J. Cell. Mol. Med. Vol 14, No 6B, 2010 pp. 1717-1725

Blockade of B-cell-activating factor suppresses lupus-like syndrome in autoimmune BXSB mice

Hanlu Ding [a], Li Wang [a, *], Xiongfei Wu [b], Jun Yan [c], Yani He [c], Bing Ni [d], Wenda Gao [e], Xuemei Zhong [f]

[a] *Department of Nephrology, Sichuan Provincial People's Hospital, Chengdu, China*
[b] *Department of Nephrology, Southwest Hospital, The Third Military Medical University, Chongqing, China*
[c] *Department of Nephrology, Daping Hospital, The Third Military Medical University, Chongqing, China*
[d] *Department of Immunology, Institute of Immunology PLA, The Third Military Medical University, Chongqing, China*
[e] *The Transplant Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA*
[f] *Department of Medicine, Boston University Medical Center, Boston, MA, USA*

Received: November 20, 2008; Accepted: May 13, 2009

Abstract

B-cell-activating factor (BAFF), a member of the tumour necrosis factor superfamily, plays a critical role in the maturation, homeostasis and function of B cells. In this study, we demonstrated the biological outcome of BAFF blockade in BXSB murine lupus model, using a soluble fusion protein consisting of human BAFF-R and human mutant IgG4 Fc. Mutation of Leu^{235} to Glu in IgG4 Fc eliminated antibody-dependent cell cytotoxicity (ADCC) and complement lysis activity, and generated a protein devoid of immune effector functions. Treatment of BXSB mice with BAFF-R-IgG4mut fusion protein for 5 weeks resulted in significant B-cell reduction in both the peripheral blood and spleen. Treated mice developed lower proteinuria, reduced glomerulonephritis and much delayed host death than untreated animals. Thus, BAFF blockade with BAFF-R-IgG4mut protein is an effective strategy to treat B-cell-mediated lupus-like pathology. Moreover, compared with other IgG isotypes with undesired effector functions, mutant IgG4 Fc should prove useful in constructing novel therapeutic reagents to block immune molecule signalling in various diseases.

Keywords: BAFF • receptor antagonist • lupus, autoimmunity • BXSB mice

Introduction

Lupus nephritis is characterized by loss of B-cell tolerance to self-antigen and the presence of abnormally activated circulating B cells [1], contributing to tissue damage by secreting autoantibodies [2]. In disease models, activated autoreactive B cells can also drive the loss of tolerance in the T-cell compartment [3]. B-cell-activating factor (BAFF, also called TALL-1, zTNF4, BlyS and TNFSF13b) is a member of the TNF superfamily that is essential for B-cell survival, proliferation and immunoglobulin secretion [4–7]. Transgenic mice overexpressing BAFF develop symptoms characteristic of systemic lupus erythematosus (SLE), with high levels of rheumatoid factors, circulating immune complexes, anti-DNA autoantibodies and immunoglobulin deposition in the kidneys. The animals eventually succumb to an immune complex-mediated, lupus-like nephritis [8–10]. Even in the absence of T-cell help, BAFF-transgenic mice that were completely deficient in T cells still develop an SLE-like disease, indicating a critical role of BAFF in B-cell autoimmunity [11]. The levels of BAFF are also elevated in the serum of patients with SLE, correlated with circulating levels of anti-dsDNA antibodies and with clinical disease activity [12–15]. Thus, inhibition of BAFF signalling is a potential therapeutic option for treating lupus nephritis.

In addition to interacting with BAFF receptor (BAFF-R, BR3, TNFRSF13C), BAFF also binds to two other receptors. One is transmembrane activator and calcium-modulator and cyclophilin ligand (CAML) interactor (TACI, TNFRSF13B), and the other is B-cell maturation antigen (BCMA, TNFRSF17). Compared with BAFF-R, these two receptors have more restricted functions: TACI controls T-cell-independent B-cell antibody response, isotype switching and B-cell homeostasis, whereas BCMA supports the survival of bone marrow plasma cells (for review, see reference [16]). Of the three receptors for BAFF, only BAFF-R is specific and the primary receptor for transmitting the BAFF-dependent B-cell survival signal, whereas TACI and BCMA also bind to the related ligand APRIL (a proliferation-inducing ligand, TNFSF13) [9, 17].

*Correspondence to: Dr. Li WANG,
Department of Nephrology,
Sichuan Provincial People's Hospital. Chengdu,
Sichuan, 610072, P. R. China.
E-mail: scwangli62@163.com

doi:10.1111/j.1582-4934.2009.00817.x

Strategies to block BAFF signalling have been tested in animal models of diseases with promising success. For example, treatment with a soluble TACI-Ig fusion protein in NZB/W F1 mice, a mouse model of lupus nephritis, inhibited the development of proteinuria and prolonged survival of the animals [9]. Adenovirus-mediated expression of TACI-Ig also blocked the development of hypergammaglobulinaemia, decreased the numbers of splenic plasma cells in C57BL/6 (B6) lpr/lpr mice and reduced the extent of glomerulonephritis and proteinuria, improved survival in MRL.lpr/lpr mice [18].

Nevertheless, because of the complexities of receptor-ligand cross-interaction as well as the species difference between human beings and mice, one should take extra caution in interpreting the experimental data. For instance, Ramanujam *et al.* compared the effects of BAFF-R-Ig, which blocks only BAFF, with those of TACI-Ig, which blocks both BAFF and APRIL, in a murine SLE model [19]. They found that both reagents similarly inhibited disease activity, but non-selective blockade with TACI-Ig further interfered with the development of T-cell-dependent IgM response, probably because of blockade on APRIL and TACI/BCMA interaction [19]. Although BAFF-R-Ig seems more specific, unlike human BAFF-R, mouse BAFF-R can also weakly bind to mouse APRIL [20], and hence high doses of soluble BAFF-R-Ig may inhibit mouse APRIL in addition to BAFF.

Another important issue when applying receptor immunoglobulin fusion proteins as cytokine antagonists is that, in most of the applications people often use human or mouse IgG1 Fc tail as the fusion partner. The IgG1 isotype has strong antibody-dependent cell cytotoxicity (ADCC) and complement activation capacity. Hence, an IgG1 fusion protein may become lytic to a cell if it binds to its cell surface ligand [21]. As BAFF is also synthesized as a membrane-bound protein [4], BAFF-R-IgG1 could kill $BAFF^+$ cells including monocytes, thus complicating the interpretation of BAFF blockade data and may cause unwanted side effects when used as a therapeutic agent. In this study, we characterized the biological activity, both *in vitro* and *in vivo*, of a human BAFF-R-IgG4mut fusion protein in BXSB murine lupus model. The ability of IgG1 to induce complement lysis was eliminated by replacing the Fc domain of human IgG1 with that of human IgG4, which does not bind C1q, the first protein in the complement cascade. Also, mutation of Leu^{235} to Glu in IgG4 Fc eliminated the remaining ADCC activity and generated a protein devoid of immune effector functions [21]. Here, we describe in detail that treatment of BXSB mice with the soluble BAFF-R-IgG4mut fusion protein decreases the lupus-like symptoms and prolongs the survival of animals. The results support the clinical potential of BAFF-R-IgG4mut as a therapeutic agent for lupus nephritis.

Materials and methods

Animals

Male BXSB mice, aged 8 weeks, 21–22 g in weight, were obtained from the Experimental Animal Laboratory of Peking University Health Science Center (Beijing, China). All animals were housed at 22°C under a 12-hr light/dark cycle under pathogen-free conditions. The animals were monitored for the development of proteinuria every 2 weeks and serum was collected every 3 weeks to measure anti-dsDNA antibody titres. Morbidity was checked three times a week. Animal care and handling was performed with the approval of the Institutional Authority for Laboratory Animal Care of the Health Science Center.

Construction of BAFF-R-IgG4mut fusion protein

The Leu^{235} to Glu mutation in hIgG4 was created by overlapping PCR with primers: IgG4mu UP: CCAGCACCTGAGTTCGAAGGGGGACCATCAGTC and IgG4mu DN: GACTGATGGTCCCCCTTCGAACTCAGGTGCTGG. The coding sequence for the extra-membrane region of hBAFF-R (Genbank: AF373846) was chemically synthesized by annealing the following four long oligos with built-in mutations V20Q and L27P to eliminate aggregates [22]. BAFF-R PciI primer: ggtaacATGtctAGGCGAGGGCCCCGGAGCCTGCG GGGCAGGGACGCGCCAGCCCCCACGCCCTGCaaCCCGGCCGA; BAFF-R (53–129) primer: CCCCCACGCCCTGCaaCCCGGCCGAGTGCTTCGACCc GCTGGTCCGCCACTGCGTGGCCTGCGGGCTCCTGCGCACG; BAFF-R (181–105) primer: GCAGCGCCGTCCTGGGCGCAGGGCTGCTGGCCCCGGC-CGGTTTCGGCCGCGGCGTGCGCAGGAGCCCGCAGGCCACG; BAFF-R XhoI primer: ctaactcgAGCGCCGCCTCGCCGGCCCCCGCGCCCACCGACTCCTGCG-GCTGCAGCGCCGTCCTGGGCGCAGGGCTG. The annealed product was digested with PciI and XhoI and cloned in-frame with hIgG4mut tail into the pSec/WG vector. The right recombinant was transfected into Flp-In Chinese hamster ovary (CHO) cells with lipofectamine reagent and stable constitutive expression cell line was generated according to the instructions of the manufacturer (Invitrogen).

Western blot of BAFF-R-IgG4mut fusion protein

BAFF-R-IgG4mut fusion protein was expressed and purified by Protein A affinity column chromatography as described previously [23]. Purity of the purified protein was assayed on a 12% SDS-PAGE gel. For Western blot analysis, the membrane with transferred protein was blocked in 1% blocking solution in TBS (150 mM NaCl in 50 mM Tris-HCl, pH 7.5) for 12 hrs at 4°C, and then incubated with goat anti-human BAFF-R polyclonal antibody (0.2 μg/mL, clone AF1162, R&D Systems, USA) overnight at 4°C. After washing three times with 0.5% Tween in TBS buffer, the blot was incubated for 1 hr with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (1:300, Sino-American Biotechnology Co., Beijing, China). Proteins were detected with DAB reagent sets (Sino-American Biotechnology Co., Beijing, China).

Bioactivity assay of BAFF-R-IgG4mut fusion protein

B cells from BALB/c spleen were purified by the Miltenyi B-cell enrichment kit (Miltenyi Biotec, USA). B cells 2×10^5 were seeded in 96-well plate and stimulated with 5 or 10 μg/ml anti-IgM (Southern Biotech, USA) in triplicates. In some wells, 2 ng/ml of mouse recombinant BAFF (R&D Systems) and serially diluted purified hBAFF-R-IgG4mut was added. Cell proliferation after 3 days was measured by ^{3}H-thymidine incorporation (0.5 μCi/well) added at the last 18 hrs of culture.

Measurement of serum BAFF levels

Serum BAFF was measured by ELISA in male BXSB animals before onset of disease (8 weeks of age) as a baseline, and then measured during disease progression. Briefly, plate-coated rabbit antimouse BAFF polyclonal antibody (20 μg/ml, eBioscience) was incubated for 1 hr at 37°C with sera samples diluted 1:100. After washing, rat antimouse BAFF monoclonal antibody (40 μg/ml, R&D Systems) as detecting antibody was then incubated for 1 hr at 37°C, the latter of which was further recognized by biotinylated antirat IgG (1:200, Zhongshan Biotechnology Co., Beijing, China) and avidin-conjugated HRP with tetramethylbenzidine as the substrate. Recombinant mouse BAFF (R&D Systems) was used as a standard (diluted from 1–1000 ng/l) to measure BAFF concentrations in 100 μl of serum samples in triplicate.

Treatment of mice with BAFF-R-IgG4mut fusion protein

Eight weeks old BXSB male mice were randomly divided into three groups (15 mice per group) and treated with phosphate buffered saline (PBS), human IgG4 (200 μg/animal; Sigma-Aldrich, USA) or 100 μg doses of BAFF-R-IgG4mut fusion protein three times a week for 5 weeks. As we observed similar results between the mice treated with PBS or human IgG4 in all the experiments, these results were combined and presented as a control group. All the above reagents were administrated intraperitoneally.

FACS analysis of lymphocytes from peripheral blood and spleen

$B220^+$ cells and $CD4^+$ and $CD8^+$ T cells in peripheral blood and spleen were analysed by fluorescence-activated cell sorting (FACS) at 10, 12 and 15 weeks of age. Except mentioned, all antibodies were from eBioscience (USA). For $B220^+$ B-cell detection, cells were stained with combinations of antigen-presenting cell (APC)-conjugated antimouse B220 and fluorescein isothiocyanate (FITC)-conjugated antimouse CD5. For T-cell detection, cells were stained with FITC-conjugated antimouse CD8, phycoerythrin (PE)-conjugated antimouse CD4 and PE-Cy5-conjugated antimouse CD3 (Coulter-Immunotech, USA). Alternatively, total T cells were presented as $B220^-CD5^+$ cells.

Measurement of proteinuria and anti-dsDNA antibody in serum

The presence of proteins in mouse urine was measured using Uristix (Ames). Blood samples were collected from the tail veins of treated BXSB mice at 9, 10, 12 and 15 weeks of age. The levels of serum IgG against double-stranded (ds) DNA was measured by ELISA using calf thymus dsDNA (Sigma) as the antigen with some modifications [24]. Briefly, 10 μg/ml of dsDNA was coated onto 96-well ELISA plates. After blocking with 2% foetal bovine serum in PBS, 50 μl of 1:100 diluted sera were added to each well and incubated for 120 min. at 37°C. The plates were then washed three times with PBS containing 0.05% Tween and incubated with 100 μl of HRP-conjugated goat antimouse IgG antibody (Sino-American Biotechnology) at 1:1000 dilution. After washing, the plates were developed with H_2O_2 and Ophenylenediamine at 4 μg/mL in phosphate-citrate buffer (pH 5.0). The reaction was stopped with 2 N H_2SO4 and absorbance at 490 nm was measured by an ELISA plate reader (Alexis, Montreal, Canada).

Assessment of nephritis

For monitoring the development of nephritis, proteinuria was measured as described previously [25], and urine protein concentrations of >100 mg/dl were considered positive. For histological evaluation of renal disease, mice were killed after treatment at 15 weeks of age. Kidneys were either fixed in 10% buffered formalin or prepared for cryostat sectioning. Formalin-fixed tissues were embedded in paraffin, sectioned and stained by the haematoxylin and eosin method. Cellularity was determined by counting the number of cells of 12 randomly selected glomeruli in each kidney cross-section. The degree of cellularity was scored from 0 to 3 by the following criteria as described [26]: grade 0, normal (35–40 cells/glomerulus); grade 1, mild (41–50 cells/glomerulus); grade 2, moderate (51–60 cells/glomerulus); grade 3, severe (>60 cells/glomerulus). The intensity of tubular interstitial inflammatory cell infiltrates was scored according to a 0–4 scale as previously described [27], where 0 represents no abnormality, and 1, 2, 3 and 4 represent mild, moderate, moderately severe and severe abnormality, respectively. For determining IgG deposition, frozen sections were fixed in acetone and stained with FITC-conjugated goat antimouse IgG mAb (Sino-American Biotechnology). The staining profiles were obtained with a fluorescent microscope (BX-50; Olympus, Tokyo, Japan) and the mean fluorescence intensity (MFI) of 15 glomeruli from each section was averaged blindly from 0 to 4+ by a pathologist who was unaware of the experimental design, as described previously [28].

Statistical analysis

Log-rank and Student's t-tests were used. Methods were noted in figure legends. Values of $P < 0.05$ were considered significant.

Results

BAFF-R-IgG4mut fusion protein blocks BAFF activity *in vitro*

We successfully constructed the expression vector for BAFF-R-IgG4mut containing the extracellular domain of human BAFF-R gene (Genbank: AF373846). Based on amino acid sequence, the mature human BAFF-R-IgG4mut protein expressed in CHO cells has a calculated molecular mass of approximately 35.6 kD. As a result of glycosylation, the recombinant protein migrates with apparent molecular weight (MW) of 40–50 kD in SDS-PAGE under reducing conditions (Fig. 1A). The authenticity of the BAFF-R moiety was confirmed by Western blot (Fig. 1B). To test the bioactivity of BAFF-R-IgG4mut protein, we stimulated mouse B cells with anti-IgM, anti-IgM plus BAFF (2 ng/ml) or anti-IgM+BAFF+BAFF-R-IgG4mut. Low dose of exogenous BAFF did not induce B-cell proliferation by itself (data not shown),

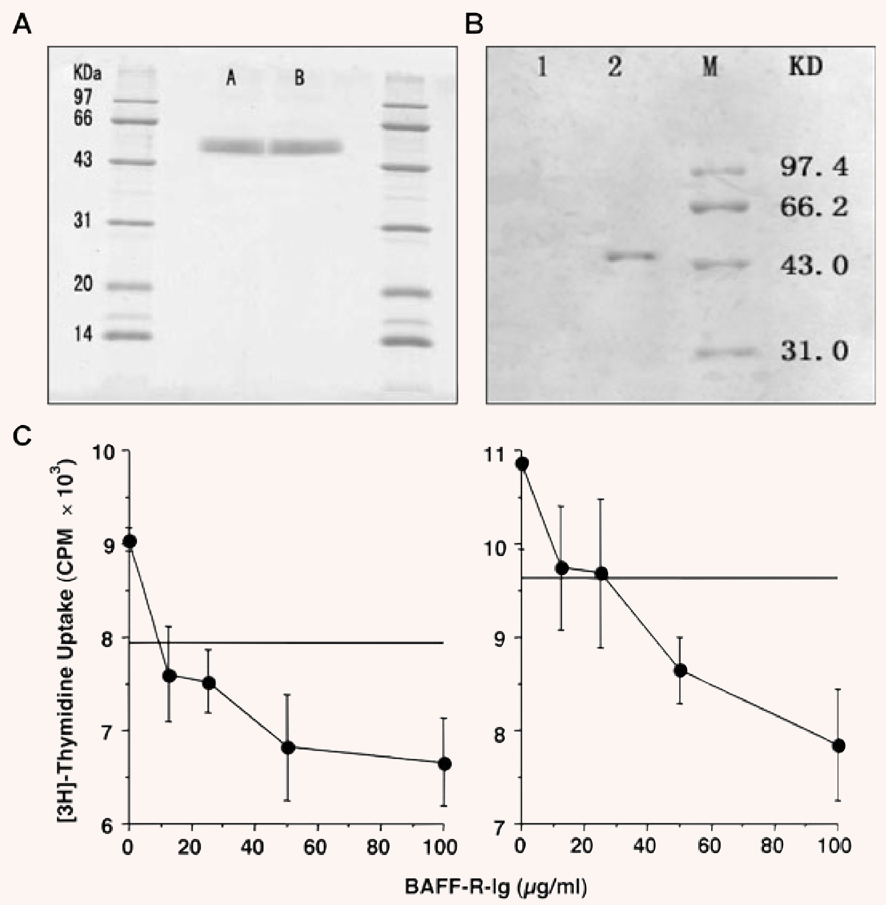

Fig. 1 Characterization of BAFF-R-IgG4mut fusion protein. (**A**) SDS-PAGE analysis. A and B both are loaded with purified BAFF-R-IgG4mut. (**B**) Western blot analysis with anti-BAFF-R mAb. 1, negative control; 2, BAFF-R-IgG4mut fusion protein; M, molecular weight marker. (**C**) *In vitro* bioactivity assay. Purified mouse splenic B cells were stimulated with 5 μg/ml (left side) or 10 μg/ml (right side) of anti-IgM in the presence of 2 ng/ml of BAFF and indicated doses of BAFF-R-IgG4mut fusion protein. B-cell proliferation after 3 days was measured by ^{3}H-thymidine incorporation in triplicates. B-cell thymidine incorporation by anti-IgM stimulation alone was labelled as a flat line in each side. BAFF-R-IgG4mut alone did not stimulate B-cell proliferation (data not shown).

but increased anti-IgM-stimulated proliferation. Clearly, BAFF-R-IgG4mut protein exerted a dose-dependent suppression of anti-IgM+BAFF stimulated proliferation (Fig. 1C). Interestingly, in almost all the cultures with BAFF-R-IgG4mut protein added, B-cell proliferation is even lower than that induced by anti-IgM alone. This suggests that during activation by anti-IgM, B cells secrete endogenous BAFF as a growth or survival factor, and this effect can also be neutralized by BAFF-R-IgG4mut protein.

BAFF-R-IgG4mut blocks the elevation of serum BAFF levels during murine lupus development

To determine if administration of BAFF-R-IgG4mut can neutralize BAFF *in vivo*, we measured serum BAFF levels in BXSB mice at 9, 10, 12 and 15 weeks of age. An average of one-fold increase in serum BAFF was detected in 70% of the animals at 10 weeks of age, and the level reached threefold over baseline in 100% of the animals by 15 weeks of age in control mice. After treatment with BAFF-R-IgG4mut, the serum BAFF levels were significantly reduced (Fig. 2), indicating this recombinant fusion protein can be used as a BAFF blocker *in vivo*.

BAFF-R-IgG4mut prevents the development of lupus-like nephritis

To assess the effects of BAFF-R-IgG4mut on the development of lupus-like nephritis, we started the treatment of BXSB mice at 8 weeks of age before serum levels of BAFF were elevated. Proteinuria (>100 mg/dl) was first detected in the control mice (PBS or IgG) at 9 weeks of age and became prominent at 12 weeks of age (Fig. 3A). At the cessation of treatment (15 weeks of age), 100% of the mice treated with PBS or IgG4 developed renal disease with proteinuria >100 mg/dl. Although 50% of the mice treated with BAFF-R-IgGmut developed proteinuria at 15 weeks of age, the disease course was significantly delayed ($P < 0.01$). As the hallmark of lupus is the development of anti-dsDNA autoantibodies, we measured by ELISA the levels of serum anti-dsDNA IgG in BXSB mice after different treatments. The results showed that BXSB mice treated with PBS or IgG4 possessed highly elevated levels of serum anti-dsDNA IgG, and there was an obvious

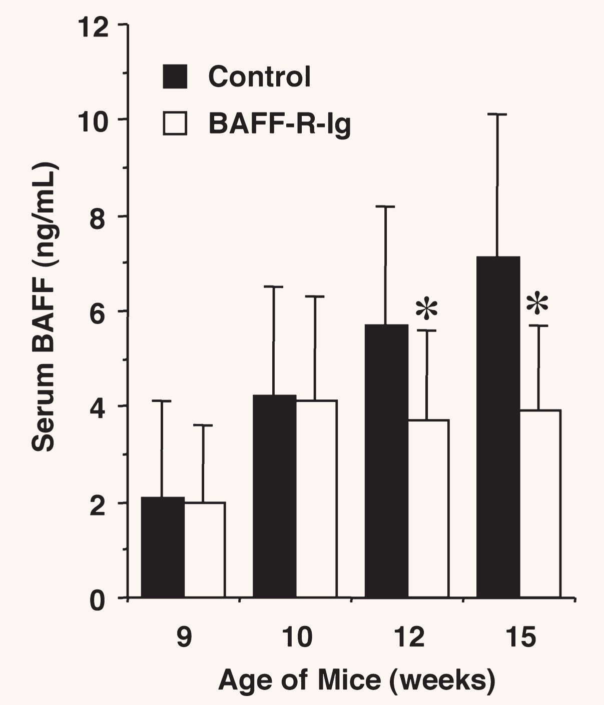

Fig. 2 BAFF-R-IgG4mut treatment inhibits the elevation of BAFF serum levels during lupus development. Serum BAFF levels in BXSB mice after different treatments were measured by ELISA. Data are presented as mean ± S.E.M. of five mice in each group. *Statistically different when BAFF-R-IgG4mut group is compared with PBS or IgG4 group by Student's t-test for independent samples ($P < 0.05$).

trend of increase in antibody titres with age. The levels of anti-dsDNA IgG were suppressed in the mice 4 weeks after the initial treatment with BAFF-R-IgG4mut and were maintained at 15 weeks of age compared with PBS or IgG treatment alone ($P = 0.035$) (Fig. 3B). Animals treated with BAFF-R-IgG4mut had 100% survival at 30 weeks of age compared with 0% survival in the control group ($P < 0.01$) (Fig. 3C). Although all animals in BAFF-R-IgG4mut-treated group eventually died of disease, this may be as a result of the neutralization of this human fusion protein by murine anti-human antibody.

Histological verification of the beneficial effects of BAFF-R-IgG4mut

The improvement of nephritis by BAFF-R-IgG4mut treatment was verified by histological examination of glomerular hypercellularity, tubular interstitial inflammatory cell infiltration and IgG deposition in renal tissue sections from mice that underwent different treatments. At 15 weeks of age, renal sections from PBS or IgG-treated mice showed mesangial hypercellularity, in conjunction with tubular interstitial inflammatory cell infiltration (Fig. 4A). The above pathological changes were diminished in BAFF-R-IgG4mut-treated mice (Fig. 4B). While there was strong deposition of IgG to glomeruli in PBS or IgG4-treated mice (Fig. 4C), the intensity of such IgG deposition was clearly inhibited in mice treated with BAFF-R-IgG4mut (Fig. 4D). In semi-quantitative analyses, BAFF-R-IgG4mut treatment showed the most significant inhibition on glomerular hypercellularity, tubular interstitial inflammatory cell infiltration and IgG deposition, compared with treatment with PBS or IgG (Fig. 4E–G). These results support the notion that blocking BAFF:BAFF-R interaction could effectively inhibit the development of lupus-like disease in mice.

Effects of BAFF-R-IgG4mut on lymphocyte composition

To study the potential mechanisms contributing to the suppression of disease in BAFF-R-IgG4mut-treated animals, we determined the percentage of B cells ($B220^+CD5^-$) and T cells ($CD3^+CD4^-CD8^+$, $CD3^+CD4^+CD8^-$) by flow cytometry at 10, 12 and 15 weeks of age. At 12 weeks of age (4 weeks after treatment), there was already a significant decrease of the percentage of B cells in the peripheral blood and spleen in animals treated with BAFF-R-IgG4mut, compared with control animals (Fig. 5A). To show this effect is B-cell specific, we used the $B220^-CD5^-$ non-T, non-B cell population as an internal calibrator, and calculated the ratios of B or T cells *versus* this calibrating population. We found that starting from 12 weeks of age, BXSB mice had increased B-cell population in both the spleen and the peripheral blood. Treatment with BAFF-R-IgG4mut fusion protein significantly suppressed B-cell expansion, while having no effect on T cells (Fig. 5B). These results indicate a role for BAFF in maintaining peripheral B-cell populations and blocking BAFF:BAFF-R has a beneficial effect on inhibiting B-cell hyperplasia in murine lupus.

Discussion

BAFF-R is expressed on T cells and a wide range of B-cell subsets, including immature, transitional, mature, memory and germinal centre B cells, as well as on plasma cells [29–32]. In this study, we used hBAFF-R-Ig instead of mBAFFR-Ig to specifically block BAFF signalling in mice. This is because mouse BAFF-R weakly binds to mouse APRIL, and hence high experimental doses of recombinant soluble mouse BAFF-R-Ig may inhibit mouse APRIL in addition to BAFF [20]. We used BXSB mice, a well-characterized mouse model that spontaneously develops an autoimmune syndrome similar to human SLE, and is characterized by hypergammaglobulinemia, autoantibody production and the development of fatal glomerulonephritis. Positive serology to self-Ags (dsDNA, ssDNA, erythrocytes and platelets) and immune complex-mediated glomerulonephritis is the hallmark of disease in BXSB mice. Histopathological changes are evident by 10 weeks of age in male

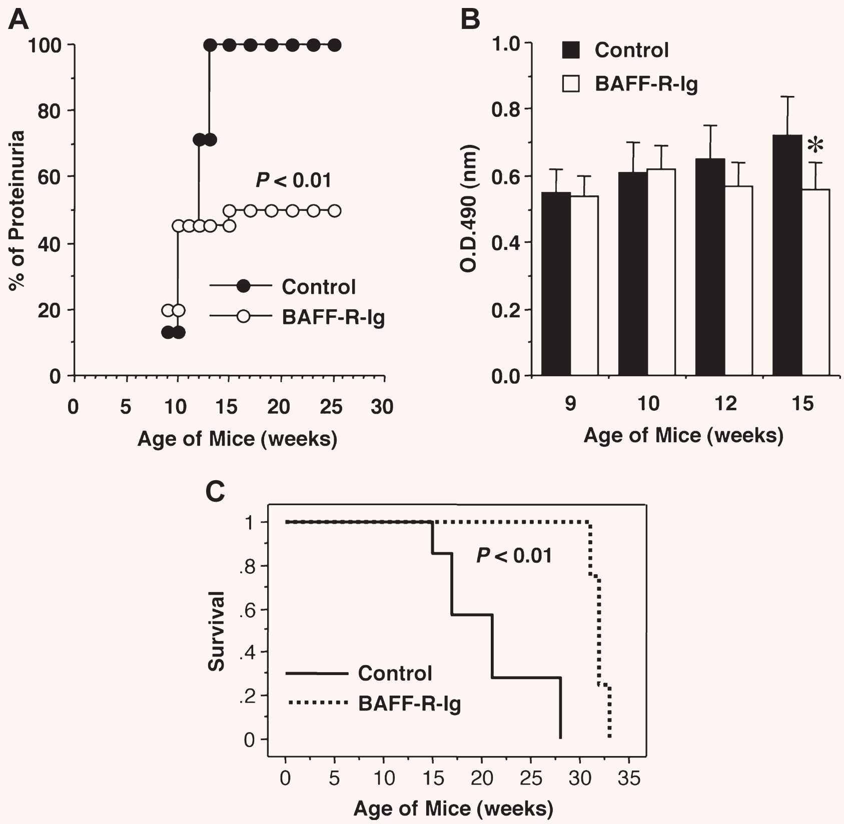

Fig. 3 BAFF-R-IgG4mut prevents the development of lupus nephritis. (**A**) Eight weeks old male BXSB mice (15 mice per group) were injected intraperitoneally with PBS or IgG4 as control, or 100 μg BAFF-R-IgG4mut three times a week for 5 weeks. The percentage of mice in each group that have developed proteinuria (>100 mg/dl) is shown. BAFF-R-IgG4mut treatment significantly delayed the onset of proteinuria by log-rank test ($P < 0.01$). (**B**) Serum levels of circulating anti-dsDNA antibodies are shown as A_{490} values from an ELISA. (**C**) BAFF-R-IgG4mut treatment significantly delayed host death by log-rank test ($P < 0.01$). Kaplan-Meier survival plot of treated animals is shown.

mice, leading to end-stage renal disease and 70% mortality by 40 weeks of age [33, 34].

The BAFF-R-IgG4mut fusion protein is functional as a blocker for BAFF, as shown by its ability to neutralize BAFF activity *in vitro*. In this study, we demonstrated that weekly treatment of BXSB mice with BAFF-R-IgG4mut fusion protein for 5 weeks resulted in significant B-cell reduction in both the peripheral blood and the spleen. Treatment with BAFF-R-IgG4mut also led to a partial suppression of serum autoantibody levels. Glomerulonephritis was reduced in conjunction with the improvement in proteinuria. This was associated with prolonged survival in treated animals. In BXSB mice, the infiltrating T cells include both $CD4^+$ and $CD8^+$ T cells that appear to be required for disease pathogenesis. However, BAFF blockade in this model has no effect on the activation and expansion of T cells, as $CD4^+$ and $CD8^+$ T-cell compositions are not different between the groups. The delayed mortality with BAFF blockade could result from reduced autoantibodies and/or from reduced numbers of B cells driving the infiltrative process [3, 35]. The results indicate that blocking B-cell extravasation is sufficient to quench autoimmune reactions and may be the major mechanism for the BAFF-R-IgG4mut fusion protein activity *in vivo*. Our data suggest that BAFF blockade may be effective as an interventional strategy, rather than being simply a preventative measure, in patients with established disease.

Recombinant fusion proteins consisting of the extracellular domain of immunoregulatory proteins and the Fc region of immunoglobulin G (IgG) are a novel class of protein therapeutics. A crucial component of these fusion proteins is the IgG domain. The Fc region of IgG1, the most commonly used isotype in fusion proteins, exerts immune effector function, such as complement lysis and ADCC. Depending on their potential application, fusion proteins targeting cancer cells or autoreactive immune cells may gain potency if they have the capability to induce cell death of their targets. This increase in potency has to be balanced with possible side effects if the ligand of the fusion protein is expressed on tissues other than the target cells. Even though the original design of certain receptor fusion proteins is to block their soluble cytokine ligands, one should bear in mind that some cytokines can be expressed in a cell-surface-bound form. For example, TNF-α is synthesized as a trans-membrane 26 kD protein that is transported to the cell surface where it is cleaved by a metalloproteinase (TACE) to release the soluble 17 kD protein [36]. TNFR-IgG1, brand name Enbrel™, can effectively block TNF-α and has been used to treat rheumatoid arthritis [37]. However, some unwanted side effects, such as increased incidence of infection and cancer, as well as ‘injection site reaction’ might be caused by Enbrel™ binding to cell-surface TNF-α and subsequent lysis of these cells *via* IgG1-mediated effector functions [37].

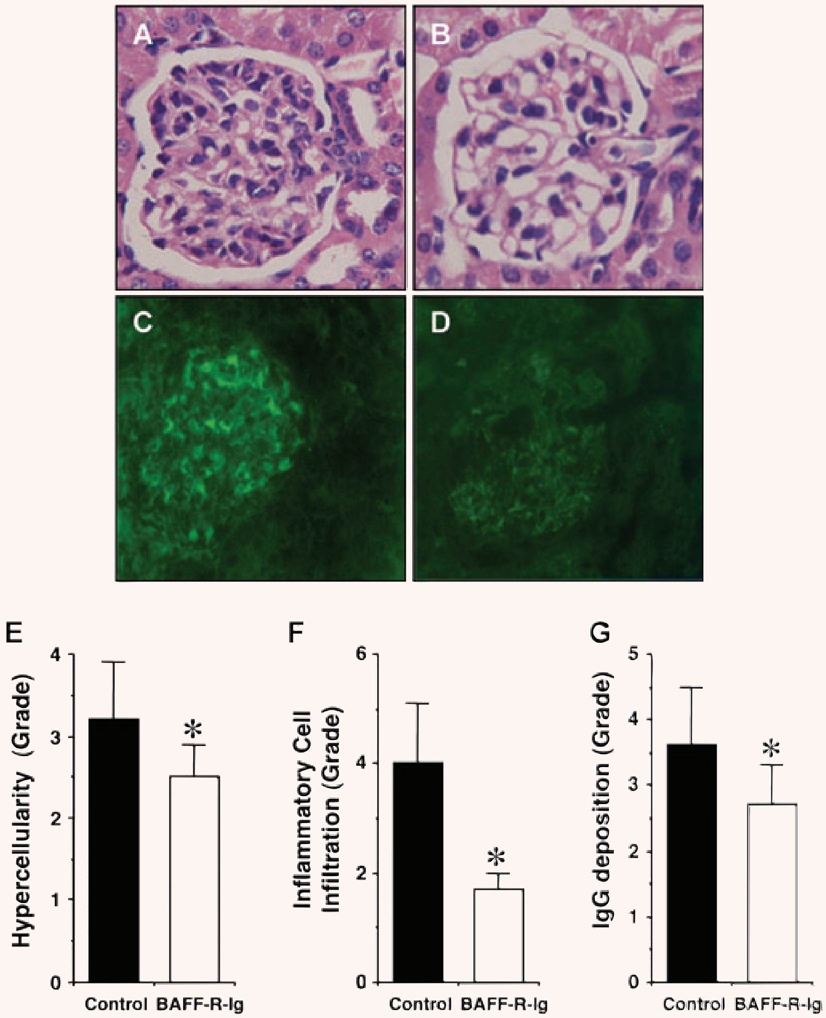

Fig. 4 Histological evaluation of renal disease in BXSB mice underwent different treatments. Kidney sections from mice at 15 weeks of age after treatment with PBS or IgG4 (**A**, **C**), or BAFF-R-IgG4mut (**B**, **D**) were examined by haematoxylin and eosin staining for histopathological changes (**A**, **B**), and by immunofluorescence for IgG deposition (**C**, **D**). Original magnification: ×400. (**E**–**G**) Semi-quantitative evaluation of renal disease in BXSB mice treated with PBS or IgG4 as control, or BAFF-R-IgG4mut fusion protein. The degree of hypercellularity of glomeruli (**E**) and the extent of tubular interstitial inflammatory cell infiltration (**F**) and IgG deposition (**G**) were calculated as described earlier. Data are presented as mean ± S.E.M. of five mice in each group. *Statistically different when compared with PBS or IgG4 group ($P < 0.05$) by Student's t-test for independent samples.

Like TNF-α, BAFF can also be expressed as a membrane-bound form on many types of immune cells [4]. Potential lysis of these cells during treatment with conventional BAFF-R-IgG1 could create a 'hole' in host immunity and may increase the incidence of infection and cancer. In our study, the ability of IgG1 fusion proteins to induce complement lysis was eliminated by replacing the Fc region of human IgG1 with that of human IgG4, which does not bind C1q, the first protein in the complement cascade [21]. ADCC is exerted by monocytes and natural killer cells when they recognize opsonized cells *via* their Fc receptors. Human IgG4 does not bind Fc γ RII and Fc γ RIII, but it does bind Fc γ RI. A crucial subdomain for this interaction has been mapped to amino acids 234–238 of IgG4 [38]. Mutation of Leu^{235} to a Glu in human IgG4 should completely abolish Fc γ RI binding and ADCC activity [21]. Thus, our study demonstrated that mutant IgG4 Fc should prove useful in constructing novel therapeutic reagents to block immune molecule signalling in various diseases.

Last, human lupus is a very complicated disease, affecting multiple organs. One should not expect any monotherapy to be the ultimate regimen. Potentially efficacious reagents might be possibly combined to achieve synergy in future therapy. Compared with untreated controls, treated animals in our study did show prolonged survival by week 30, although they all died by week 33. This sudden onset of host death is more likely because of the insufficient treatment and/or intrinsic disease mechanism, but not the toxicity of BAFF-R-IgG4mut, as histological analysis did not reveal any sign of liver abnormality after treatment with BAFF-R-IgG4mut (data not shown). It is possible that the full human sequence of BAFF-R-IgG4mut induced murine anti-human neutralizing antibodies that made its therapeutic benefit less

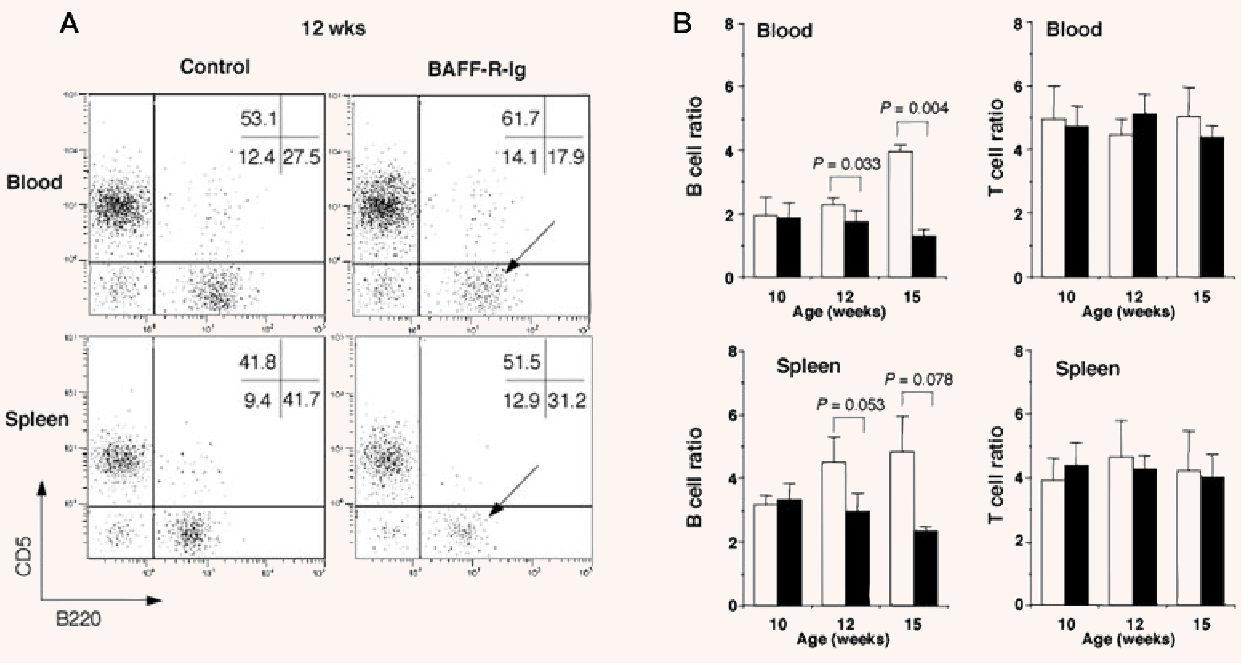

Fig. 5 BAFF-R-IgG4mut significantly suppresses B-cell expansion in the spleen and the peripheral blood of BXSB mice. (**A**) At 12 weeks of age, the percentages of viable B ($B220^+CD5^-$) and T ($B220^-CD5^+$) cells in the peripheral blood (upper panels) and the spleen (lower panels) of mice in each group were determined by flow cytometry. (**B**) The ratios of B ($B220^+CD5^-$) or T ($B220^-CD5^+$) cells *versus* the non-T non-B population ($B220^-CD5^-$) in the peripheral blood (upper panels) and the spleen (lower panels) of mice in each group were presented as bar graphs over time. Control groups, open bar; BAFF-R-IgG4mut groups, solid bar. Statistical significance was calculated by Student's t-test.

prominent. This is not without a precedent. In a study by Charles Dinarello's group [39], injection of human α-1-antitrypsin (hAAT) in mice induced anti-hAAT after 18 days. The authors further showed that it was the neutralizing antibody that limited the beneficial effect of hAAT, as pre-vaccination with hAAT abolished its protective effect on transplanted allogeneic islets, and rejection-caused hyperglycaemia correlated well with the existence of anti-hAAT titres. In our study, we did not inject BAFF-R-IgG4mut for $>$5 weeks, as once the anti-human antibodies are induced, the again introduced human reagent will be of limited effect. In addition, formation of mouse antibody:human antigen complex and its deposition may accelerate the disease. This issue can be controlled by using murine BAFF-R-Ig with escalating doses to cover longer period of time in future animal studies, but will not become an issue in human trials.

Acknowledgements

We thank Man Jiang for the technical assistance in purifying BAFF-R-IgG4mut protein. This study was supported by grants from the National Natural Science Foundation of China 30400214 (to H. D.) and from American Heart Association 0730298N (to X. Z.).

The authors declare no competing financial interests.

References

1. **Klinman DM, Shirai A, Ishigatsubo Y, *et al.*** Quantitation of IgM- and IgG-secreting B cells in the peripheral blood of patients with systemic lupus erythematosus. *Arthritis Rheum.* 1991; 34: 1404–10.
2. **Mitchison NA, Wedderburn LR.** B cells in autoimmunity. *Proc Natl Acad Sci USA.* 2000; 97: 8750–1.
3. **Chan OT, Hannum LG, Haberman AM, *et al.*** A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *J Exp Med.* 1999; 189: 1639–48.
4. **Moore PA, Belvedere O, Orr A, *et al.*** BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science.* 1999; 285: 260–3.
5. **Mackay F, Schneider P, Rennert P, *et al.*** BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol.* 2003; 21: 231–64.
6. **Mackay F, Browning JL.** BAFF: a fundamental survival factor for B cells. *Nat Rev Immunol.* 2002; 2: 465–75.

7. **Mackay F, Silveira PA, Brink R.** B cells and the BAFF/APRIL axis: fast-forward on autoimmunity and signaling. *Curr Opin Immunol.* 2007; 19: 327–36.
8. **Mackay F, Woodcock SA, Lawton P, *et al.*** Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J Exp Med.* 1999; 190: 1697–710.
9. **Gross JA, Johnston J, Mudri S, *et al.*** TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. *Nature.* 2000; 404: 995–9.
10. **Khare SD, Sarosi I, Xia XZ, *et al.*** Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice. *Proc Natl Acad Sci USA.* 2000; 97: 3370–5.
11. **Groom JR, Fletcher CA, Walters SN, *et al.*** BAFF and MyD88 signals promote a lupuslike disease independent of T cells. *J Exp Med.* 2007; 204: 1959–71.
12. **Zhang J, Roschke V, Baker KP, *et al.*** Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. *J Immunol.* 2001; 166: 6–10.
13. **Cheema GS, Roschke V, Hilbert DM, *et al.*** Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. *Arthritis Rheum.* 2001; 44: 1313–9.
14. **Stohl W, Metyas S, Tan SM, *et al.*** B lymphocyte stimulator overexpression in patients with systemic lupus erythematosus: longitudinal observations. *Arthritis Rheum.* 2003; 48: 3475–86.
15. **Becker-Merok A, Nikolaisen C, Nossent HC.** B-lymphocyte activating factor in systemic lupus erythematosus and rheumatoid arthritis in relation to autoantibody levels, disease measures and time. *Lupus.* 2006; 15: 570–6.
16. **Bossen C, Schneider P.** BAFF, APRIL and their receptors: structure, function and signaling. *Semin Immunol.* 2006; 18: 263–75.
17. **Thompson JS, Bixler SA, Qian F, *et al.*** BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science.* 2001; 293: 2108–11.
18. **Liu W, Szalai A, Zhao L, *et al.*** Control of spontaneous B lymphocyte autoimmunity with adenovirus-encoded soluble TACI. *Arthritis Rheum.* 2004; 50: 1884–96.
19. **Ramanujam M, Wang X, Huang W, *et al.*** Similarities and differences between selective and nonselective BAFF blockade in murine SLE. *J Clin Invest.* 2006; 116: 724–34.
20. **Bossen C, Ingold K, Tardivel A, *et al.*** Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *J Biol Chem.* 2006; 281: 13964–71.
21. **Taylor L, Bachler M, Duncan I, *et al.*** In vitro and in vivo activities of OX40 (CD134)-IgG fusion protein isoforms with different levels of immune-effector functions. *J Leukoc Biol.* 2002; 72: 522–9.
22. **Pelletier M, Thompson JS, Qian F, *et al.*** Comparison of soluble decoy IgG fusion proteins of BAFF-R and BCMA as antagonists for BAFF. *J Biol Chem.* 2003; 278: 33127–33.
23. **Gao W, Demirci G, Strom TB, *et al.*** Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. *Transplantation.* 2003; 76: 994–9.
24. **Ravirajan CT, Sarraf CE, Anilkumar TV, *et al.*** An analysis of apoptosis in lymphoid organs and lupus disease in murine systemic lupus erythematosus (SLE). *Clin Exp Immunol.* 1996; 105: 306–12.
25. **Knight JG, Adams DD, Purves HD.** The genetic contribution of the NZB mouse to the renal disease of the NZB x NZW hybrid. *Clin Exp Immunol.* 1977; 28: 352–8.
26. **Kinoshita K, Tesch G, Schwarting A, *et al.*** Costimulation by B7–1 and B7–2 is required for autoimmune disease in MRL-Faslpr mice. *J Immunol.* 2000; 164: 6046–56.
27. **Watson ML, Rao JK, Gilkeson GS, *et al.*** Genetic analysis of MRL-lpr mice: relationship of the Fas apoptosis gene to disease manifestations and renal disease-modifying loci. *J Exp Med.* 1992; 176: 1645–56.
28. **Zheng SG, Wang JH, Koss MN, *et al.*** CD4+ and CD8+ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. *J Immunol.* 2004; 172: 1531–9.
29. **Ye Q, Wang L, Wells AD, *et al.*** BAFF binding to T cell-expressed BAFF-R costimulates T cell proliferation and alloresponses. *Eur J Immunol.* 2004; 34: 2750–9.
30. **Gorelik L, Cutler AH, Thill G, *et al.*** Cutting edge: BAFF regulates CD21/35 and CD23 expression independent of its B cell survival function. *J Immunol.* 2004; 172: 762–6.
31. **Avery DT, Kalled SL, Ellyard JI, *et al.*** BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. *J Clin Invest.* 2003; 112: 286–97.
32. **Ng LG, Sutherland AP, Newton R, *et al.*** B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. *J Immunol.* 2004; 173: 807–17.
33. **Andrews BS, Eisenberg RA, Theofilopoulos AN, *et al.*** Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *J Exp Med.* 1978; 148: 1198–215.
34. **Izui S, Ibnou-Zekri N, Fossati-Jimack L, *et al.*** Lessons from BXSB and related mouse models. *Int Rev Immunol.* 2000; 19: 447–72.
35. **Shlomchik MJ, Madaio MP, Ni D, *et al.*** The role of B cells in lpr/lpr-induced autoimmunity. *J Exp Med.* 1994; 180: 1295–306.
36. **Beutler B, Cerami A.** The biology of cachectin/TNF–a primary mediator of the host response. *Annu Rev Immunol.* 1989; 7: 625–55.
37. **Wong M, Ziring D, Korin Y, *et al.*** TNFalpha blockade in human diseases: mechanisms and future directions. *Clin Immunol.* 2008; 126: 121–36.
38. **Woof JM, Partridge LJ, Jefferis R, *et al.*** Localisation of the monocyte-binding region on human immunoglobulin G. *Mol Immunol.* 1986; 23: 319–30.
39. **Lewis EC, Shapiro L, Bowers OJ, *et al.*** Alpha1-antitrypsin monotherapy prolongs islet allograft survival in mice. *Proc Natl Acad Sci USA.* 2005; 102: 12153–8.