown). Interestingly, APRIL transcripts did not show any obvious changes in the SP of arthritic mice (data not shown). Thus, we focused in the present study on the characterization of dysregulated BAFF expression during CIA development. Among the receptors for BAFF, expression of BAFF-R was markedly increased in splenic IgM⁺ B cells from arthritic mice whereas BCMA was down-regulated and levels of TACI mRNA remained unchanged (Fig. 1B). In marked contrast, no alteration of these receptor transcripts was observed among BM IgM⁺ B cells from both normal and arthritic mice.

To determine whether increased BAFF mRNA expression led to elevated levels of serum BAFF protein in CIA mice, we used a sandwich ELISA to measure BAFF serum concentrations. Compared with control animals, serum levels of soluble BAFF were significantly higher in CIA mice at the acute phase of arthritis (11.7 ± 1.8 ng ml⁻¹ versus 4.7 ± 0.8 ng ml⁻¹, *P* < 0.001) and at the chronic stage (9.8 ± 2.8 ng ml⁻¹ versus 4.7 ± 0.8 ng ml⁻¹, *P* < 0.001).

Early studies suggested a direct pathogenic role for CII-specific antibodies in the initiation and progression of CIA, which has been further supported by recent evidence that immunization with monoclonal anti-CII antibodies can induce arthritis in mice (18, 19). Since BAFF is involved in peripheral B cell proliferation and activation (20), we examined whether increased levels of BAFF in CIA mice are associated with

increased anti-CII antibody production. An ELISA for anti-CII antibodies showed a statistically significant correlation between the increased BAFF production and elevated levels of anti-CII antibodies in the sera of CIA mice at both acute and chronic stages of arthritis (Fig. 2, $P < 0.001$). These data suggest that increased BAFF expression may be possibly involved in the pathogenesis of CII-induced arthritis.

Increased BAFF expression by DCs and macrophages in CIA mice

To characterize the dynamic change of cell populations in lymphoid organs during arthritis induction, we analyzed the

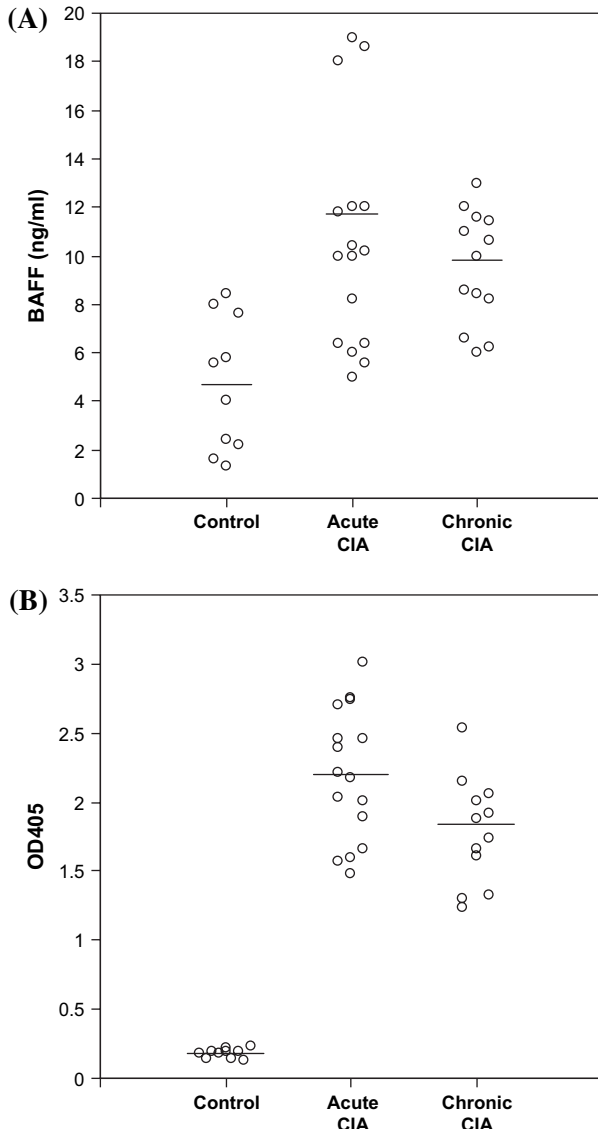


Fig. 2. Elevated levels of BAFF and anti-CII antibody production in the sera of CIA mice. Serum concentrations of soluble BAFF (A) and anti-CII antibody (B) from adjuvant-treated controls and CIA mice at the acute and chronic stages were measured by ELISA. Levels of BAFF and anti-CII antibodies were profoundly increased in the sera of CIA mice, especially, to a greater extent, from samples of CIA mice at the acute stage. The horizontal bars indicate the mean value of each group and the P value was determined by t -test.

number and frequency of various subsets of immune cells in the SP of arthritic mice. The cellularity was significantly increased in the SP of acute arthritic mice as compared with adjuvant-treated controls ($128.1 \pm 17.6 \times 10^6$ versus $80.1 \pm 9.1 \times 10^6$, $P < 0.01$; $n = 24$). However, the total number of splenocytes returned to the normal value in chronic CIA mice ($86.5 \pm 10.2 \times 10^6$; $n = 14$). As shown in Fig. 3(A), splenic B220⁺ B cell population size was significantly increased by 2-fold whereas CD4⁺ T cell compartment was moderately enlarged in acute CIA mice. Interestingly, the CD11c⁺MHC⁺ DC population also showed a 2-fold expansion in the SP of acute CIA mice and returned to control value in chronic arthritic mice (Fig. 3B). In contrast, CD11b⁺ macrophage and Gr-1⁺ granulocyte populations did not show any apparent change. In acute CIA mice, the splenic IgM⁺HSA^{high} immature B cell population was slightly expanded whereas the total number of IgM⁺HSA^{low} mature B lymphocytes was greatly increased by 2.5-fold (Fig. 3C). Further analysis of splenic B cell populations revealed that transitional type 1 B cell population (IgM^{high}IgD^{low}) were moderately expanded while transitional type 2 B cells (IgM^{high}IgD^{high}) and mature B cells (IgM^{low}IgD^{high}) showed a pronounced 2-fold increase in absolute numbers at the acute stage of CIA (Fig. 3D). However, all splenic B cell subsets were only moderately increased in chronic CIA mice. In order to identify the cellular source of BAFF production, various cell populations were purified from the SP of acute and chronic CIA mice, as well as control animals, for semi-quantitative RT-PCR analysis. As shown in Fig. 4(A), CD11c⁺ DCs, purified from CIA mice at an acute stage of arthritis, showed markedly increased BAFF expression, whereas during the chronic stage BAFF expression levels in DCs resembled those of normal controls. In contrast, while Mac-1⁺ macrophages purified from the SP of acute phase CIA mice displayed only moderately up-regulated BAFF expression, BAFF mRNA levels were highly elevated in macrophages at a chronic stage of the disease (Fig. 4B). Gr-1⁺ granulocytes from CIA mice showed no significant change in BAFF mRNA expression at any stage of disease (data not shown).

Macrophages were among the most abundant infiltrating inflammatory cells in inflamed joints, as revealed by flow cytometric analysis of purified cell populations from joint tissue (data not shown). To verify whether levels of BAFF gene expression are locally increased during induction of autoimmune arthritis, joint tissues from control and diseased CIA mice were prepared for mRNA analysis. BAFF expression was markedly up-regulated in the joint tissues of both acute and chronic CIA mice (Fig. 4C). Fluorescent dye-conjugated mAb against BAFF and fluorescence microscopy were used to verify BAFF expression at the protein level. As shown in Fig. 5, splenic DCs purified from acute stage of CIA mice displayed strong immunofluorescent labeling of BAFF protein, whereas DCs from control mice or chronic stage CIA mice showed very weak staining for BAFF, consistent with our findings by RT-PCR analysis (Fig. 4). To ascertain whether BAFF expression is differentially expressed by DC subsets at various developmental stages, BM-derived immature DCs and LPS-stimulated mature DCs were prepared and examined for BAFF mRNA expression. As shown in Fig. 6, only low levels of BAFF

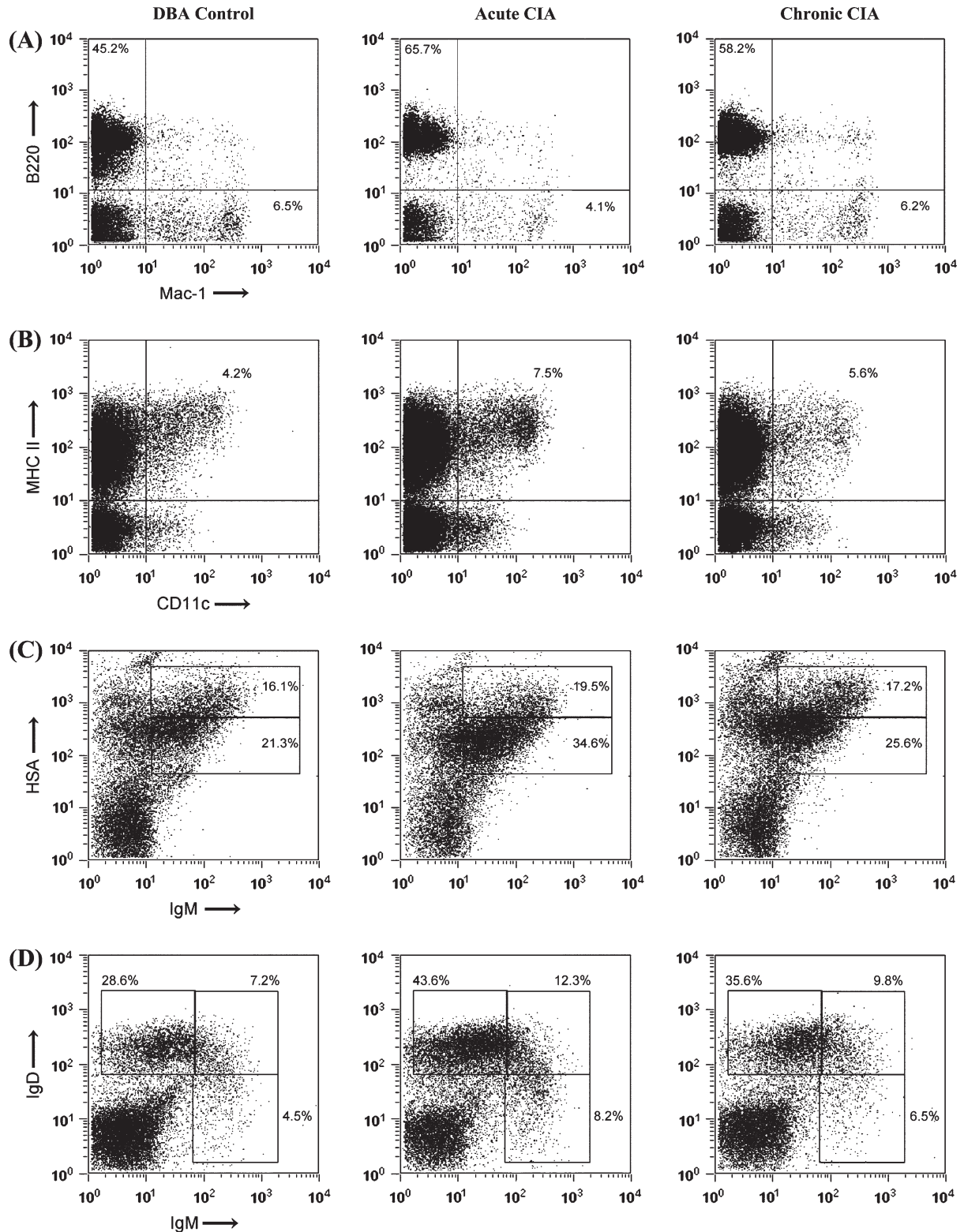


Fig. 3. Enlarged B cell and DC populations in the SP of acute CIA mice. (A) Phenotypic analysis of splenic cell populations from control and CIA mice at both acute and chronic stages by flow cytometry. Incidences of B220⁺ B lymphocytes and Mac-1⁺ macrophages (A), MHC-II⁺CD11c⁺ DCs (B) and B cell subsets (C and D) were indicated in flow cytometry plots. (E) The total cell numbers of IgM⁺ B cells, CD4⁺ T cells and CD11c⁺ DCs in the SP of acute CIA mice were significantly increased as compared with the normal value from controls (mean \pm SD; * P < 0.05, ** P < 0.01). (F) Further analysis of B cell sub-populations revealed markedly expanded transitional B cells (IgM^{high}IgD^{low}, IgM^{high}IgD^{high}) as well as IgM⁺HSA^{low} mature B cells in the SP of acute CIA mice (mean \pm SD; * P < 0.05, ** P < 0.01).

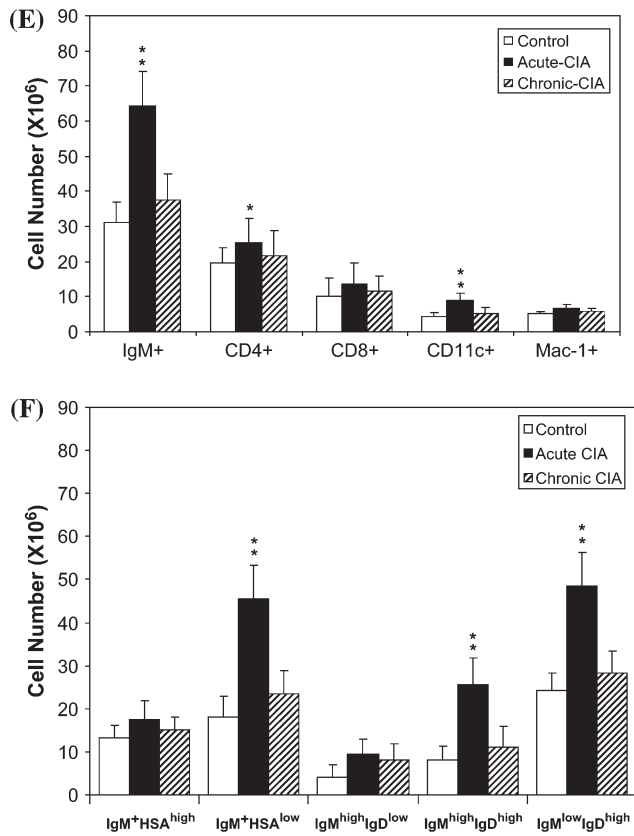


Fig. 3. Continued.

expression were detected in immature DCs, whereas BAFF mRNA was greatly increased upon maturation with LPS stimulation. Similar results were obtained from CII-pulsed mature DCs (Fig. 6). Taken together, these findings suggest that DCs and macrophages may possibly contribute to the BAFF overproduction at the acute and chronic stages of arthritis development, respectively.

Enlarged splenic B cell pool with suppressed apoptosis in CIA mice

To ascertain if enhanced B cell survival contributes to the enlarged B cell pool in the SP of acute CIA mice, we analyzed apoptotic kinetics of splenic B cells using a sensitive short-term culture system (16). After 10 h incubation without serum, B cells from CIA mice displayed a significantly lower apoptotic incidence than cells from control mice ($34.6 \pm 7.3\%$ versus $58.3 \pm 10.3\%$, $P < 0.01$). These data suggest that suppressed B cell apoptosis may contribute to the enlarged B cell pool in the SP of acute CIA mice.

To study the role of BAFF in regulating B cell survival, splenic cell suspensions prepared from acute CIA mice were cultured with or without the presence of BAFF. It was found that BAFF significantly enhanced the survival of IgM⁺ B cells in short-term cultures, and this protective effect was almost completely blocked by a soluble decoy receptor BCMA-Fc (Fig. 7A). To directly evaluate the effect of BAFF on B cell apoptosis, freshly

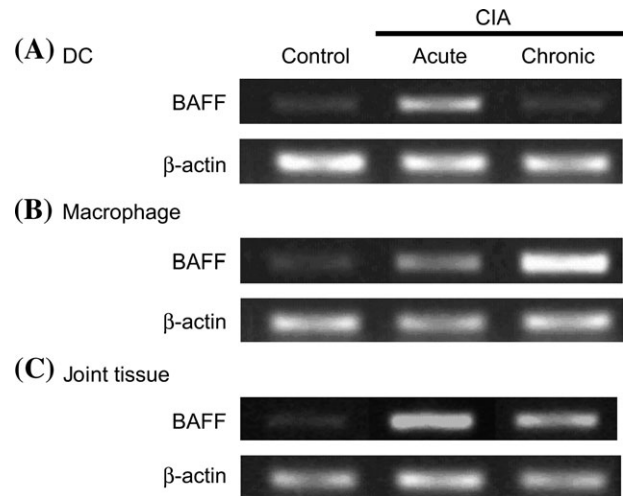


Fig. 4. Expression of BAFF in DCs and macrophages by semi-quantitative RT-PCR analysis. CD11c⁺ DCs and CD11b⁺CD11c⁻ macrophages were purified by cell sorting from adjuvant-treated control and CIA mice both at the acute and chronic stages (purity >96%). (A) Levels of BAFF expression were dramatically increased in DCs from CIA mice at the acute stage and reduced to the control levels at the chronic stage. (B) BAFF expression was moderately increased in macrophages from CIA mice at the acute phase and reached much higher levels at the chronic stage. (C) In local joint tissue, levels of BAFF expression were markedly increased in both acute and chronic CIA mice.

prepared SP cell suspensions were cultured in RPMI medium either with or without BAFF (200 ng ml^{-1}) for 10 h and analyzed by flow cytometry. Recombinant BAFF markedly suppressed apoptosis of splenic B cells in control and CIA mice (Fig. 7B). These results indicate that BAFF over-expression during the CIA induction may have the effect of increasing the peripheral B cell pool by suppressing B cell apoptosis.

DC-secreted BAFF in enhancing the proliferation of B cells from CIA mice

Since we had detected increased levels of BAFF-R expression in splenic B cells from CIA mice, we sought to examine the effect of BAFF on the proliferation of peripheral B cells. Splenic B cells purified from control and acute CIA mice were cultured with anti-IgM and/or BAFF for 72 h. Compared with normal controls, splenic B cells from arthritic mice showed a 1.5-fold increase in proliferation rates in response to both anti-IgM and BAFF stimulation (Fig. 8). Moreover, treatment with anti-IgM and BAFF together showed synergistic effects on B cell proliferation, most marked on B cells from CIA mice. Treatment with BCMA-Fc, a soluble receptor for BAFF, specifically blocked BAFF-induced B cell proliferation (Fig. 8). To directly evaluate if increased BAFF production by DCs contributes to enhanced peripheral B cell proliferation in CIA induction, we purified splenic DCs from control and arthritic mice and co-cultured them with splenic B cells from normal mice for 72 h. In the presence of anti-IgM stimulation, the B cell proliferation rate was significantly higher when B cells were co-cultured with DCs from CIA mice (Fig. 9). Furthermore, treatment with BCMA-Fc greatly reduced B cell proliferation induced by normal DCs and, to an even greater extent, in cultures of

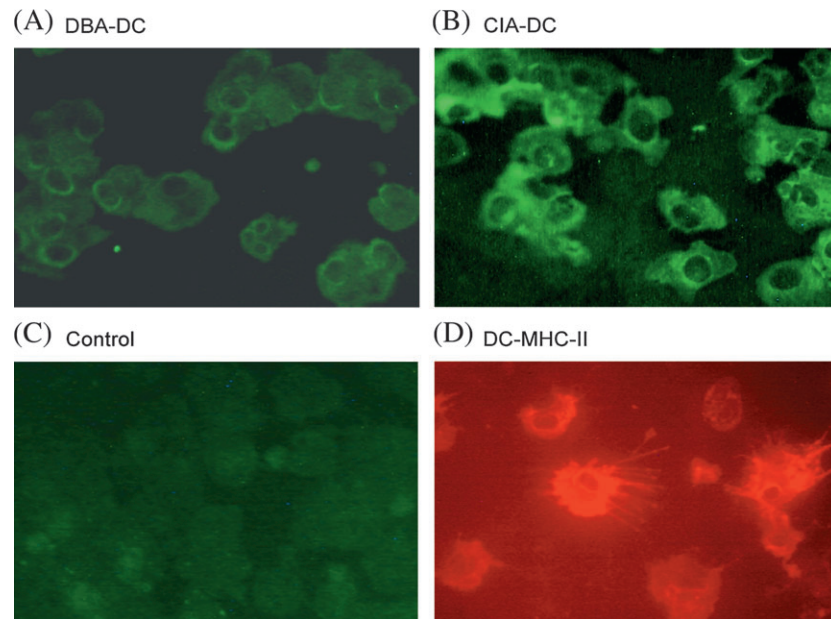


Fig. 5. Expression of BAFF in DCs by fluorescence microscopy. Sorting-purified splenic CD11c⁺ DCs were fixed after cytopsin preparation and immunostained with FITC-anti-BAFF mAb. DCs from adjuvant-treated control mice (A) displayed very weak staining whereas DCs obtained from acute CIA mice (B) showed intensive labeling of BAFF protein. (C) DCs from CIA mice stained with isotypic control antibody. (D) All purified DCs showed high levels of MHC-II expression. Images of one representative cytopsin preparation from four separate sorting experiments for DC purification are shown ($\times 200$).

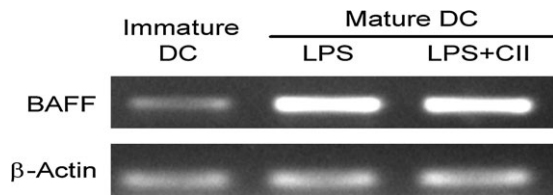


Fig. 6. Increased levels of BAFF transcripts upon DC maturation. BM-derived DCs were generated as described in Methods and prepared for semi-quantitative RT-PCR analysis. Immature DCs showed lower levels of BAFF expression whereas LPS-stimulated mature DCs displayed profoundly increased BAFF expression. Similar levels of up-regulated BAFF transcripts were detected in CII-pulsed mature DCs.

B cells with DCs from CIA mice. These data provide supportive evidence that DC-produced BAFF plays an important role in driving B cell proliferation during the development of autoimmune arthritis in the CIA experimental model.

Discussion

In the present study, we have characterized the expression patterns of BAFF and its receptors during the development of autoimmune arthritis. Our data suggest that increased BAFF production may contribute to the onset and progression of autoimmune arthritis by enhancing the survival and proliferation of peripheral B cells. Moreover, we have found that BAFF expression in DCs and macrophages are highly increased at the acute and chronic stages of arthritis

development, respectively. Thus, our data reveal an important mechanistic role for BAFF in the regulation of B cell maturation and survival and, especially, in the pathogenesis of systemic autoimmune arthritis.

The developmental process of B cells is characterized by a delicate balance between cell production by proliferation and cell loss by apoptosis (21, 22), and our previous studies have demonstrated that apoptosis is an important mechanism in modulating normal B lymphopoiesis (21, 23). Current views hold that dysregulated apoptosis appears to underlie the breakdown of immune tolerance and the development of autoimmunity. It has been well established that the TNF family cytokines play a crucial role in the development of the immune system and the maintenance of lymphocyte homeostasis. BAFF, in particular, has been implicated in the regulation of peripheral B cell maturation, proliferation and survival, as evidenced by the specific loss of mature B cells, but not other cell lineages, in mice treated with TACI-Ig (15, 24). Several studies have reported that BAFF enhances the survival of newly generated immature B cells from BM and splenic B cells at the transitional stage (25–27). Recently acquired data show that serum levels of BAFF are elevated in human autoimmune disorders such as SLE, RA and Sjogren's syndrome (10, 28–30), and gene-targeting studies have demonstrated a potent role for BAFF in the development of systemic autoimmune diseases. Transgenic expression of BAFF in mice leads to enlargement of the SP and lymph nodes and increased numbers of peripheral B cells (4). This B lymphoplasia is often accompanied by auto-antibody production and hyperglobulinemia (4). With advancing age, BAFF transgenic mice develop overt signs of SLE including proteinuria, glomerulopathy and damaged renal function. A recent report further

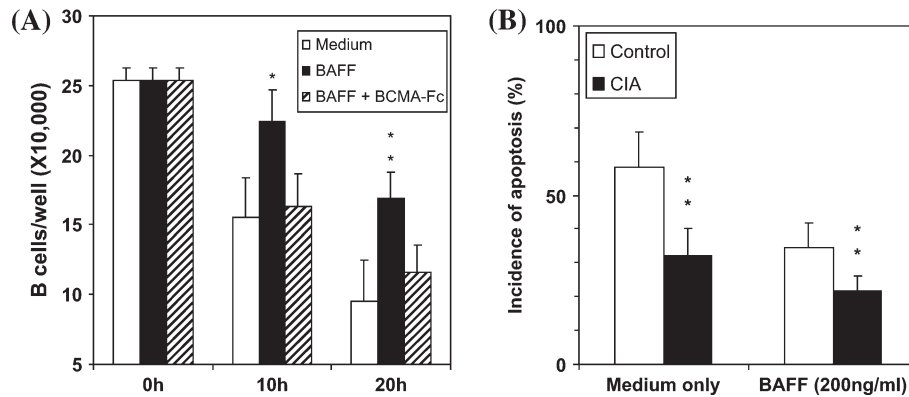


Fig. 7. Effect of BAFF on B cell survival in CIA mice. (A) Splenic cell suspensions from acute CIA mice were cultured in 2% FCS/RPMI-1640 medium either with BAFF (200 ng ml⁻¹) or BAFF plus BCMA-Fc for various time intervals (10 and 20 h). Viable cell numbers from each well of triplicate cultures were counted, followed by immunophenotypic labeling and flow cytometric analysis. BAFF significantly enhanced the survival of IgM⁺ B cells; and the protective effect of BAFF was almost completely blocked by BCMA-Fc treatment. Similar results were obtained from a repeat experiment. (B) Freshly prepared splenic cell suspensions from adjuvant-treated control and acute CIA mice were cultured for 10 h with serum-free RPMI medium in the presence or absence of BAFF (200 ng ml⁻¹) and stained with FITC-anti-IgM and PE-Annexin V for the detection of apoptotic B cells by flow cytometry. Splenic IgM⁺ B cells from CIA mice displayed much lower apoptotic incidence as compared with controls. Apoptotic incidence of B cells was greatly reduced when cultured with recombinant BAFF protein. Data represent four separate experiments (mean \pm SD; ***P* < 0.01).

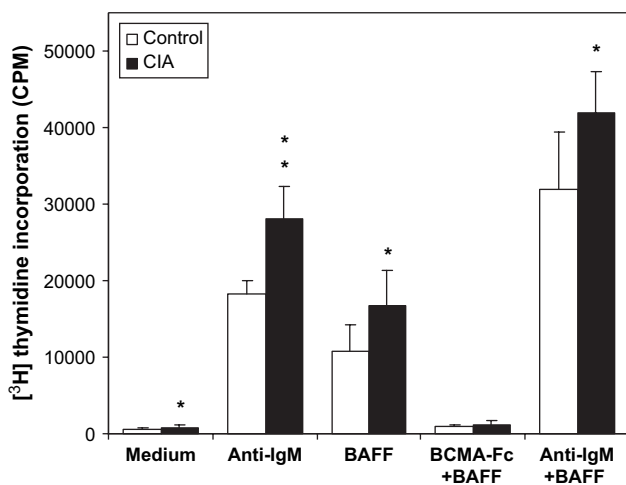


Fig. 8. Effect of BAFF on B cell proliferation. Sorting-purified splenic IgM⁺ B cells from control and acute CIA mice were cultured for 72 h with 10% FCS/RPMI in the absence and presence of anti-IgM (10 μ g ml⁻¹), BAFF (100 ng ml⁻¹), BCMA-Fc (100 ng ml⁻¹) plus BAFF and anti-IgM plus BAFF. Cells were incubated for the final 16 h with [³H]TdR. The results are expressed as the mean thymidine incorporation from triplicate cultures \pm SD. Similar results were obtained from a repeat experiment.

revealed that aged BAFF transgenic mice develop a condition that is secondary to their lupus-like disease and has profound similarities to Sjogren's syndrome in humans, featuring filtration of large numbers of B cells and dramatic up-regulation of BAFF expression in inflamed salivary glands (29). Moreover, elevated levels of serum BAFF in mice genetically prone to spontaneous development of SLE (NZB \times NZW F1 and MPL-lpr/lpr mice) have been found to correlate with disease severity and progression (15, 31).

To obtain a better mechanistic understanding of the role of BAFF in the pathogenesis of systemic autoimmune arthritis, we established the expression pattern of this protein in CIA mice and investigated the function of BAFF in regulating B cell proliferation and survival in these animals. During the induction of autoimmune arthritis, highly up-regulated BAFF expression is detected in the SP as well as in local inflamed joint tissue, whereas BAFF mRNA levels show no obvious changes in the BM of diseased animals. Moreover, elevated levels of BAFF in the sera of CIA mice are closely correlated with profoundly increased concentrations of anti-CII antibodies. These data suggest that dysregulated BAFF expression in the SP and joint tissue may be involved in the progression of autoimmune arthritis.

The splenic B cell pool of acute CIA mice is substantially enlarged, and our phenotypic analyses have revealed that total numbers of splenic transitional type 2 B cells (IgM^{high}IgD^{high}) and mature B cells (IgM^{low}IgD^{high}) are significantly increased by 2-fold. Moreover, acute CIA mice display markedly higher levels of serum BAFF than chronic CIA ones. Interestingly, the changes in B cell sub-populations of acute CIA mice are consistent with data generated from BAFF transgenic mice showing that the peripheral B cell pool is greatly expanded, especially the transitional type 2 and mature B cells (4). Peripheral B cells from CIA mice display significantly higher proliferative capacity upon anti-IgM and BAFF stimulation. In addition, recombinant BAFF can markedly suppress apoptosis of CIA splenic B cells. The findings that increased BAFF production promotes B cell survival and correlates with auto-antibody production support a pathogenic role for BAFF in the development of autoimmune arthritis by direct effect on B cells. Intriguingly, splenic B cell sub-populations only show a moderate expansion at the chronic stage of CIA compared with the controls despite the elevated serum levels of BAFF protein (Fig. 2), suggesting that the complex process of B cell maturation and survival is subject to

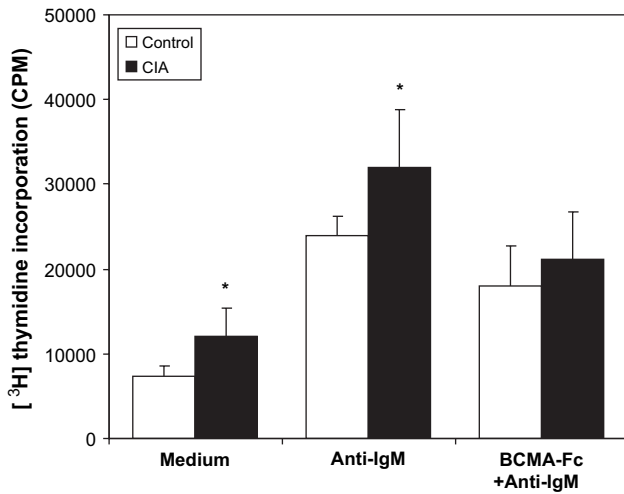


Fig. 9. DC-induced proliferation of B cells from CIA mice. Sorting-purified CD11c⁺ MHC-II⁺ DCs and IgM⁺ B cells from the SP of control and acute CIA mice were cultured in a Transwell system in the absence and presence of anti-IgM (10 µg ml⁻¹), BAFF (100 ng ml⁻¹) and BAFF plus BCMA-Fc for 3 days. Cells were incubated for the final 16 h with [³H]TdR. The results are expressed as the mean thymidine incorporation from triplicate cultures. The data are presented as the mean ± SD from one of two independent experiments with similar results.

different effector mechanisms operating at various stages of CIA pathogenesis. The functional implication of increased BAFF levels in the progression of CIA to the chronic state waits to be determined. Recently, treatment with TACI-Fc has been shown to substantially inhibit inflammation, bone and cartilage destruction and disease development in CIA mice. However, it has remained unclear whether this effect is mediated by blocking the function of either BAFF or APRIL or both ligands (32). Although APRIL has also been found to enhance the proliferation of peripheral B and T cells in cultures (7), we have not detected any significant changes in APRIL expression during CIA induction (our unpublished results). Of note, our recent studies have found that immunized DBA mice treated with recombinant BAFF displayed a particularly early onset of arthritis induction with an increased severity of clinical symptoms and joint tissue pathology (our unpublished results). Thus, our results point to a potent role of BAFF in autoimmune responses. Further studies are underway in our laboratory to address the possible involvement of APRIL in the development of experimental arthritis.

It is well documented that BAFF is expressed by myeloid lineage cells such as monocytes, DCs and granulocytes, but the cellular source of BAFF overproduction during the onset and progression of autoimmune arthritis has been unclear (17, 33). Using cell sorting-purified splenic cell populations from control and CIA mice, we have now revealed that DCs appear to be the main cellular source for the highly increased BAFF production at the acute stage of arthritis development, whereas macrophages are a predominant source for BAFF expression at the chronic stage of the disease. Recent studies have demonstrated that blood-derived CD11c⁺ DCs are the primary cells that efficiently capture and transport pathogens

to the SP and provide critical survival signals (34). These signals, which can be inhibited by TACI-Fc, induce antigen-specific marginal zone B cells to differentiate into IgM-secreting plasmablasts (34). Thus, increased levels of BAFF expression in substantially enlarged splenic DC population from acute CIA mice may be a pathological trigger of autoimmune development resulting from a normal response to foreign antigen or auto-antigen. Our data from co-cultures of purified splenic DCs and B cells provide further evidence that DC-secreted BAFF mediates B cell activation at the acute stage of experimental arthritis (Fig. 9) and that splenic DCs purified from CIA mice enhance B cell proliferation more profoundly than DCs from control mice (Fig. 8). Moreover, DC-induced B cell proliferation can be specifically, though partially, blocked with treatment by BCMA-Fc (Fig. 9). These results suggest that DCs in CIA mice generate a primary signal for B cell proliferation and antibody production and contribute, in this way, to the onset of the disease. Since BCMA-Fc is also a soluble decoy receptor for APRIL, it needs to be determined whether APRIL is involved in mediating the interaction between DCs and B cells.

In CIA mice at the chronic stage, macrophages are the predominant cell type for BAFF overproduction (Fig. 4B). Studies by Kim *et al.* (35) have shown that macrophages and lymphocytes infiltrate the synovium and sometimes form germinal centers in joint tissue of RA patients. We have also observed that macrophages are among the most abundant infiltrating inflammatory cells in the inflamed joint tissue of CIA mice (data not shown). As BAFF is known to activate both B and T cells, increased BAFF production by macrophages in local tissue may lead to activation of infiltrated B cells and T cells, resulting in lymphocyte-mediated damage in inflamed joints (20, 36, 37). Indeed, recent immunohistochemical studies have revealed that fibroblast-like synoviocytes from inflamed joints of patients with RA express functional BAFF in response to pro-inflammatory cytokines (38). Previous studies showed that G-CSF stimulated granulocytes are a predominant source of functional BAFF (39). However, levels of BAFF mRNA expression remain unchanged in Gr-1⁺ granulocytes from arthritic mice (our unpublished results).

Among the three receptors for BAFF, we have found that only BAFF-R expression is significantly up-regulated in the splenic IgM⁺ B cells of CIA mice. Although our data cannot rule out the possibility that the altered composition of splenic B cell subsets, especially the enlarged population of mature B cells, may be attributed to the increased BAFF-R expression, emerging evidence has demonstrated an essential role for BAFF-R in signaling BAFF function on B cell maturation and survival. B cell receptor (BCR) ligation has been shown to selectively up-regulate expression of BAFF-R (40). Therefore, up-regulation of BAFF-R mRNA in B cells of arthritic mice might result from auto-antigen engagement via BCR on autoreactive B cells. A recent study by Ng *et al.* (41) has demonstrated that BAFF-R is the principal BAFF-R facilitating BAFF co-stimulation of circulating B and T cells, and newly emerged evidence indicates that BAFF over-expression rescues self-reactive B cells from peripheral deletion (42). It is plausible to reason from these findings that, in the presence of self-antigen and raised levels of BAFF, signals emanating from BCR and BAFF-R may synergistically elicit proliferation and

enhance survival of autoreactive B cells. Several reports suggest that the action of BAFF-R signaling in B cells is to enhance proliferation and survival by up-regulating anti-apoptotic factors such as Bcl-2, A1 and Bcl-xL (40, 43). More recently, Bim, a BH-3-only proapoptotic protein and a key regulator of lymphocyte homeostasis, has been shown to be a critical player in depletion of autoreactive B cells and a barrier against autoimmune disease (44, 45). Interestingly, we have detected significantly decreased expression of Bim in splenic B cells of CIA mice while levels of other Bcl-2 family gene expression remain unaltered (our unpublished results). Thus, it remains to be elucidated whether BAFF signaling regulates B cell survival by directly modulating the function of Bim.

Taken together, our findings on the CIA mouse model support a pathogenic role of BAFF and B cells in the development of experimental arthritis. We have identified the cellular source for BAFF overproduction and its correlation with disease progression. In view of the complex mechanisms underlying the pathogenesis of autoimmune arthritis, further characterization of BAFF-mediated humoral and cellular immune reactions should provide a fuller understanding of the molecular mechanisms involved in the development of autoimmune arthritis and may contribute to potential therapeutic interventions for human RA.

Acknowledgements

We thank Dr Dennis G. Osmond (McGill University, Montreal, Canada) for critical reading of the manuscript. This work was supported by grants to L.L. from the Research Grants Council of Hong Kong Special Administrative Region, China (HKU7447/03M), the National Natural Science Foundation of China and Research Grants Council of Hong Kong Joint Research Scheme (N_HKU722/04) and the National Key Basic Research Program of China (2001CB510002).

Abbreviations

APRIL	a proliferation-inducing ligand
BAFF	B cell activation factor
BAFF-R	B cell activation factor receptor
BCR	B cell receptor
BCMA	B cell maturation antigen
BM	bone marrow
CIA	collagen-induced arthritis
CII	collagen type II
DC	dendritic cell
[³ H]TdR	[³ H]thymidine
IFA	incomplete Freund's adjuvant
RA	rheumatoid arthritis
RT	reverse transcription
SLE	systemic lupus erythematosus
SP	spleen
TAC1	transmembrane activator and CAML interactor
TNF	tumor necrosis factor
TAE	Tris acetate EDTA

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